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VOLTAGE-DEPENDENT BLOCK OF LOCUST MUSCLE GLUTAMATE CHANNELS BY CHLORISONDAMINE

BY M. L. J. ASHFORD*, P. BODEN†, R. L. RAMSEY, M. S. P. SANSOM, H. SHINOZAKI‡ AND P. N. R. USHERWOOD

Department of Zoology, University of Nottingham, University Park, Nottingham NG7 2RD

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SUMMARY

Chlorisondamine reversibly reduced the amplitude of the neurally evoked twitch of locust retractor unguis muscle in a dose-dependent manner and also blocked agonist-induced contractions. Depolarizations elicited by ionophoresis of L-glutamate were also reduced in amplitude by chlorisondamine, but there was no effect of this drug on desensitization. Neurally evoked synaptic currents were reduced in amplitude, their rise time was decreased and their decay phase made biphasic (or more complex) by chlorisondamine ($>10^{-5}$ mol l^{-1}). These effects of chlorisondamine were voltage-dependent, such that at very hyperpolarized potentials (−120 mV and −140 mV) there was an apparent reduction in the degree of block. Single-channel studies indicate that chlorisondamine acts to block the channel both in the open and the closed form and that these actions are dependent on concentration and voltage. The actions of chlorisondamine are discussed in relation to the basic kinetic models of channel block.

INTRODUCTION

Although detailed information is available on the kinetics of drug-modified acetylcholine-activated ionic channels (Lambert, Durant & Henderson, 1983), comparable information is lacking for non-cholinergic channels such as those activated by excitatory amino acids. A well-defined channel suitable for such analysis is the glutamate receptor channel found in locust leg muscle. The properties of channels gated by junctional and extrajunctional L-glutamate D-receptors of insect muscle (Usherwood & Cull-Candy, 1974) have been studied using the techniques of

* Present address: Department of Pharmacology, University of Cambridge, Hills Road, Cambridge.

† Present address: Parke-Davis Research Unit, Addenbrooke's Hospital Site, Hills Road, Cambridge.

‡ Present address: Tokyo Metropolitan Institute of Medical Science, Bunkyo-Ku, Tokyo 113, Japan.

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noise analysis (Anderson, Cull-Candy & Miledi, 1976) and single-channel recordings (Patlak, Gration & Usherwood, 1979; Gration & Usherwood, 1980; Cull-Candy, Miledi & Parker, 1981; Ashford *et al.* 1984*b*,*c*; Kerry *et al.* 1987*a*). Recent studies have shown that certain drugs, especially those bearing a net positive charge, block these cationic channels. Yamamoto & Washio (1979, 1983) reported that (+)-tubocurarine causes a voltage-dependent block of postjunctional glutamate receptor channels of mealworm (*Tenebrio molitor*) muscle when the channels are open, a finding subsequently confirmed by Cull-Candy & Miledi (1983) for locust muscle. These studies on synaptic currents have recently been extended to single extrajunctional D-receptor channels by Kerry *et al.* (1985, 1987*b*). Tubocurarine blocks this channel in a concentration- and voltage-dependent fashion with a low unblocking rate ($1{\cdot}53\times10^{-2}\,ms^{-1}$). Streptomycin also postsynaptically blocks glutamatergic synapses on locust muscle and shortens the life-time of the channel gated by extrajunctional glutamate receptors (GluR) (Gration & Usherwood, 1980). However, charged drugs do not always act only as open-channel blockers in this system. Trimetaphan, in addition to being an open-channel blocker, also facilitates desensitization of locust muscle GluR (Ashford *et al.* 1987).

In this paper we describe the effects of chlorisondamine (a diquaternary amine tetrachloroisoquinoline) on locust nerve–muscle junctions and on extrajunctional glutamate D-receptor channel complexes studied using a variety of experimental approaches, including single-channel recordings. Previous investigations have shown that this compound blocks the open channels gated by GluR on the postjunctional membrane of crayfish (Shinozaki & Ishida, 1983), lobster and crab muscle (Lingle, Eisen & Marder, 1981) and that this block is voltage-dependent. In addition, it has been suggested that chlorisondamine can induce a stable, closed but blocked state of acetylcholine receptor (AChR) channels of crustacean muscle (Lingle, 1983*a*) and amphibian acetylcholine receptor channels (Neely & Lingle, 1986). In the present study we show that chlorisondamine transiently blocks the open channel gated by GluR of locust muscle but that the voltage-dependence of this block is the reverse of that expected for a positively charged drug. In addition, we present evidence for a second, possibly more significant, effect of chlorisondamine on the locust GluR channel which may be interpreted as closed-channel block. A brief account of this work has been published elsewhere (Ashford *et al.* 1984*a*).

MATERIALS AND METHODS

Retractor unguis and extensor tibiae nerve–muscle preparations from two species of adult locust (*Schistocerca gregaria* and *Locusta migratoria*) were used (Usherwood & Machili, 1968; Clark, Gration & Usherwood, 1979; Ashford *et al.* 1987). Similar results were obtained from the two species. The composition of the saline was (in $mmol\,l^{-1}$): NaCl, 180; KCl, 10; $CaCl_2$, 2; Hepes, 10; pH 6·8. For focal recording of excitatory postsynaptic potentials (EPSPs) and voltage-clamped excitatory postsynaptic currents (EPSCs), the calcium concentration was reduced to $1{\cdot}5\,mmol\,l^{-1}$ and magnesium chloride was added until the amplitude of the

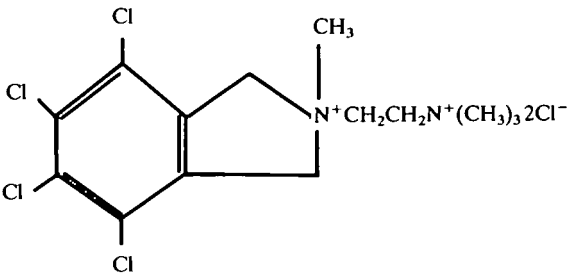


Fig. 1. The structure of chlorisondamine chloride.

postsynaptic response was reduced to 10–15 mV (final $MgCl_2$ concentration was 25–35 mmol l^{-1}). Improvement in the quality of the voltage clamp was obtained by replacing the NaCl in standard locust saline with sodium isethionate (isethionate saline). Chlorisondamine chloride (Fig. 1) was dissolved directly in the locust saline. Details of the voltage-clamp and ionophoresis procedures have been described previously (Ashford *et al.* 1987).

