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The interaction between hexamethonium and tubocurarine on the rat neuromuscular junction

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1 The ability of hexamethonium (C6) to reverse the neuromuscular blocking action of tubocurarine (Tc) has been reinvestigated at the voltage clamped endplate of the omohyoid muscle of rat. The possibility that a weak anticholinesterase action of C6 could contribute to the paradoxical potentiation of the peak amplitude of the endplate response has been examined.

2 C6 (50–200 μM) caused an increase in the amplitude of nerve-evoked endplate currents (e.p.cs) recorded in the presence of 0.6 μM Tc. The effect decreased with hyperpolarization of the muscle fibre. Irreversible inhibition of acetylcholinesterase resulted in a loss of the anti-curare effect of C6.

3 C6 did not cause an increase in e.p.c. amplitude when acetylcholine (ACh) receptors were blocked irreversibly by α-bungaratoxin. When transmission was blocked by increased Mg^{2+} concentration, C6 (50–400 μM) reduced the amplitude of e.p.cs without appreciably affecting their time course.

4 C6 caused a decrease in the amplitude of miniature endplate currents (m.e.p.cs) the effect being slightly increased when the fibre was hyperpolarized. An e-fold increase in the effectiveness of C6 occurred with approximately 58 mV hyperpolarization. High concentrations (> 400 μM) affected the time course of m.e.p.cs in a manner suggestive of open channel block, but this was not evident at 200 μM, the concentration that was most effective in reversing Tc block.

5 When tested against responses to short ionophoretic pulses of agonists, C6 was less effective against ACh (EC_{50} ca. 300 μM) than against carbachol (CCh) (EC_{50} 100 μM). When cholinesterase was irreversibly inhibited, C6 blocked responses to both agonists equally (EC_{50} ca. 100 μM).

6 The effectiveness of C6 in blocking the action of CCh was reduced 10 fold in the presence of 0.6 μM Tc, implying that the two antagonists compete for the same binding site.

7 C6 (50–200 μM) in the presence of Tc (0.6 μM) increased the response to ionophoretically applied ACh but not that to CCh.

8 C6 was equipotent in blocking m.e.p.cs and responses to ionophoretically applied ACh whereas Tc was more potent against the exogenously applied agonist.

9 C6 was a weak inhibitor of acetylcholinesterase activity in rat muscle homogenates (EC_{50} 1.5 mM).

10 The results are discussed in terms of the kinetic hypothesis advanced by Ginsborg & Stephenson (1974) to account for the Tc reversal phenomenon. It is concluded that this theory can explain most of the effect on e.p.cs, but that the weak anticholinesterase action of C6 is also a factor, particularly in the reversal of Tc block of ionophoretic responses.

Introduction

Stephenson & Ginsborg have reported, and analysed mathematically, circumstances where one antagonist can partially reverse the action of another antagonist (Stephenson & Ginsborg, 1969; Ginsborg & Stephenson, 1974). For this phenomenon to occur, the two blocking agents must be competitive antagonists, one of which, e.g. tubocurarine (Tc), dissociates from the receptor relatively slowly and the other, e.g. hexamethonium (C6) rapidly. A paradoxical potentiation of this type has been reported when

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C6 is applied to curarized mammalian neuromuscular preparations, and the results interpreted in terms of the Ginsborg-Stephenson hypothesis (Ferry & Marshall, 1971; 1973; Blackman *et al.*, 1975). If the preparation is equilibrated with Tc, and tested with agonist pulses that are short relative to its dissociation time, then application of C6, which dissociates more rapidly, in addition to Tc, can partially *restore* the response to agonist, instead of it blocking further. This happens because a proportion of the relatively stable Tc-receptor complexes became replaced by C6-receptor complexes that can dissociate during the agonist test pulse, and the response increases even though addition of C6 *reduces* the fraction of unoccupied receptors.

A modification of this hypothesis (see Colquhoun, 1975) accounts for the reversal phenomenon in terms of 'buffered diffusion' of the antagonists. If an antagonist has a high affinity for the receptors, the majority of antagonist molecules within the synaptic cleft will be bound, and only a very small proportion will be free. Thus, if Tc has a dissociation constant equal to 0.1 μM (Waud *et al.*, 1973) and the effective receptor concentration within the cleft is 0.4 mM (Fambrough & Hartzell, 1972), then at equilibrium with 1 μM Tc only about 1 in 400 Tc molecules will be free within the cleft. Introduction of a competing substance, such as acetylcholine (ACh), will cause only a very small rapid reduction of Tc occupancy, even if the dissociation rate constant for Tc is large, because any decrease in Tc occupancy will be opposed by the 'buffering' effect resulting from the large increase in free Tc concentration. Equilibration would eventually occur, if the acetylcholine were not removed from the synaptic cleft, as the excess Tc diffused out of the cleft, but this will be too slow to permit effective equilibration during the rising phase of the endplate current (e.p.c.). With an antagonist of lower potency, such as C6, the buffering effect will be much less marked, so receptor occupancy by C6 can decrease sufficiently rapidly when ACh is applied to allow an approach to a competitive equilibrium during the e.p.c. Addition of C6 to a preparation equilibrated with Tc might then cause a paradoxical potentiation of the e.p.c. even though diffusion, rather than dissociation of Tc from receptors, is rate-limiting.

At ganglionic nicotinic receptors the blocking action of C6 is increased by hyperpolarization of the cell (Blackman, 1959; Ascher *et al.*, 1979; Rang, 1982), and there is good evidence that this effect is due to an action on receptor-associated ionic channels rather than directly on the ACh receptor itself (Rang, 1982). A similar though less dramatic ionic channel-blocking action has been reported for C6 at the motor endplate (Milne & Byrne, 1981). These authors demonstrated a voltage-dependent reduction in the time to peak of the e.p.c. in the presence of C6 and decamethonium which is indicative of occlusion of open ion channels. At the neuromuscular junction Tc has been found to have ion channel-blocking effects also (Manalis, 1977; Katz & Miledi, 1978; Colquhoun *et al.*, 1979), although its main mechanism of action, especially in lower concentrations, is by competition with ACh for the nicotinic receptor (Colquhoun *et al.*, 1979).

Thus, it is clear that C6 and Tc are not strictly competitive antagonists at the ACh receptor, which raises the question of whether additional, or other, mechanisms may be responsible for the anti-curare action of C6 at the mammalian neuromuscular junction. This effect has been investigated at the voltage clamped endplate of rat omohyoid muscle and the results are presented in this paper. The degree of reversal of Tc blockade by C6 is sensitive to membrane potential, suggesting that the two blocking agents may be binding to different sites; blockade produced by low concentrations of Tc alone was not voltage-dependent. Results obtained during ionophoretic application of agonists, and in preparations where acetylcholinesterase (AChE, E.C. 3.1.1.7), was irreversibly inhibited reveal that the anti-curare action of C6 could be due, at least in part, to a weak anticholinesterase effect of C6.

Methods

Electrophysiological recordings were made from the omohyoid muscle of rat as described by Dreyer *et al.*, (1976). Young rats (Lister hooded strain of either sex, 30–40 g) were killed with chloroform; the thorax was opened and the animals were bled by perfusion with Krebs solution through the left ventricle. The omohyoid muscle, with its motor nerve, was removed with surrounding structures and dissected free in Krebs solution. Pieces of the scapula and hyoid bone and a 10 mm segment of motor nerve remained attached. Overlying connective tissue was removed and surface fibres were cut and teased away to expose endplates on the underlying fibres. The muscle was pinned as a thin sheet on a bed of Sylgard resin (Dow-Corning elastomer 184) and it was continuously superfused with normal Krebs solution at approximately 3 ml min^{-1} (chamber volume approximately 1 ml). The composition of normal Krebs solution was (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and it was constantly gassed with 95% O_2/5% CO_2. In some experiments neuromuscular transmission was blocked by increasing the $MgSO_4$ concentration to 7.5–10 mM and reducing the $CaCl_2$ concentration to 1.2 mM without altering other ion concentrations. The motor nerve was drawn into a suction electrode for stimulation. The bath temperature was main-

tained at 20°C by passing the Krebs solution through a water jacket circulated with water from a refrigerating/heating unit (Churchill); the temperature could be checked with a microthermistor probe placed close to the muscle. For recording miniature endplate currents (m.e.p.cs) the temperature was maintained at 18°C.

Recording of m.e.p.cs and e.p.cs

Endplate regions were located visually by Zeiss-Normarski differential interference optics (water immersion objectives × 40) for the placement of microelectrodes. Current changes across the endplate region were measured by a two microelectrode voltage clamp arrangement similar to that of Dionne & Stevens (1975); current was measured as the voltage drop across a 1 MΩ resistor placed in series with the current-passing electrode. Both current-passing and voltage-recording electrodes were filled with 3M KCl and had resistances of 8–15 MΩ. High frequency noise was eliminated by feeding signals through a low-pass filter (Barr & Stroud) with a cut-off frequency of 2 kHz (roll-off 48 dB/decade). Signals were stored on magnetic tape (Racal Store 4 – FM) for subsequent computer analysis.

To record e.p.cs the motor nerve was stimulated with single supramaximal stimuli (0.1–0.2 ms duration) at a frequency of 0.5 Hz. Neuromuscular transmission was blocked with either Tc (0.6 μM) or elevated Mg^{2+}. In experiments where e.p.cs were to be studied in the presence of Tc at concentrations less than 0.6 μM, a balance between the desired concentration of Tc and an increased Mg^{2+} concentration was found which could effect blockade of neuromuscular transmission. Attempts were made to record control e.p.cs from untreated (drug-free) muscles in normal Krebs solution by stretching the muscle to overcome the twitch response evoked by nerve stimulation; however, it was found that the resting input impedance of the fibres dropped and the cells did not withstand prolonged impalement with two microelectrodes. Therefore, all control e.p.cs have been recorded in the presence of Tc, elevated Mg^{2+} or Tc/Mg^{2+} combination. In experiments in which acetylcholinesterase was inhibited irreversibly, muscles were superfused with Krebs solution containing methanesulphonyl fluoride (MSF) at a concentration of 15 mM for 20 min before the addition of Tc or other drugs.

Irreversible ACh receptor blockade was produced by α-bungarotoxin. It proved difficult to achieve adequate blockade, since addition of α-bungarotoxin to the superfusion medium tended to obliterate endplate responses in superficial fibres before blocking the twitch of the deeper fibres. Very thin muscle preparations in which several fibre layers were removed were used and miniature endplate potentials (m.e.p.ps) were monitored continuously as the medium containing α-bungarotoxin (0.3 μg ml^{-1}) was superfused. When m.e.p.ps were no longer detectable the muscle was washed in inhibitor-free Krebs solution. Any residual muscle twitch in response to nerve stimulation was then blocked by elevation of the Mg^{2+} concentration to about 8 mM.

A series of control e.p.cs or m.e.p.cs were recorded at membrane potentials of − 60, − 80, and − 100mV. The holding potential was usually set at − 60 mV and adjusted manually to record responses at other potentials. Drugs were added to the superfusion medium and 5–7 min were allowed for equilibration before endplate responses were recorded. Successive drug applications (increasing concentrations) were tested on most cells, allowing for equilibration between recordings. Where possible, the drug was washed out and control responses were recorded again.

Ionophoretic responses

ACh and carbachol (CCh) were applied ionophoretically to the endplate by means of a current pump similar to that described by Dreyer & Peper (1974). Ionophoretic pipettes had resistances of 100 MΩ and contained 1M ACh or CCh as chlorides. Ejecting currents were usually 5–7 nA and of 20 ms duration. In some experiments, double-barrelled ionophoretic pipettes containing 0.1 M ACh and CCh were used. Ejection pulses (approx. 20 nA) of 20 ms duration were applied, alternating between barrels, every 3 s. Positioning of the pipette at the endplate was considered acceptable when the rise time of the endplate current was approximately 20 ms. Signals were passed through a low-pass filter with a cut-off frequency of 900 Hz. Only cells in which responses returned to pre-drug controls upon washing were acceptable.

Analysis of electrophysiological data

Signals stored on magnetic tape were analysed by computer. For e.p.cs, the stimulus artifact was used as a trigger for a computer programme which captured and stored events and also monitored synaptic failures. A series of 15 (Tc-blocked) or 60 (Mg^{2+} blocked) consecutive e.p.cs, digitized with 128 points at 5000 Hz, were captured. By means of editing and averaging programmes, the rising phase of each e.p.c. was aligned and the responses successively averaged. In experiments where AChE was inhibited irreversibly, e.p.cs were digitized at 3000 Hz, 256 points, to accommodate the greatly prolonged decay phase. Where appropriate, the averaged e.p.cs were fitted by a non-linear least squares method (see Colqhoun *et al.*, 1979) to obtain estimates of peak amplitudes

and rate constant(s) for the exponential decay phase. M.e.p.cs were captured in a similar way, except that the m.e.p.c. itself served as a trigger, and a stretch of pre-trigger baseline was included with each captured event.