In all experiments in which single-channel currents were recorded, the extensor tibiae muscles were pretreated with 3×10^{-6} mol l^{-1} concanavalin A for 30 min to block D-receptor desensitization (Mathers & Usherwood, 1976, 1978; Patlak *et al.* 1979). Patch-clamp electrodes were made using a modified vertical microelectrode puller and the two-step process described by Hamill *et al.* (1981). The electrodes were filled with isethionate saline containing 10^{-4} mol l^{-1} sodium L-glutamate either alone or with the test concentration of chlorisondamine. The drug was not added to the bathing solution. Fresh pipettes were used for each concentration of drug. In each experiment the muscle fibre was voltage-clamped with a conventional two-microelectrode clamp, and channel recordings were made from extrajunctional D-receptors only (H-receptor-mediated chloride conductances were not observed). Single-channel currents were recorded with a bandwidth of d.c. to 3 kHz and stored on tape using an analogue recorder (Racal Store 4DS). All experiments were undertaken at room temperature (21–24°C). Values quoted in the text and Tables represent means ± S.E.M.; the differences between means were analysed by an unpaired Student's *t*-test ($P < 0{\cdot}05$ taken as significant).

Data analysis

Analogue records were fed into a PDP 11/34 computer from the tape recorder, digitized with a sample frequency of 20 kHz and stored on disk. Each event was displayed on a Tektronix graphics terminal. A set of x–y cursors was used to measure the relevant parameters, i.e. rise time, amplitude and decay time of EPSP and EPSC. Details of the measurement procedures are presented by Ashford *et al.* (1987).

Single-channel parameters were determined by an automated procedure using a window comparator and a PDP 11/34 computer (for details see Gration *et al.* 1982; Kerry *et al.* 1987*a*).

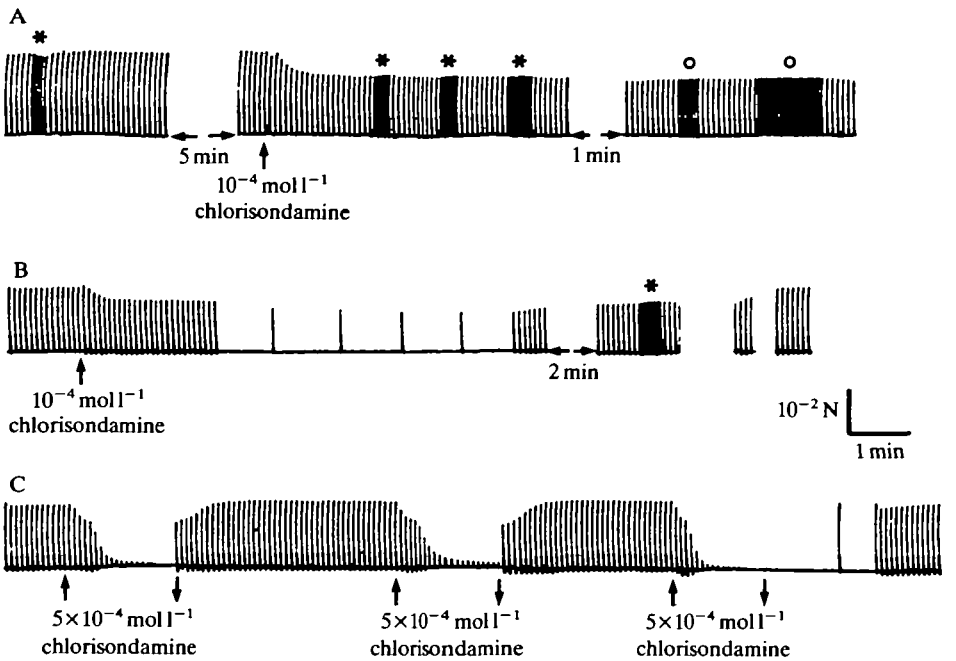


Fig. 2. Depression of neurally evoked twitch contractions of locust retractor unguis muscle by chlorisondamine. The drug was dissolved in locust saline and superfused over the preparation. (A) 10^{-4} mol l^{-1} chlorisondamine reduced the amplitude of the twitch by about 30% when the retractor unguis nerve was stimulated maximally at 0·2 Hz. An increase in stimulation frequency to either 4 Hz (○) or 6 Hz (•) produced a slight but consistent recovery of the twitch; in the absence of drug the twitch amplitude was reduced slightly at 6 Hz (•). If the preparation was rested in the chlorisondamine-containing saline for a brief period (approx. 1 min) then the first few twitches after the rest period were reduced slightly in amplitude compared with twitches before the rest period. The effect of stimulation frequency on the twitch amplitude in drug-containing saline is better illustrated in B which shows recordings from a second preparation. 10^{-4} mol l^{-1} chlorisondamine again reduced the twitch amplitude by about 30%. There was a further reduction when the stimulation frequency was lowered to about 1 stimulus min^{-1}. The twitch amplitude recovered slightly when the stimulation frequency was raised again to 0·2 Hz and there was a further slight increase when it was raised to 6 Hz (•). Recovery of the twitch was complete within 1 min following removal of the drug (final two traces shown in B). (C) 5×10^{-4} mol l^{-1} chlorisondamine almost completely abolished the twitch, which recovered very rapidly upon removal of the drug, and was not dependent upon stimulation of the preparation throughout the recovery period.