Acetylcholinesterase assays

C6 was tested as an inhibitor of AChE of rat diaphragm homogenates by the spectrophotometric method of Ellman *et al.*, (1961). Endplate regions were dissected from diaphragms of rats and homogenized (20% w/v) in 0.1 M phosphate buffer, pH 8.0, by means of a Polytron blender. Homogenates were centrifuged at 10,000 *g* for 20 min and supernatants, containing enzyme, were assayed. Measurements of the change in optical density (at 412 nm) versus time were made at 20–22°C. The assay mixture contained 0.1 M phosphate buffer, pH 8.0, 0.32 mM dithiobisnitrobenzoic acid, 0.48 mM acetylthiocholine iodide as substrate, and enzyme; C6 was tested as an inhibitor over the concentration range 0.2 to 3.2 mM, with and without 0.6 μM Tc present. AChE activity was also measured with edrophonium bromide, 0.1 to 5.0 μM, as inhibitor.

Materials

Drugs used were (+)-tubocurarine chloride (Koch-Light), hexamethonium dibromide (Sigma), methanesulphonyl fluoride (Aldrich), acetylcholine chloride (Sigma), carbamylcholine chloride (Sigma), edrophonium bromide (Roche) and α-bungarotoxin (Sigma).

Computer stimulations

We are very grateful to Dr D.M.J. Quastel and Dr P. Pennefather, University of British Columbia, for providing us with their m.e.p.c simulation programme (Pennefather & Quastel, 1981), modified to allow for simulation of the effect of two antagonists applied simultaneously. In this programme, which is very similar to that developed by Wathey *et al.*, (1979), the area of the synaptic cleft is represented as a series of concentric annular compartments (10 in our calculations), within each of which the drug concentrations are assumed to be uniform. Transfer of drug between compartments is calculated on the basis of diffusion equations, and within each compartment the binding and dissociation of drug molecules and receptors, and the hydrolysis of ACh, is calculated from mass action equations, integrated at intervals (usually 0.005 ms) that are short in relation to the rates of diffusion and binding. The m.e.p.c. is initiated by depositing a quantum (10000 molecules) and ACh in the central compartment. Each channel is assumed to be controlled by two receptor sites, and the amplitude of the m.e.p.c. at any moment is taken to be proportional to the total number of sites doubly-occupied with ACh molecules.

The parameters used to define the rates of binding, diffusion and hydrolysis of ACh, the number of receptors per unit volume of the cleft, and the rate of hydrolysis by AChE are based on those used by Pennefather & Quastel (1981), the details being given in the legend to Figure 7. The programme was run in Fortran on a PDP 11/03 computer.

Results

Reversal of tubocurarine block by hexamethonium

Tc (0.6 μM) reliably produced complete neuromuscular block and the mean amplitude of e.p.cs recorded in its presence was 2.71 ± 0.34 nA at − 60mV (11 muscles). E.p.cs decayed with a simple exponential time course and hyperpolarization in the range − 60 mV to − 100 mV increased both the amplitude and the time constant of e.p.cs, as described by many previous authors (for example, Takeuchi, 1963; Magleby & Stevens, 1972; Dionne & Stevens, 1975).

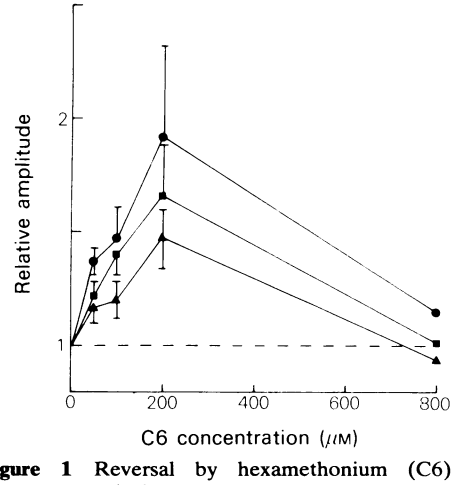

Figure 1 Reversal by hexamethonium (C6) of tubocurarine (Tc)-induced reduction of e.p.c. amplitude. The ordinates show e.p.c. amplitude in the presence of C6 and 0.6 μM Tc, expressed relative to the amplitude recorded in the presence of 0.6 μM Tc alone. Values exceeding 1.0 therefore represent an increase in amplitude produced by C6. E.p.cs were recorded at holding potentials of − 60 mV (●), − 80 mV (■) and − 100 mV (▲). The number of cells tested at each C6 concentration were: 50 μM (6), 100 μM (4), 200 μM (3), 800 μM (1). Error bars show s.e.mean. Note that hyperpolarization reduces the magnitude of the reversal phenomenon at each C6 concentration.

Table 1 Time constant of decay of e.p.c. (τ) in presence of hexamethonium relative to control

Hexamethonium (M)	*Membrane potential* (mV) −60	−80	−100
50	1.02±0.06(6)*	1.11±0.06(6)	1.15±0.06(6)
100	1.14±0.05(4)	1.21±0.06(4)	1.27±0.09(4)
200	1.05±0.17(3)	1.19±0.20(3)	1.30±0.28(3)
800	1.20(1)	1.54(1)	2.03(1)

* $\frac{\tau \text{ with hexamethonium}}{\tau \text{ without hexamethonium}}$ ±s.e.mean (n); tubocurarine present at 0.6 μM.

The effect of C6 (50–800 μM) on the amplitude of e.p.cs recorded in the presence of 0.6 μM Tc is shown in Figure 1. At −60 mV, addition of 200 μM C6 roughly doubled the e.p.c. amplitude; a higher concentration (800 μM) was much less effective. Hyperpolarization of the fibre in the range −60 mV to −100 mV reduced the effectiveness of C6 in reversing the action of Tc.

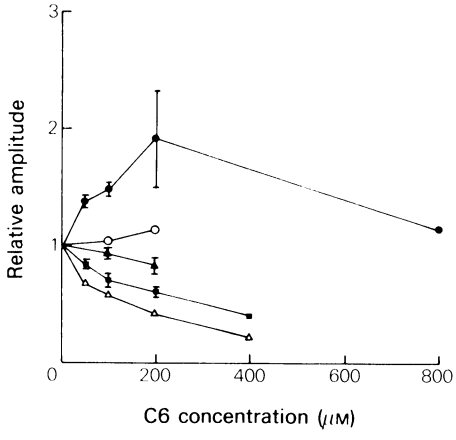

Figure 2 Effect of tubocurarine (Tc) concentration on the reversal phenomenon. Ordinates are peak e.p.c. amplitude in the presence of hexamethonium (C6), expressed relative to the amplitude recorded in the absence of C6 at different concentrations of Tc. The Tc concentrations were: 0.0 (△), 0.05–0.1 μM (▲), 0.2 μM (○), 0.6 μM (●). The calcium concentration was reduced to 1.2 mM, and extra magnesium was added (7.5–10 mM) in order to prevent twitching at the lower Tc concentrations. The line shown with closed squares (■) gives results for reduction of m.e.p.c. amplitude by C6, with normal Krebs solution containing no Tc. The results for 0.6 μM Tc are redrawn from Figure 1. At Tc concentrations less than 0.6 μM, C6 had no effect on, or reduced, the e.p.c. amplitude. Block by increased Mg^{2+} on its own (△) was not antagonized by C6. Error bars show s.e.mean for 3–6 observations.

C6 had only a small effect on the decay time course of e.p.cs in these experiments. The decay phase remained a simple exponential, with the time constant slightly, but consistently, prolonged in the presence of C6 (Table 1).

Lower concentrations of Tc were also tested, with added magnesium to reduce the quantal content of e.p.cs and prevent muscle twitching. Figure 2 shows that C6 failed to increase e.p.c. amplitude under these conditions, producing instead a further block, which became more marked when the fibre was hyperpolarized. There were no clearcut changes in the decay kinetics. These results are consistent with the finding of Ferry & Marshall (1973), who showed that C6 cannot reverse the neuromuscular block produced by magnesium.

The action of hexamethonium on m.e.p.cs

In order to understand more fully the nature of the C6-Tc interaction it was important to analyse the action of C6 itself in rather more detail. In particular, the contribution of the channel-blocking action, described by Milne & Byrne (1981), needed to be assessed; if this was the preponderant mechanism by which C6 causes block of endplate responses, a different explanation of the Tc reversal phenomenon from that proposed by Blackman *et al.*, (1975) would be needed.

C6 reduced m.e.p.c. amplitude, the EC_{50} being about 300 μM when the cell was clamped at −60 mV and 150 μM at −100 mV.

The effect of C6 on m.e.p.c. peak amplitude is shown as a function of membrane potential in Figure 3. To assess the voltage-dependence of the reduction in peak amplitude the value of Λ (V) − 1 for various membrane potentials (V) was calculated (see Ascher *et al.*, 1979; Rang, 1982).

$$\text{where } \Lambda = \frac{\text{Amplitude in absence of inhibitor}}{\text{Amplitude in presence of inhibitor}}$$

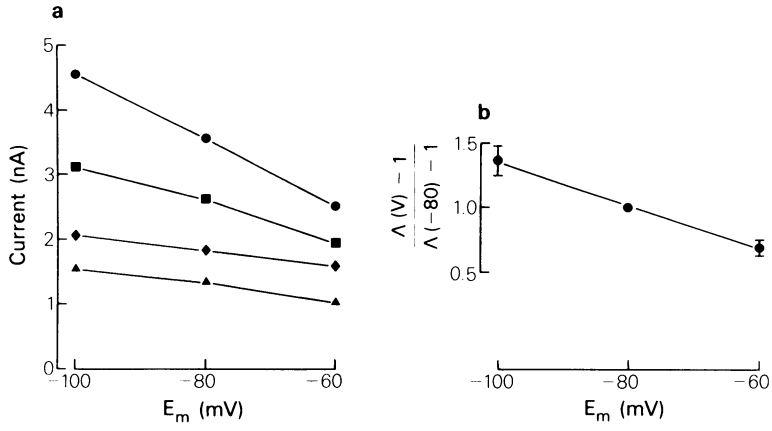

Figure 3 Voltage-dependence of the effect of hexamethonium (C6) on m.e.p.c amplitude. (**a**) Current-voltage relationship for m.e.p.cs as a function of C6 concentration. Control (●); 50 μM; 200 μM (◆); 400 μM (▲). (**b**) Blocking effect, expressed as $(\Lambda(V) - 1/(\Lambda(-80) - 1)$, as a function of membrane potential, calculated for C6 concentrations in the range 50–400 μM. Each point is the mean (± s.e.mean) of 16 measurements. The characteristic voltage, estimated from the slope of the line, is 58 mV.

The results for C6 (50–400 μM) normalized with respect to $\Lambda(-80) - 1$ are shown in Figure 3b. Based on results obtained over the holding potential range −60 to −100 mV, an e-fold change in $\Lambda - 1$ was produced with a potential change (the characteristic voltage) of about 58 mV. This is comparable to the result obtained by Rang (1982) for the reduction of e.s.c. amplitude in parasympathetic ganglia by C6 (2–30 μM). Studies of the voltage-dependent channel-blocking properties of other drugs have revealed characteristic voltages in the range of 25 to 50 mV (Ascher *et al.*, 1978; 1979; Colquhoun *et al.*, 1979; Colquhoun & Sheridan, 1981).