RESULTS

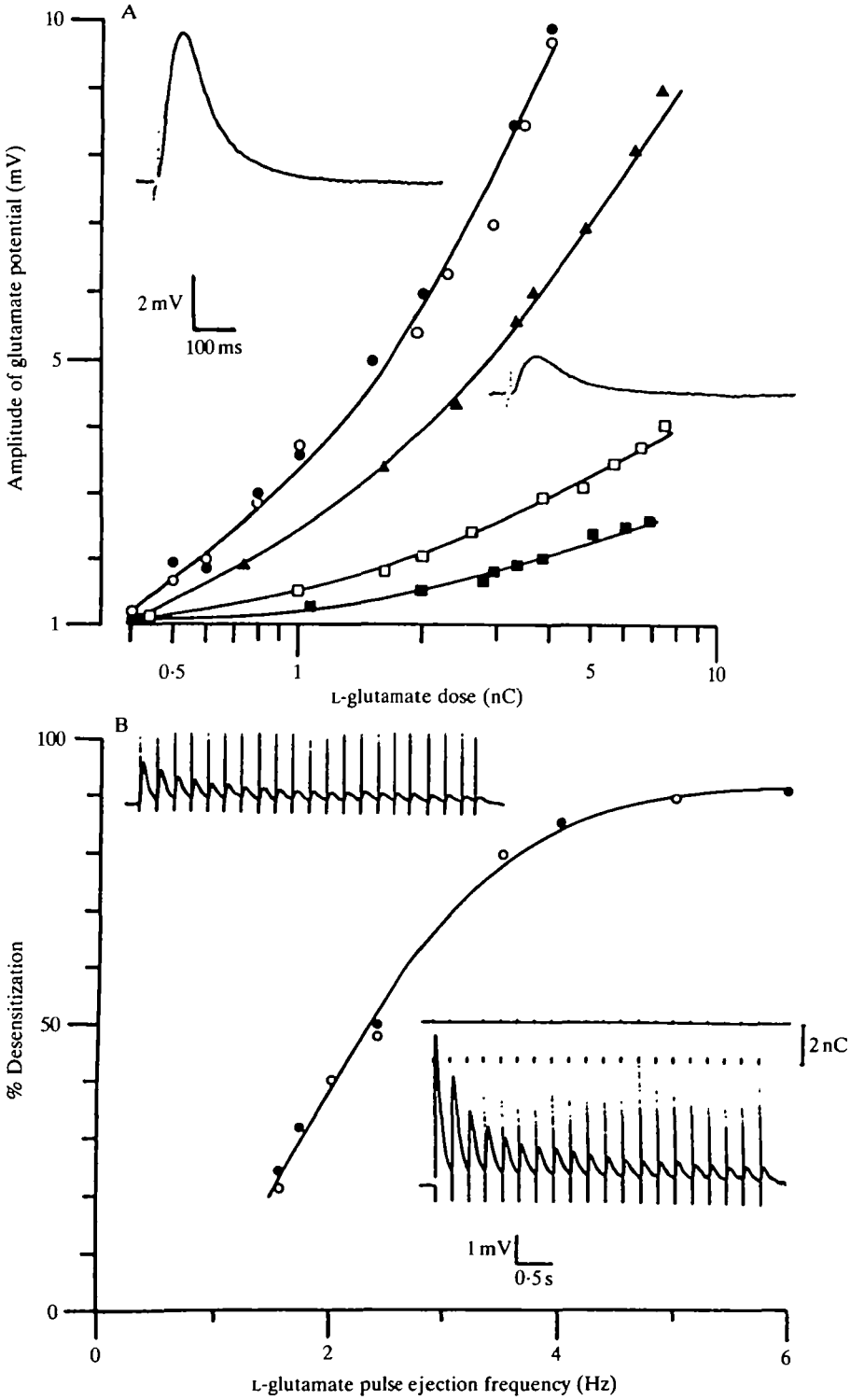
At concentrations $>5\times10^{-5}$ mol l^{-1} chlorisondamine reduced the amplitude of the neurally evoked twitch contraction of locust retractor unguis muscle (Fig. 2A); at 5×10^{-4} mol l^{-1} the twitch was almost abolished (Fig. 2C). Somewhat surprisingly, for a drug which is thought to block open cationic channels, a small increase in twitch amplitude was observed when the stimulation frequency was increased (from 0·2 Hz

to 6 Hz) (Fig. 2B). Fig. 2 also shows that a major part of the inhibition of the twitch by this drug was independent of recent stimulation history. Chlorisondamine (5×10^{-4} mol l^{-1}) completely blocked the response of the retractor unguis muscle to bath-applied L-quisqualic acid (10^{-4} mol l^{-1}) and L-glutamatic acid (10^{-4} mol l^{-1}), both of which act postsynaptically in this system (Usherwood & Machili, 1968; Usherwood & Cull-Candy, 1974). The rate of recovery of the twitch following removal of chlorisondamine from the muscle bath was the same whether or not the muscle was stimulated repetitively throughout the wash-off period (Fig. 2C).

Ionophoretic studies

Even at concentrations as high as 10^{-3} mol l^{-1}, chlorisondamine had no effect on the resting potential and input conductance of locust extensor tibiae muscle, but at concentrations $\geq10^{-6}$ mol l^{-1} it reduced the amplitude of depolarizations (glutamate potentials) elicited by ionophoretic application of L-glutamate to single excitatory junctional sites on this muscle. The effect of chlorisondamine on the glutamate potential was dose-dependent; a 50% reduction in amplitude being obtained with about 3×10^{-5} mol l^{-1} chlorisondamine (Fig. 3A). With 10^{-4} mol l^{-1} chlorisondamine the glutamate potential was reduced in amplitude by about 80%, but its time course either remained unchanged (e.g. Fig. 3A inset) or exhibited a slight prolongation (data not shown). Qualitatively similar results were obtained by Lingle *et al.* (1981) and Shinozaki & Ishida (1983) for the action of chlorisondamine on glutamate potentials of crustacean excitatory nerve–muscle junctions. The differing sensitivities of the neurally evoked twitch response and the ionophoretic glutamate response to chlorisondamine may be accounted for by inaccessibility to the drug of the junctions deep within the muscle.

Successive depolarizations elicited by ionophoretic pulse application of L-glutamate to an excitatory junction decline in amplitude owing to desensitization when the pulse repetition frequency is >2 Hz. The onset rate of desensitization, determined by using trains of ionophoretic pulses, is dependent upon the amount of glutamate applied per ionophoretic pulse and the pulse ejection frequency (Clark *et al.* 1979). Chlorisondamine (50 μmol l^{-1}) had no effect on the desensitization onset rate (Fig. 3B) (see also Lingle *et al.* 1981). Clark *et al.* (1979) and Anis, Clark, Gration & Usherwood (1981) have shown that recovery from desensitization is usually an exponential process, which is independent of the level of desensitization induced by glutamate. Chlorisondamine at a concentration of 50 μmol l^{-1} had no effect on the rate of recovery from desensitization (Fig. 3C) of the postjunctional GluR at superficially located nerve–muscle junctions (Mathers & Usherwood, 1976, 1978). Pretreatment of a locust muscle preparation with concanavalin A (2×10^{-6} mol l^{-1}) blocked desensitization but did not influence inhibition of the glutamate potential by chlorisondamine at these sites. These results indicate that chlorisondamine does not influence desensitization of locust muscle GluR. Studies of the effects of this drug on ionophoretic glutamate responses of crab and lobster muscle (Lingle *et al.* 1981) and of crayfish muscle (Shinozaki & Ishida, 1983) have led to the same conclusion.