The time course of m.e.p.cs (both rising and decay phases) was not consistently altered by C6 at concentrations up to 200 μM. At higher concentrations (400 μM and above) the decay phase was often split into a fast and a slow exponential component, as found by Milne & Byrne (1981) for concentrations in the range 750–1500 μM. This type of kinetic effect is characteristic of drugs that block open ionic channels, but it did not appear to contribute significantly to the action of C6 in the concentration-range that was effective in producing Tc reversal. The voltage-dependent reduction of m.e.p.c. amplitude must therefore be attributed to a mechanism other than open channel block, a conclusion that Milne & Byrne (1981) also reached. Indeed studies of channel-blocking drugs on the endplate (Adams, 1977; Masukawa & Albuquerque, 1978; Tiedt *et al.*, 1979; Adams & Feltz, 1980a, b) and on autonomic ganglion cells (Ascher *et al.*, 1979; Rang, 1982) have generally concluded that their blocking effects are too large to be accounted for solely in terms of open channel block, and imply the existence of voltage-dependent block at some other site, often postulated to be the closed channel. In the case of C6 acting on the endplate, this additional site appears to be mainly responsible for its blocking action. Results presented later suggest that this site is the receptor itself, though it was unexpected to find that the effect shows appreciable voltage-sensitivity, since the binding of other competitive antagonists such as Tc (Jenkinson, 1960; Colquhoun *et al.*, 1979) and gallamine (Colquhoun & Sheridan, 1982) has been shown to be insensitive to membrane potential. Colquhoun & Rang (1976) showed that C6 retards the binding of α-bungarotoxin to receptors in membrane fragments from normal and denervated rat muscle (K_I ca 100 μM) which suggests that it can bind to receptors.

Effect of hexamethonium on endplates blocked with α-bungarotoxin

The Ginsborg-Stephenson hypothesis attributes the potentiating action of C6 to its ability to reduce the fraction of receptors occupied by the slowly-dissociating antagonist, Tc. On the basis of this hypothesis, C6 should not be able to reverse the blocking effect of α-bungarotoxin, which combines irreversibly with the receptors. If, on the other hand, its potentiating effect results from a weak anticholinesterase action, it might be expected to reverse the blocking action of α-bungarotoxin as well as that of Tc. Experiments with very thin muscle preparations blocked with α-bungarotoxin showed that C6 caused further depression of e.p.c. amplitude

Table 2 Effect of hexamethonium and edrophonium on e.p.c. amplitude during blockade with α-bugarotoxin

Drug	*Conc.* (μM)	*Amplitude of e.p.c. relative to control* (at −60 mV)
Hexamethonium	50	0.75 ±0.061(3)*
	100	0.598±0.013(3)
	200	0.391±0.038(5)
Edrophonium	0.2	1.30 ±0.16 (4)
	0.5	1.57 (2)

* mean ± s.e.mean (n).

(Table 2), which is in contrast to its effect on Tc-blocked endplates. The potency of C6 in blocking e.p.cs in α-bungarotoxin-treated muscle was similar to its potency in blocking m.e.p.cs or responses to ionophoretic pulses of ACh (see later section). This result strongly suggests that the reversibility with which Tc binds to receptors is an essential factor in the mechanism of the C6-reversal phenomenon, just as the Ginsborg-Stephenson hypothesis implies. The effect of low concentrations of edrophonium was also tested on these α-bungarotoxin-treated muscles to determine the effect of weak cholinesterase inhibition. At 0.2 and 0.5 μM edrophonium caused a marked increase in e.p.c. amplitude (Table 2). These concentrations produced, respectively, 12% and 20% inhibition of cholinesterase activity in muscle homogenates. The effect of edrophonium on e.p.c. amplitude in these experiments agrees with the results of Pennefather & Quastel (1981) who showed that cholinesterase inhibition is nearly as effective in increasing m.e.p.c. amplitude when receptors are irreversibly inactivated as when they are reversibly blocked.

The results presented so far appear to be broadly consistent with the Ginsborg-Stephenson hypothesis, but we have also investigated the possibility that a weak anticholinesterase action of C6 in addition to its receptor blocking effect could account for part of its effect in reversing Tc block. The finding that C6 enhances endplate responses only in the presence of moderately high concentrations of Tc (and not in Mg^{2+}-blocked preparations) is consistent with the properties of anticholinesterases, whose effect on m.e.p.c. amplitude is normally slight but increases progressively as the number of available receptors is reduced (see Pennefather & Quastel, 1981). We have confirmed that a low concentration of edrophonium (1 μM) caused only a very small increase (6 ± 1%; $n = 4$) in e.p.c. amplitude in Mg^{2+}-blocked muscles, while increasing the time-constant of decay by 51 ± 2%; 1 μM edrophonium blocked AChE activity in muscle homogenate by 32%. In muscles blocked by Tc (0.7 μM) the same concentration of edrophonium increased e.p.c. amplitude by 71 ± 21% ($n = 5$) and the decay time-constant by 14 ± 14%. According to the analyses of Wathey *et al.*, (1979), Rosenberry (1979) and Pennefather & Quastel (1981), this is because the fraction of released ACh molecules 'captured' by receptors is normally high (about 80% according to Pennefather & Quastel). Only when the number of available receptors, and hence the fraction of ACh molecules captured by receptors, is reduced can cholinesterase inhibition substantially increase this fraction and hence increase the amplitude of the postsynaptic response.

The following experiments were intended to give evidence about the contribution of a putative anticholinesterase action of C6 to the Tc reversal phenomenon.

Lack of effect of hexamethonium in anticholinesterase-treated muscles

Muscles were treated with MSF (see methods) to produce irreversible cholinesterase inhibition, and exposed to 1.5–1.8 μM Tc to block transmission. Under these conditions C6 (100–200 μM) had no appreciable effect on e.p.c. amplitude, the relative e.p.c. amplitude (200 μM C6 − 60 mV holding potential) being 1.01 ± 0.08 ($n = 5$) compared with e.p.cs recorded in the presence of Tc alone.

Effects of tubocurarine and hexamethonium on response to ionophoretically applied acetylcholine and carbachol

If the Tc reversal effect of C6 relies in part on an anticholinesterase effect certain predictions can be made: (1) C6 should be less effective in blocking ACh responses than CCh responses, because its blocking effect against ACh will be opposed by simultaneous cholinesterase inhibition. This difference should be abolished when cholinesterase activity is blocked. (2) The reversal phenomenon should not occur when CCh is used as agonist.