A
10
5
1
Amplitude of glutamate potential (mV)
2 mV
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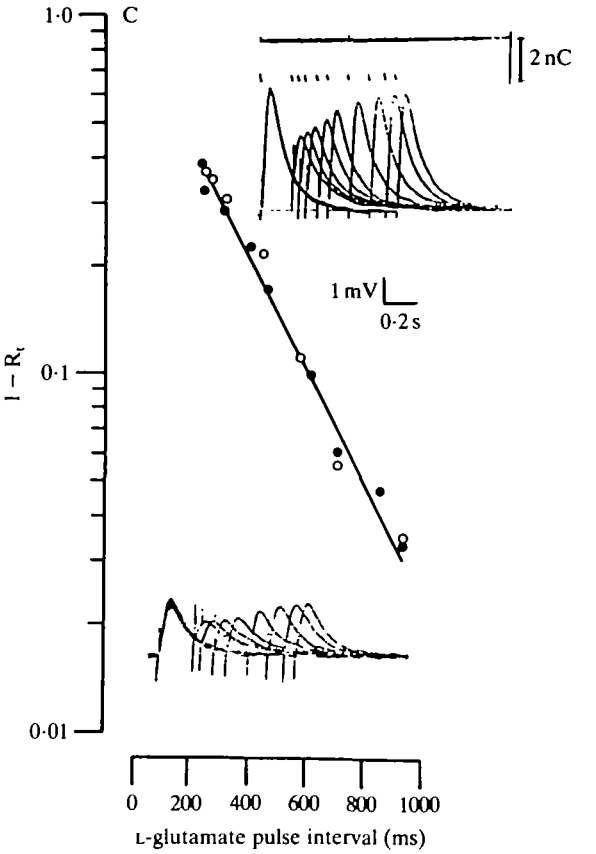


Fig. 3. (A) Effect of chlorisondamine (▲, 2×10^{-5} mol l^{-1}; □, 5×10^{-5} mol l^{-1} and ■, 10^{-4} mol l^{-1}) on the dose–response relationship for L-glutamate ionophoresis at a superficial nerve–muscle junction on an extensor tibiae muscle fibre. The dose–response relationship in drug-free saline before chlorisondamine application (○) was similar to that recorded 30 min after termination of the chlorisondamine treatment (●). L-glutamate was pulsed onto the nerve–muscle junction at a frequency of 0·1 Hz. Solid lines through data points drawn by eye. Inserts show glutamate potential in the absence (top) and in the presence of 5×10^{-5} mol l^{-1} chlorisondamine during application of 2 nC of L-glutamate. (B) Effect of chlorisondamine on the onset of desensitization at a superficial nerve–muscle junction studied by applying trains of glutamate pulses; pulse frequencies in a train were varied between 1·5 and 6 Hz. Desensitization onset in drug-free saline (○) is compared with that seen in 5×10^{-5} mol l^{-1} chlorisondamine (●). Ordinate, % desensitization (given by the peak amplitude of the tenth glutamate potential in a train expressed as a percentage of that of the first potential in the train); abscissa, frequency at which glutamate potentials were elicited. Inserts show trains of glutamate potentials in the absence (bottom) and in the presence of drug (top). Identical pulses of L-glutamate to those monitored in the upper trace of the bottom inset were applied in the absence and presence of drug. (C) Recovery from desensitization in drug-free saline (○) and in saline containing 5×10^{-4} mol l^{-1} chlorisondamine (●) observed by examining the influence of varying the interval between a conditioning glutamate potential and a subsequent test potential on the relative amplitudes of the two responses ($1 - R_t$). The rates of recovery from desensitization were identical in both cases. Line fitted by eye. Inserts are examples of data obtained in the absence (top) and presence (bottom) of drug, which were used to construct the graph. Identical pulses of L-glutamate (monitored in upper trace of top inset) were applied in the presence and absence of drug.

Table 1. *The effects of chlorisondamine on the rise times of extracellularly recorded EPSPs*

Experiment	Treatment	Rise time (ms)
1	Control	0·97 ± 0·04
	2×10^{-4} mol l^{-1} chlorisondamine	0·76 ± 0·03*
2	Control	0·86 ± 0·01
	2×10^{-4} mol l^{-1} chlorisondamine	0·69 ± 0·01*

Between 20 and 25 currents were analysed to obtain each value.
Values are mean ± S.E.; *$P < 0·001$ when compared with control data.

The effects of chlorisondamine on neurally evoked synaptic events

Extracellular, focally recorded synaptic potentials

The amplitudes of extracellular EPSPs and miniature EPSPs, recorded at a muscle membrane potential of about −60 mV, were reduced by chlorisondamine, such that at drug concentrations $\geq10^{-4}$ mol l^{-1} miniature EPSPs were difficult to distinguish from noise. The EPSP rise time (20 to 80 % peak EPSP amplitude) decreased by about 20 % in 2×10^{-4} mol l^{-1} drug (Table 1) and its decay (80 % to 20 % peak EPSP amplitude) usually became biphasic, with an early fast phase, which was faster than the control decay, and a late slow phase, which was slower than the control decay (see Fig. 7). However, the decays of many EPSPs recorded in drug-containing saline were complicated by the presence of a 'shoulder' (see below).

Voltage-clamp studies

The amplitude of the EPSC (recorded intracellularly under voltage-clamp) was linearly related to membrane potential with an extrapolated reversal potential of +4 ± 1·9 mV (S.D.; $N = 6$) (Fig. 4A) (see also Anwyl & Usherwood, 1974; Ashford *et al.* 1987). Chlorisondamine (5×10^{-5} mol l^{-1}) depressed the EPSC amplitude (Fig. 4A). The relationship between EPSC amplitude and membrane potential was distinctly non-linear when chlorisondamine was present in the muscle bath, with the drug being less effective at hyperpolarized membrane potentials (Fig. 4A). The effectiveness of chlorisondamine in depressing the EPSC can be expressed in terms of $\Lambda - 1$, where Λ is the ratio of EPSC amplitude in the absence of chlorisondamine to that in its presence (see Ascher, Large & Rang, 1979). $\Lambda - 1$ is clearly dependent upon drug concentration (Fig. 4B). Its relationship to membrane potential is less clear, although with 5×10^{-4} mol l^{-1} chlorisondamine it was significantly reduced in value when the muscle was hyperpolarized beyond −80 mV.

In the absence of chlorisondamine the EPSC rise time (20 % to 80 % peak EPSC amplitude) varied between 0·6 and 1 ms (data from five preparations) and showed

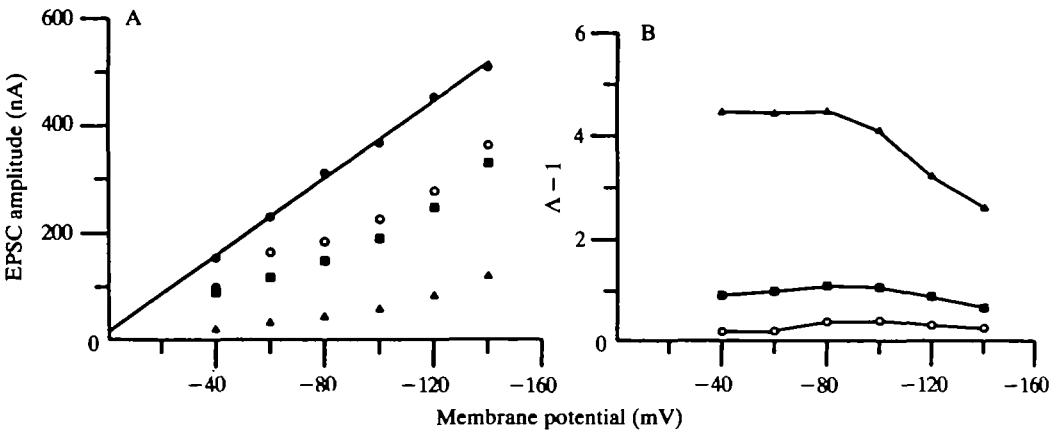


Fig. 4. (A) Dependence of the EPSC amplitude on membrane potential in the absence (●) and presence of chlorisondamine at concentrations of 5×10^{-5} mol l^{-1} (○); 10^{-4} mol l^{-1} (■) and 5×10^{-4} mol l^{-1} (▲). The straight line drawn through the control data points was fitted by regression analysis. All points in this and in subsequent figures are the mean ± S.E.M. (shown only where bigger than symbol) of at least 20 currents for each potential. (B) The voltage-dependence of the effectiveness of the drug (expressed as $\Lambda - 1$, see text) for three concentrations of chlorisondamine; 5×10^{-5} (○), 10^{-4} (■) and 5×10^{-4} mol l^{-1} (▲). Note that hyperpolarization beyond −80 mV generally reduced the effectiveness of the drug and this was especially evident for the highest concentration tested.

little voltage-dependence. At the muscle resting potential of −60 mV, chlorisondamine (5×10^{-6} mol l^{-1}) reduced the rise time in a concentration-dependent manner (Fig. 5A). Hyperpolarization of the muscle resulted in a further reduction in rise time for drug concentrations $\leq10^{-5}$ mol l^{-1} (data not shown), but for high concentrations the rise time returned towards the control value when the membrane potential was increased (Fig. 5B). The decay (80 % to 20 % peak EPSC amplitude) of the voltage-clamped EPSC of locust extensor tibiae muscle is usually monophasic and exponential (Anwyl & Usherwood, 1974, 1975; Ashford *et al.* 1987). When 5×10^{-5} mol l^{-1} chlorisondamine was present in the bathing medium and the extensor tibiae muscle fibre was clamped at either −40 mV or −60 mV the EPSC decay was usually biphasic, comprising an early fast phase, which was faster than the control decay, and a late slow phase, which was slower than the control decay (combined these were often >90 % of peak EPSC amplitude). However, for some EPSCs under these conditions the early phase was absent and the response decayed monophasically, with a decay time constant similar to that of the slow phase of biphasic decays. For EPSCs with biphasic decays, hyperpolarization reduced the amplitude of the fast phase relative to the slow phase (Fig. 6) such that at holding potentials ≥-100 mV all decays in chlorisondamine were monophasic (Table 2). There was no detectable voltage-sensitivity of the decay of control EPSCs, but in the presence of chlorisondamine hyperpolarization decreased the decay rate constant for monophasically decaying EPSCs and the rate constant of the slow phase of

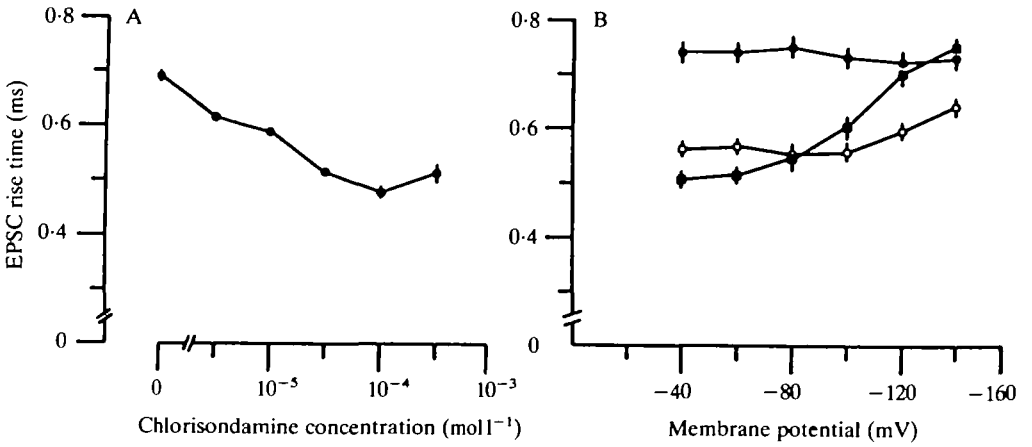


Fig. 5. (A) Dependence of the rise time of EPSCs recorded at a membrane potential of −60 mV on the concentration of chlorisondamine. Data are from a single voltage-clamp experiment. Note that the rise time is reduced significantly by low concentrations of chlorisondamine (5×10^{-6} mol l^{-1}) but that there is no further decrease in rise time above 10^{-4} mol l^{-1}. (B) Relationship between EPSC rise time and holding potential for control (●), 5×10^{-5} mol l^{-1} (○) and 10^{-4} mol l^{-1} chlorisondamine (■). At holding potentials close to the resting potential of locust muscle (approx. −60 mV) chlorisondamine reduces the rise time, but when the membrane is hyperpolarized the EPSC rise time increases towards control values.

biphasically decaying events. These observations are in agreement with the effect of hyperpolarization on the slow phase of chlorisondamine-induced biphasic decays of EPSCs recorded from crustacean muscle by Lingle *et al.* (1981). They also reported that hyperpolarization increased the rate constant for the initial fast decay phase of the biphasically decaying EPSC in these systems, but in our studies the rate constant for the fast decay phase decreased with hyperpolarization, such that the rate constants for the fast and slow phases converged at −100 mV. The membrane potential change required to produce an e-fold change in the time constant of the slow decay phase was 170·8 ± 6·5 mV (S.E.M. for seven experiments). For any given membrane potential the time constant of monophasic decays and the slow phase of biphasic decays increased with chlorisondamine concentration (Fig. 7). There was no difference between the amplitudes of EPSCs with monophasic decays and those with biphasic decays, which suggests that monophasically decaying events are not a consequence of a reduction in EPSC amplitude (i.e. simply through loss of the fast decay component).