Both of these predictions were borne out experimentally. A series of experiments was performed in

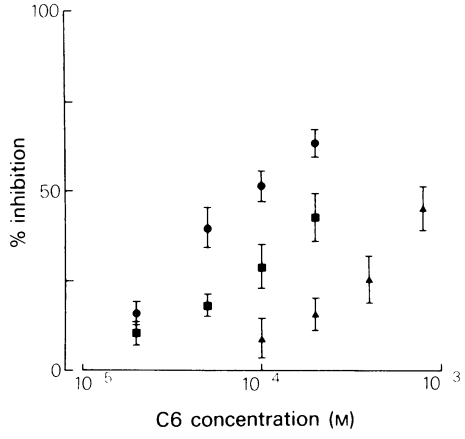

Figure 4 Inhibition of endplate response to ionophoretically applied agonists by hexamethonium (C6). Acetylcholine (ACh) (●); carbachol (CCh) (■); CCh in the presence of 0.6 μM tubocurarine (Tc) (▲). Points are the mean (with s.e.mean) of results from at least three cells. The holding potential was − 60 mV, C6 was 3.6 times as effective at blocking CCh, compared with its potency in blocking ACh. Its effectiveness against CCh was reduced 10 fold in the presence of 0.6 μM Tc.

which ACh or CCh was applied ionophoretically to the endplate in the form of a 20 ms pulse. The position of the ionophoretic pipette was adjusted so that the rise-time of the response under control conditions, to either agonist, was about 20 ms, and the amplitudes of the control responses were also similar (normally 50–80 nA at − 60 mV holding potential). Figure 4 shows that C6 was more effective, by a factor of about 3.6, in blocking CCh responses than ACh responses. Quantitatively similar results were obtained when ACh and CCh were ionophoresed alternately onto the endplate from a double-barrelled pipette. In parallel experiments Tc (50 nM) produced an equal blocking effect on ACh and CCh responses. ACh responses were reduced by $45 \pm 4\%$ ($n = 5$) while CCh responses were reduced by 47% and 51% ($n = 2$). When AChE was inactivated by MSF, the differential potency of C6 with respect to ACh and CCh was abolished. The EC_{50} for C6 against either agonist was about 130 μM, similar to the EC_{50} of 100 μM for C6 when CCh was applied with or without AChE inhibition. Its blocking action showed very little voltage-sensitivity, so it is unlikely that open channel block was significant. It can be seen from Figures 5 and 6 that the Tc-reversal phenomenon was clearly present when ACh was the agonist but not with CCh as the agonist. With ACh pulses applied to a fibre in the presence of 0.6 μM Tc, C6 was almost as effective in potentiating the response as it was in potentiating e.p.cs under the same conditions (the e.p.c. data from Figure 1 are redrawn in Figure 6 for comparison). With CCh as agonist, however, the effect of C6 was a purely depressant one.

Two additional points of interest can be deduced from the results shown in Figure 4: (a) The effective-

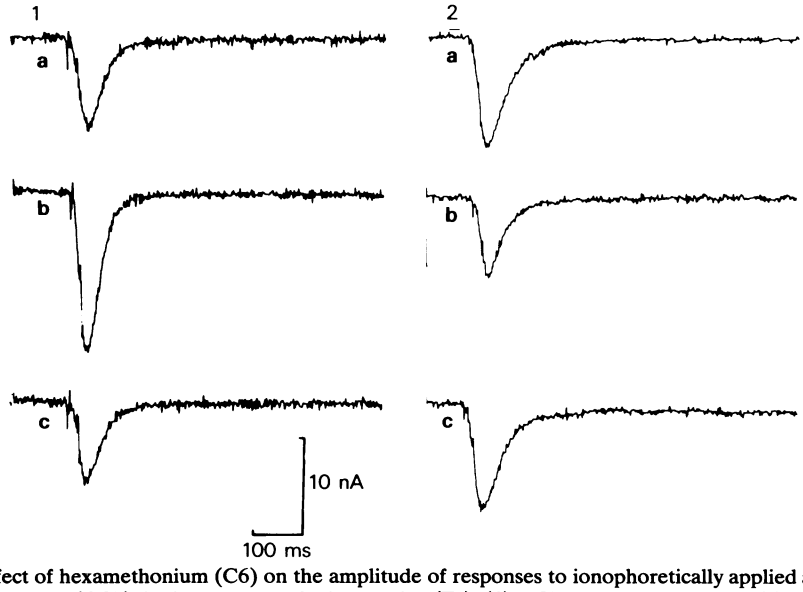

Figure 5 Effect of hexamethonium (C6) on the amplitude of responses to ionophoretically applied acetylcholine (ACh) and carbachol (CCh), in the presence of tubocurarine (Tc), (**1**) ACh responses before C6 (a), in presence of 400 μM C6 (b), after washing out C6(c). (**2**) CCh responses, as in (**1**); Tc (0.6 μM) was present throughout, and the holding potential was − 60 mV. Note that C6 increased the response to ACh, but reduced the response to CCh.

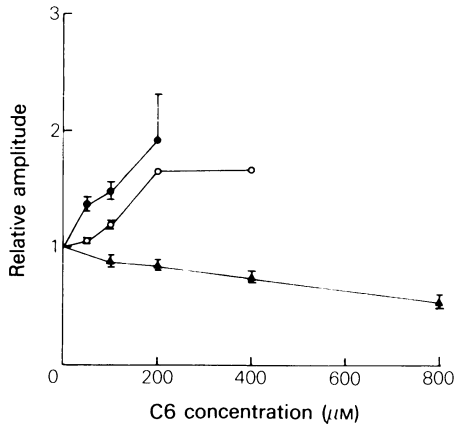

Figure 6 Comparison of effect of hexamethonium (C6) on the amplitude of endplate responses to ionophoretic acetylcholine (ACh, ○), ionophoretic carbachol (CCh, ▲) and nerve stimulation (●), in the presence of 0.6 μM tubocurarine (Tc) (redrawn from Figure 1). The holding potential was − 60 mV. Note that C6 caused a similar degree of Tc reversal with e.p.cs or responses to ionophoretic ACh, but inhibited responses to CCh. Error bars show s.e.mean for 3–6 observations.

ness of C6 in blocking the action of CCh is reduced by a factor close to 10 in the presence of 0.6 μM Tc. This is strong evidence that the drugs compete for the same blocking site, and the magnitude of the shift of the C6 curve seen in Figure 4 is consistent with published estimates of the affinity of Tc and C6 for the receptors. The EC_{50} for C6 against CCh responses is 100 μM, which agrees with the figure obtained by Colquhoun & Rang (1976) from measurements of its potency in retarding α-bungarotoxin binding. The estimated dissociation constant (K_{Tc}) for Tc at mammalian endplates is 100 nM (Lu, 1970; Waud *et al.*, 1973). If the two drugs compete for the receptors and the magnitude of the CCh response is proportional to the fraction of unoccupied receptors then the shift in the C6 curve as plotted in Figure 4 should correspond to a dose ratio of $([Tc]/K_{Tc}) + 1 = 7$. The actual shift is about 10, which is close to the predicted value; the difference may reflect uncertainty in the estimate of K_{Tc}, for the measurements of Pennefather & Quastel (1981), confirmed by a few similar measurements of our own, suggest that K_{Tc} is closer to 50 nM than 100 nM.