A major complication was the appearance of 'shoulders' on the decays of some EPSCs recorded in chlorisondamine, similar to those seen in the extracellular EPSP studies. These occurred most frequently, but not exclusively, with a drug concentration of 10^{-4} mol l^{-1} and at membrane potentials between −80 and −100 mV, and complicated the EPSC decay time course (Fig. 8). With the distributed innervation which characterizes locust muscle fibres the time course of the EPSC is determined mainly by currents conducted from distant synapses. It could be argued, therefore,

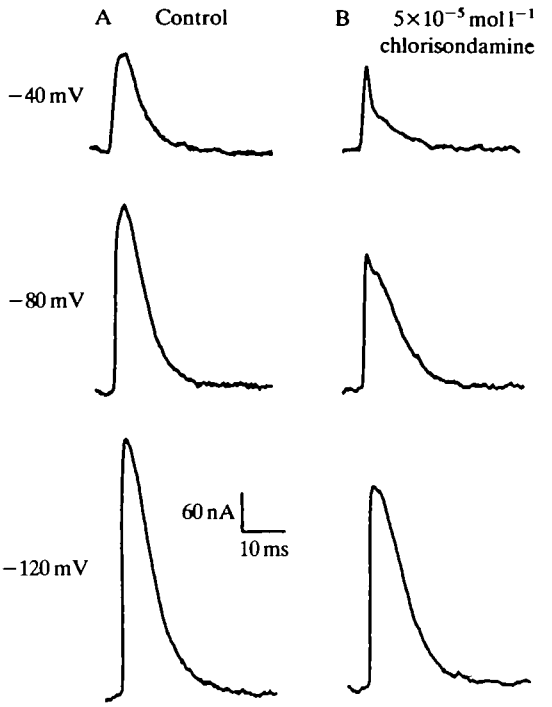


Fig. 6. Sample EPSCs recorded under voltage-clamp from a locust extensor tibiae muscle fibre at different potentials in drug-free saline (A) and in saline containing 5×10^{-5} mol l^{-1} chlorisondamine (B). The preparation was equilibrated in the drug-containing saline for 10 min before the data in B were recorded. Note the biphasic decay of the EPSC at −40 mV in chlorisondamine.

that these complex decays are artefacts introduced by the voltage-clamp recording system. However, this seems unlikely since they were never seen in the absence of chlorisondamine in either the EPSC or the EPSP experiments. In any event, the decay of the extracellular EPSP should not have been influenced by the cable properties of the fibres yet shoulders were seen on some EPSPs recorded in chlorisondamine. No shoulders could be observed on the ionophoretic responses to glutamate, nor were the responses biphasic, presumably because they lasted much longer and chlorisondamine could undergo a number of block–unblock cycles in this time. One explanation for the complex decays is that chlorisondamine acts presynaptically at locust glutamatergic nerve–muscle junctions to cause a delayed release of transmitter in addition to that which immediately follows the arrival of the nerve impulse. However, this delayed release of transmitter would have to be influenced in some way by the potential difference across the muscle fibre membrane, since the shoulders on the EPSC decays occurred with decreasing latency as the muscle fibre was hyperpolarized. [We have not investigated whether chlorisondamine acts presynaptically at locust nerve–muscle junctions, but Shinozaki & Ishida (1983) have shown that it has no effect on transmitter release at crayfish

Table 2. *Effects of membrane potential and drug concentration on the EPSC decay phase in the presence of chlorisondamine*

Chlorisondamine ($mol\,l^{-1}$)	V (mV)	I_2/I_1*	% Biphasic
5×10^{-5}	−40	0·75 ± 0·11	100
	−60	0·20 ± 0·02	45
	−80	0·15 ± 0·01	28
	−100	—	0
5×10^{-5}	−40	0·49 ± 0·04	80
	−60	0·27 ± 0·01	70
	−80	0·21 ± 0·02	35
	−100	—	0
5×10^{-5}	−40	0·56 ± 0·03	100
	−60	0·29 ± 0·02	90
	−80	0·15 ± 0·02	60
	−100	—	0
10^{-4}	−40	0·59 ± 0·03	90
	−60	0·30 ± 0·02	75
	−80	0·21 ± 0·02	30
	−100	—	0
10^{-4}	−40	0·67 ± 0·04	100
	−60	0·20 ± 0·02	45
	−80	—	0

I_1 and I_2 are the relative amplitudes of the slow and fast components, respectively, of the EPSC decay.

The percentage of biphasically decaying events was determined from at least 25 EPSCs at each membrane potential.

* Mean ± S.E.

nerve–muscle junctions.] An alternative explanation is that in addition to blocking the open GluR channel chlorisondamine also blocks the GluR channel in its closed state (i.e. in the absence of transmitter) and that reversal of this block is accelerated by the nerve–muscle transmitter (in a manner similar to that for block by chlorisondamine on open acetylcholine-activated channels as described by Neely & Lingle, 1986). This implies that the shoulders on the EPSC decay are secondary postsynaptic conductance increases during synaptic transmission which result from transmitter-induced unblocking of GluR channels.

Single-channel studies of extrajunctional GluR

The GluR of extrajunctional membrane of locust leg muscle exhibits complex kinetic properties (Ashford *et al.* 1984*b*,*c*; Kerry *et al.* 1987*a*). Analysis of channel dwell-time distributions has revealed a minimum of three open states and four closed states when the channel is gated by $10^{-4}\,mol\,l^{-1}$ L-glutamate (Fig. 10A,B), and autocorrelation analysis has indicated at least three pathways linking the open and

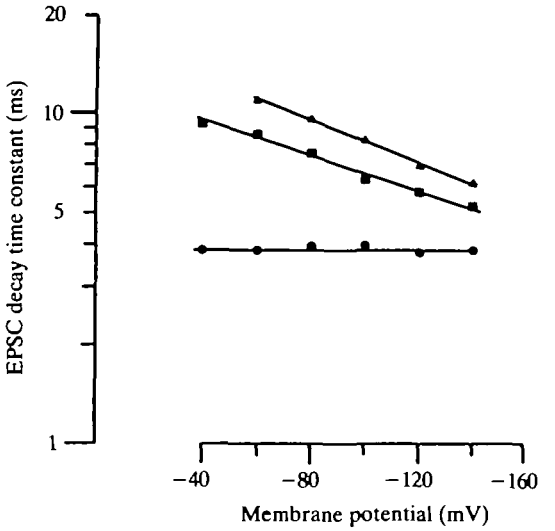


Fig. 7. Dependence of the time constant of decay of EPSC on membrane potential in the drug-free saline (●) and in the presence of 10^{-4} (■) and 5×10^{-4} mol l^{-1} (▲) chlorisondamine. The lines are fitted by regression analysis, and the slope values are 167·3 mV (■) and 136·3 mV (▲). All points are the means ± S.E.M. (bars are generally smaller than the symbols). Between 20 and 25 currents were analysed for each potential, all of which exhibited monophasic decays.