(b) C6 is as effective in blocking m.e.p.cs as in blocking responses to ionophoretically applied ACh. This is not true for Tc (see Wathey *et al.*, 1979; Pennefather & Quastel, 1981;) Pennefather & Quastel found that Tc is about four times as potent in blocking CCh responses (EC_{50} ca. 50 nM) as in blocking m.e.p.cs (EC_{50} 186 ± 9 nM), and we have found (see above) that it does not discriminate between ACh and CCh.

The kinetic argument put forward by Pennefather & Quastel (1981) satisfactorily explains the difference in EC_{50} for Tc when measured with exogenous and quantally-released agonist. The same argument would, however, predict an even greater difference with C6. This is because its affinity for receptors is about 1000 times lower than that of Tc so there is a much greater likelihood of C6 vacating the receptors during the rising phase of the m.e.p.c., making the block less effective. The finding that the EC_{50} for C6 is the same for ionophoretic ACh and for m.e.p.cs is, therefore, further evidence that it is exerting an anticholinesterase as well as a receptor blocking action.

Inhibition of anticholinesterase in muscle homogenates by hexamethonium

C6 was found to be a weak inhibitor of AChE in homogenates of rat diaphragm; the concentration causing 50% blockade was 1.5 mM. At 200 μM, the concentration which produced maximum reversal of Tc blockade, C6 decreased AChE activity in the homogenate by about 10%. Tc (0.6 μM) did not have an inhibitory effect on AChE, nor did it alter the degree of inhibition by C6.

Computer simulations

Simulation of m.e.p.cs in the presence of the competitive antagonists was carried out according to the theoretical model of Pennefather & Quastel (1981). This model envisages the quantal packet of ACh dumped at time zero in the central compartment of a series of 10 concentric rings. Diffusion between these concentric compartments is calculated, and the binding of ACh to receptors and hydrolysis by AChE within each compartment are calculated as a function of time. Each channel is assumed to be opened by the simultaneous binding of two ACh molecules. No cooperativity of ACh binding was assumed. Values for the various binding rate constants, are given in the legend to Figure 7, and are very similar to those used by Pennefather & Quastel (1981). The object of the simulation was to test whether the phenomenon of Tc reversal could be modelled, and to see whether allowance for the anticholinesterase activity of C6 affected the results appreciably. Figure 7 shows that the Tc reversal effect can be simulated, though there are certain quantitative differences between the behaviour of the model and the observed results. In the absence of Tc, C6 reduces m.e.p.c. amplitude, but as Tc is added in increasing concentrations, the reversal phenomenon becomes apparent (Figure 7a; compare with Figure 2). The model, however, requires a very

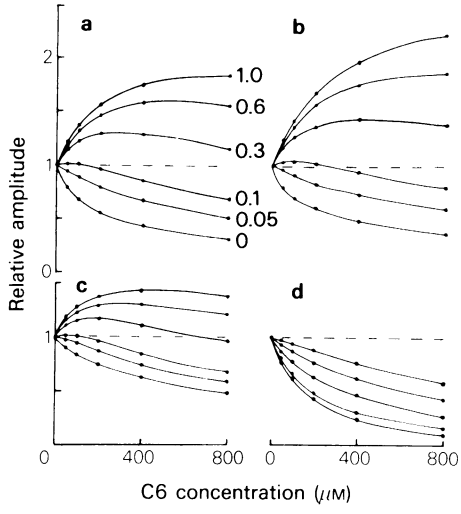

Figure 7 Computer simulation, based on model developed by Pennefather & Quastel (1981), to show effect on peak amplitude of m.e.p.c. of two competitive antagonists applied simultaneously. Each curve shows the relationship between peak m.e.p.c. amplitude (relative to the amplitude in the absence of hexamethonium, C6) and C6 concentration, at a given concentration of tubocurarine (Tc) (shown in μM on the curves in panel **a**). The Tc concentrations in the other panels are the same as those in panel (**a**) except that the curve for 0.05 μM Tc is omitted in (**d**). (**a**), (**b**) and (**c**) assume that only one molecule of Tc or C6 can bind to each receptor dimer, and that this prevents the binding of ACh to either of the two sites. (**d**) assumes that two antagonist molecules can bind to each dimer, and that hybrid occupancy states, with the two sites occupied by different species, can exist. In all cases the fraction of open channels is assumed to equal the fraction of dimers to which two ACh molecules are bound: (**a**), (**d**) AChE fully active; (**b**) AChE inhibited partially by C6 ($K_I = 1500$ μM); (**c**) AChE fully inhibited.

Rate constants for drug binding used in (**a**), (**b**) and (**c**) were as follows:

Reaction	*Forward rate constant* ($M^{-1}s^{-1}$)	*Backward rate constant* (s^{-1})
ACh + R	2×10^8	5×10^4
ACH + ACh-R	10^8	10^3
Tc + R	4×10^8	10
C6 + R	2×10^8	10^4

Rate constants for drug binding used in (**d**) were as follows:

ACh + R	2×10^8	5×10^4
ACh + ACh-R	10^8	10^3
ACh + Tc-R, ACh + C6-R	10^8	5×10^4
Tc + R	2×10^8	10
Tc + Tc-R	10^8	20
Tc + ACh-R, Tc + C6-R	10^8	10
C6 + R	10^8	10^4
C6 + C6-R	5×10^7	2×10^4
C6 + ACh-R, C6 + Tc-R	5×10^7	10^4

Other parameters (receptor density, AChE activity, etc.) were as used by Pennefather & Quastel (1981). The diffusion coefficient was assumed to be 0.6 cm^2s^{-1} for all three ligands. Reducing this to 0.3 cm^2s^{-1} made very little difference to the plots shown above, though it prolonged the m.e.p.cs appreciably.

Note that the reversal phenomenon (an increase in the relative amplitude in the presence of C6) is predicted in (**a**), (**b**) and (**c**) but not in (**d**). Analysis of the m.e.p.c. time-course (not shown) showed only small changes in rise-time or decay rate in the presence of the antagonists, except when AChE was inhibited.

high concentration of C6 ($> 2000\,\mu$M) before the reversal phenomenon disappears, whereas experimentally it disappeared at $800\,\mu$M. Allowing for a weak anticholinesterase effect of C6 (K_i $1500\,\mu$M) increases the magnitude of the reversal phenomenon (Figure 7b); however, the effect still occurs with the model, though it is considerably reduced, when cholinesterase is fully inactivated (Figure 7c). Experimentally, we found that C6 ($200\,\mu$M) had no effect on e.p.c. amplitude in the presence of $0.6\,\mu$M Tc in MSF-treated muscles.

Varying some of the quantitative parameters of the model, such as the diffusion coefficient or AChE activity, made only a small difference to its behaviour in relation to the reversal phenomenon.

With the receptor density used in Figure 7, varying the rate constants for the binding and dissociation of Tc also made very little difference. This is because the receptor density (effective concentration 0.4 mM) is so large in relation to the Tc concentration ($< 1\,\mu$M) that any dissociation of Tc molecules is immediately limited by the resulting large rise in the concentration in the synaptic cleft. Thus, the fraction of receptors occupied by Tc molecules will not decrease appreciably during the rising phase of the e.p.c., no matter how large its dissociation rate constant, because of this 'buffering' effect. This is, of course, not true for C6, where the free concentration is comparable to the effective receptor concentration, so that the buffering effect is much less important.

In testing different versions of the model we found that the predicted Tc reversal effect with C6 depended critically on the reaction mechanism that was assumed for the blocking drugs. If it is assumed that the two receptors controlling each channel bind antagonist molecules independently, so that singly and doubly occupied species can exist, together with hybrid occupancy states, the reversal phenomenon is much less pronounced, because it is only the receptors that are doubly occupied with C6 molecules and not the more numerous C6-Tc hybrids that can be rapidly vacated during the rising phase of the m.e.p.c. Figure 7d shows that when two-site binding of the antagonists is incorporated, with no restriction on the formation of hybrid occupancy states, the reversal phenomenon seen in Figure 7b is completely absent.

Discussion

The observations presented in this paper in general confirm those of Ferry & Marshall (1973) and Blackman *et al.*, (1975), but suggest that part at least of the Tc reversal phenomenon results from a weak anti-ChE action of C6. The evidence for this comes partly from the direct measurement of AChE inhibition in rat muscle homogenates, but more significantly from the comparison of its action in antagonizing the effects of ionophoretically applied ACh and CCh (Figures 4 and 6). On ChE-intact endplates, C6 was considerably less effective in blocking the action of ACh than CCh. The pipette placement was adjusted so that the two agonists produced responses of very similar time course, so that it is unlikely that systematic differences in the local concentration profile could account for the differential sensitivity. The fact that Tc blocked both agonists equally is also relevant. Since ChE inactivation removed the differential effect of C6, the most obvious interpretation is that its blocking action on ACh responses is partly opposed by an antiChE effect. This action could be enough to explain the Tc reversal seen with ionophoretically applied ACh, without invoking the Ginsborg-Stephenson mechanism.

When Tc and C6 were applied simultaneously to an endplate being stimulated by ionophoretic CCh pulses, the interaction was quantitatively consistent with the effects of two antagonists competing at the receptor sites (Figure 4) with no evidence of potentiation by C6. Thus it is likely that the relatively slow rise-time of the ionophoretic response permits a competitive equilibrium to be achieved, or alternatively that the agonist concentration reached is low enough that it produces, even at equilibrium, only a very small shift in the antagonist occupancy, so that the necessary conditions for the Ginsborg-Stephenson mechanism to operate are not realised.

The interaction of Tc and C6 in relation to e.p.cs is evidently more complex. A combination of receptor block and a weak anti-ChE effect can produce the degree of potentiation observed experimentally according to the theoretical model used in deriving Figure 7b, but there are certain features of the observations that are not well accounted for. The main ones are: (a) that an increase of C6 concentration beyond $400\,\mu$M causes a sharp reduction of the e.s.c amplitude that is not predicted by the model; (b) that hyperpolarization reduces considerably the magnitude of the Tc reversal phenomenon. The probable explanation for both of those results is that C6 has an additional, voltage-dependent, site of block in addition to its receptor blocking action, which becomes more pronounced at high C6 concentrations or at hyperpolarized potentials. If it were the receptor blocking action that showed voltage-sensitivity, hyperpolarization would be expected to enhance, rather than diminish, the Tc reversal, akin to the effect of increasing the C6 concentration, but, as Figure 1 shows, this is not what happens. The same explanation may account for the fact that ChE inhibition completely abolished the Tc reversal phenomenon, whereas the theoretical model predicts that it should reduce it without fully abolishing the effect. If an additional mechanism of block is operating, this could account for the discrepancy.

It seems unlikely that this putative additional mechanism of block, which is needed to account for the voltage-dependence of the action of C6 tested on its own, as well as for the discrepancies noted above, represents open channel block of the type described by Milne & Byrne (1981), for it occurs under conditions where C6 has no obvious effect on the kinetics of the decay phase of the e.p.c. There are, however, many reports of voltage-dependent blocking, not associated with open channel block, produced by various agents (e.g. tetraethylammonium, piperocaine, quinacrine, histrionicotoxin) on the endplate (see Albuquerque *et al.*, 1974; Adler *et al.*, 1979; Tiedt *et al.*, 1979; Adams & Feltz, 1980 a,b; Farley *et al.*, 1981), for which the most usual explanation is binding to closed ionic channels, thus affecting the amplitude but not the time course of endplate currents.

In summary, we suggest that the Tc reversal seen with C6 at the mammalian endplate results from (a) a competitive interaction at the receptors as postulated by Ginsborg & Stephenson (1974) and (b) a weak anticholinesterase effect. The former mechanism appears to be more important in relation to synaptically released ACh, and the latter in relation to ionophoretic responses. It is not possible to say from our results whether the Ginsborg-Stephenson effect occurs because Tc dissociates relatively slowly, or because of buffered diffusion, for we are still ignorant of the true rates of association and dissociation of antagonists at endplate receptors (see Colquhoun & Sheridan, 1982). In addition to these effects, C6 also produces a voltage-dependent blocking effect unrelated to receptor block, as well as an open channel blocking action, which is effective only at high concentrations.

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