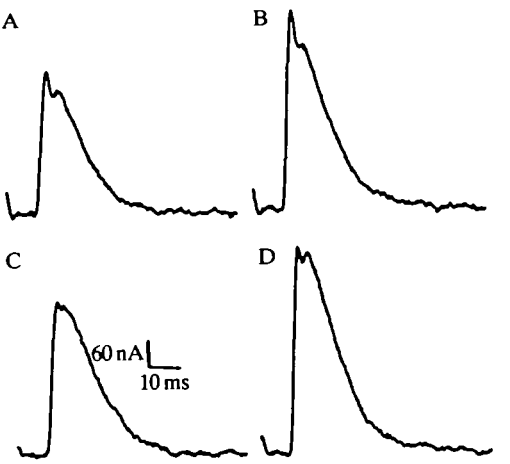


Fig. 8. Sample EPSCs recorded in saline containing 5×10^{-5} mol l^{-1} chlorisondamine (A,B,D) and 10^{-4} mol l^{-1} chlorisondamine (C) at clamped membrane potentials of −80 mV (A,B), −100 mV (C) and −120 mV (D) from locust extensor tibiae muscle fibres, showing complex decays.

closed states. Our preliminary studies of the effects of chlorisondamine on the GluR channel have been restricted to two drug concentrations (5×10^{-5} and 10^{-4} mol l^{-1}) and membrane potentials of -100 and -140 mV. Kerry *et al.* (1987*a*) have indicated that at least 10 000 channel openings and closings, preferably from a single GluR, are required to obtain a good account of the kinetics of the GluR channel for a given agonist concentration. In the present studies, this would have entailed recording 10 000 channels for each concentration of chlorisondamine and for each muscle fibre holding potential. Unfortunately since such an heroic undertaking was not possible (see also Kerry *et al.* 1987*b*), only a limited quantitative evaluation of our single-channel studies with chlorisondamine has been attempted. Nevertheless, a semi-quantitative view of the action of this drug on the extrajunctional D-receptor channel of locust muscle has been obtained which confirms and clarifies the information that was collected in macrosystem studies.

Fig. 9 compares recordings of single GluR channels in the presence and absence of 5×10^{-5} mol l^{-1} chlorisondamine. With drug in the patch pipette in addition to 10^{-4} mol l^{-1} L-glutamate, channel openings appeared as bursts of short pulses suggestive of open-channel block with high blocking and unblocking rates (Neher & Steinbach, 1978). We have not determined whether the duration of the channel in its open state (including the blocked times) is increased, as would be predicted by the open-channel block model, although our subjective impression is that this is likely. Despite the appearance of these bursts of openings, the frequency (f_o) of channel openings fell below the level normally expected for 10^{-4} mol l^{-1} L-glutamate (Table 3). The differences between recordings in the presence and absence of drug are reflected in the respective dwell-time (open and closed) distributions (Fig. 10). Fig. 10 compares the open-time distributions, obtained at a holding potential of -100 mV, in the absence of drug (Fig. 10A) and in the presence of 5×10^{-5} mol l^{-1} chlorisondamine (Fig. 10C) and 10^{-4} mol l^{-1} chlorisondamine (Fig. 10E). With chlorisondamine (5×10^{-5} mol l^{-1}) in the patch pipette the distribution contains two rather than three components, resulting from the loss of long openings and it is also characterized by a higher proportion of brief openings compared with the control distribution (Fig. 10A). The calculated mean open time (m_o) of the GluR channel fell dramatically in 5×10^{-5} mol l^{-1} chlorisondamine (Table 3), presumably because of the disruption of channel openings by what are probably transient blocking events. In addition to reducing f_o, 5×10^{-5} mol l^{-1} chlorisondamine reduced the percentage open time (%OT) (Table 3). These results cannot be explained by rapid block and unblock of the open channel alone. A more plausible explanation is that, in addition to blocking the open channel, chlorisondamine binds to the closed GluR channel either at its glutamate binding site(s) (competitive antagonism) or at a site which prevents gating of the channel (closed-channel block). The studies reported earlier in this paper suggest that the latter explanation is the more likely. The closed time distribution in 5×10^{-5} mol l^{-1} chlorisondamine (Fig. 10D) shows an increased proportion of brief events compared with the distribution obtained in the absence of drug (Fig. 10B), although the apparent rate constant (σ) for this component (β_1) of the distribution is smaller than that of controls (Table 4). The closed-time

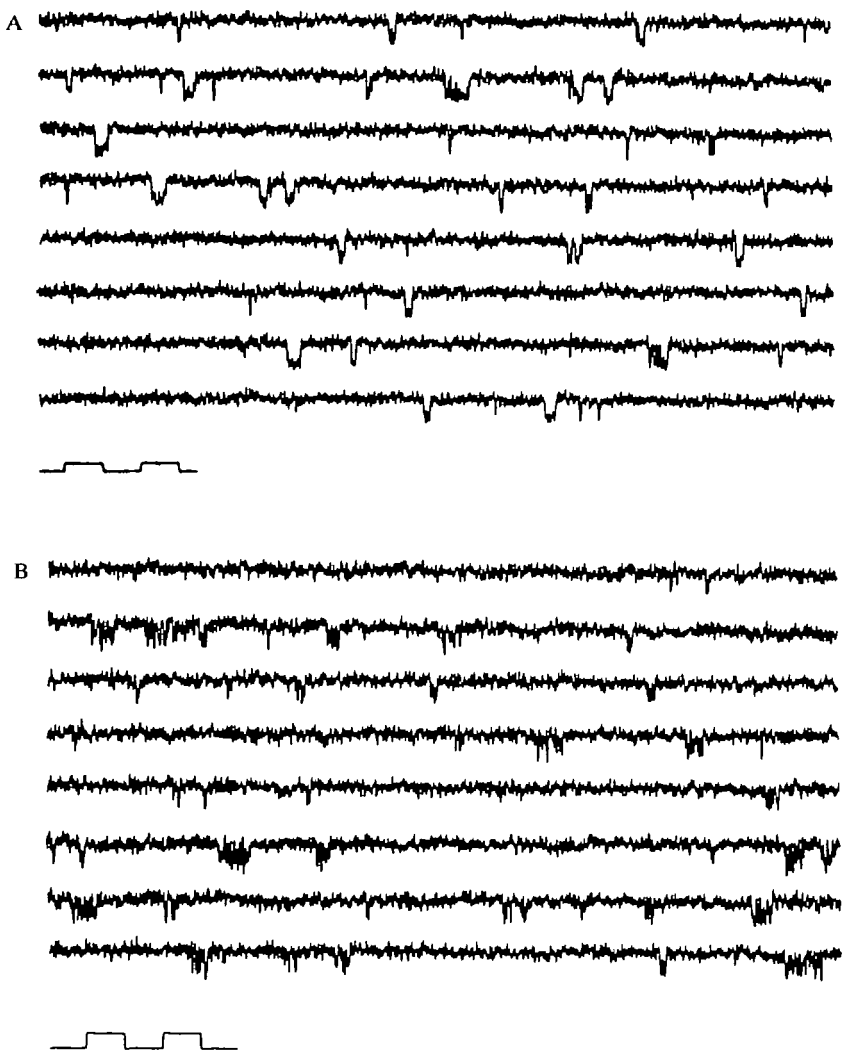


Fig. 9. Single-channel records of glutamate-gated currents recorded from locust extensor tibiae muscle fibres. In A the patch pipette contained 10^{-4} mol l^{-1} sodium L-glutamate. In B the pipette contained 10^{-4} mol l^{-1} glutamate plus 5×10^{-5} mol l^{-1} chlorisondamine. Note the presence of many more brief closings during gating of channels in B compared with A, presumably caused by rapid block and unblock of open channels by chlorisondamine. The muscle fibres were voltage-clamped at −100 mV. Data were filtered at 3 kHz. Calibrations for both A and B are 10 pA and 10 ms; also note the reduction in the ampitude of the single-channel currents in the presence of the drug (B).

distribution in the presence of drug also exhibits an increase in the proportion of long closings. Chlorisondamine also increased the calculated mean closed time (m_c).

When the concentration of chlorisondamine in the patch pipette was raised to 10^{-4} mol l^{-1}, m_c and f_o remained depressed but m_o and %OT increased. In the case of m_o this was to a value somewhat in excess of that obtained in the absence of

Table 3. *Summary of single-channel data recorded from GluR in the absence (10^{-4} mol l^{-1} sodium L-glutamate alone) and presence (with 10^{-4} mol l^{-1} sodium L-glutamate) of chlorisondamine*

Chlorisondamine concentration (mol l^{-1})	Mean open time (ms)	Mean closed time (ms)	Open channel probability (%)	Open channel frequency (s^{-1})
0	1·20	18·0	$6·3\times10^{-2}$	52·1
5×10^{-5}	0·33	51·2	$6·4\times10^{-3}$	19·4
10^{-4}	1·53	52·0	$2·9\times10^{-2}$	18·7

All of the data were obtained at a holding potential of −100 mV and are not corrected for loss of brief openings and closings (see Kerry *et al.* 1987*a*).

chlorisondamine (Table 3). At first sight, this could be interpreted to mean that an increase in drug concentration decreases the probability of open-channel block. However, these changes in m_o and %OT were accompanied by an apparent fall in the conductance of the open GluR channel (about 30 % down compared with controls) (Fig. 9). This conductance decrease would be expected if the open-channel blocking and unblocking rates in 10^{-4} mol l^{-1} chlorisondamine were so high that the resulting blocking transients could no longer be resolved. Such a change would also account for the slight increase in m_o beyond its control value. If the brief blocking transients during channel openings were not resolved it is not surprising that the open-time distribution for 10^{-4} mol l^{-1} chlorisondamine (Fig. 10E) was not greatly different from controls (Fig. 10A). However, the closed-time distribution (Fig. 10F) in 10^{-4} mol l^{-1} chlorisondamine differed from controls (Fig. 10B) by the presence of longer than normal closing events, which confirms our view that open-channel block is not the only mode of action of chlorisondamine on locust muscle GluR. The presence of long closings in 10^{-4} mol l^{-1} chlorisondamine probably explains why %OT remained below control values. A quantitative account of GluR channel open- and closed-time distributions in the presence and absence of chlorisondamine is presented in Table 4.

The voltage-dependence of GluR channel kinetics was studied qualitatively in the presence of 5×10^{-5} mol l^{-1} chlorisondamine over the voltage range −100 mV to −140 mV. In the absence of drug, the kinetics were not influenced by membrane potential. However, with chlorisondamine in the patch pipette along with L-glutamate, hyperpolarization beyond −100 mV apparently reduced the proportions of brief open and closed events and the calculated mean open and closed times were shifted towards control values.

More information is required before it will be possible to present a quantitative account of the long channel closed times recorded in chlorisondamine. Although it seems reasonable to propose that they represent block times associated with closed-channel block of GluR we cannot comment on their dependence on chlorisondamine concentration or on their quantitative relationship to membrane potential.

DISCUSSION

It is rarely possible to gain insight into the molecular basis for the action of drugs on a nerve–muscle system by studying their effects on the neurally evoked muscle twitch. However, the results of our studies of chlorisondamine action on the twitch contraction of locust leg muscle are illuminating in a number of respects. For example, they show clearly that if this drug blocks the open channel gated by postjunctional GluR, as one might anticipate from previous studies of chlorisondamine action on arthropod muscle GluR (Lingle *et al.* 1981; Shinozaki & Ishida, 1983), then the unblocking rate for this process must be high, since repetitive stimulation of the muscle did not lead to a progressive run-down in twitch amplitude. A similar argument must be invoked to account for the lack of interaction between successive glutamate potentials evoked during ionophoresis of glutamate at single nerve–muscle junctions on extensor tibiae muscle fibres. In terms of its proposed channel unblocking rate chlorisondamine would clearly differ from other open-channel blocking drugs recently tested on locust nerve–muscle preparations, such as δ-philanthotoxin (Clark *et al.* 1982) and argiotoxin (636) (Usherwood, 1986). The slight recovery of the twitch amplitude recorded in chlorisondamine when the stimulus frequency was raised was unexpected. In isolation this observation could be explained on the basis of one or more presynaptic or postsynaptic actions of the drug, but in conjunction with other data reported here it adds weight to the conclusion that chlorisondamine blocks the closed GluRs and that this can be reversed by transmitter (L-glutamate). Although the results of our ionophoretic studies give only a qualitative appreciation of chlorisondamine action they support the idea that chlorisondamine is a channel blocker by showing clearly that it antagonizes the junctional GluR channel complex of locust muscle and that most, if not all, of this antagonism is non-competitive.

A better insight into the site and mode of action of chlorisondamine is provided by studies of the voltage-clamped EPSC. The drug-induced reduction in amplitude of this current is compatible with closed-channel block, although if this is true then the voltage-dependence of this change requires an explanation. Despite the problems of interpreting changes in EPSC amplitudes in muscle fibres with distributed innervations, the possibility that chlorisondamine also blocks the open GluR channel is supported by the observed reduction in EPSC rise time and the appearance of biphasic decays when this drug was introduced into the bathing medium. The appearance of biphasic decays is usually explained by the sequential model for open-channel block first proposed by Adams (1976, 1977):

$$\mathrm{A} + \mathrm{C} \underset{a}{\overset{b}{\rightleftharpoons}} \mathrm{O} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (\mathrm{O})\mathrm{B}\,,$$

where A is agonist, and C, O and O(B) represent the closed, open and blocked states of the receptor channel, respectively, b and a are the opening and closing rate constants, and k_1 and k_{-1} are the blocking and unblocking rate constants. This scheme states that the open-channel state can be terminated by two reaction

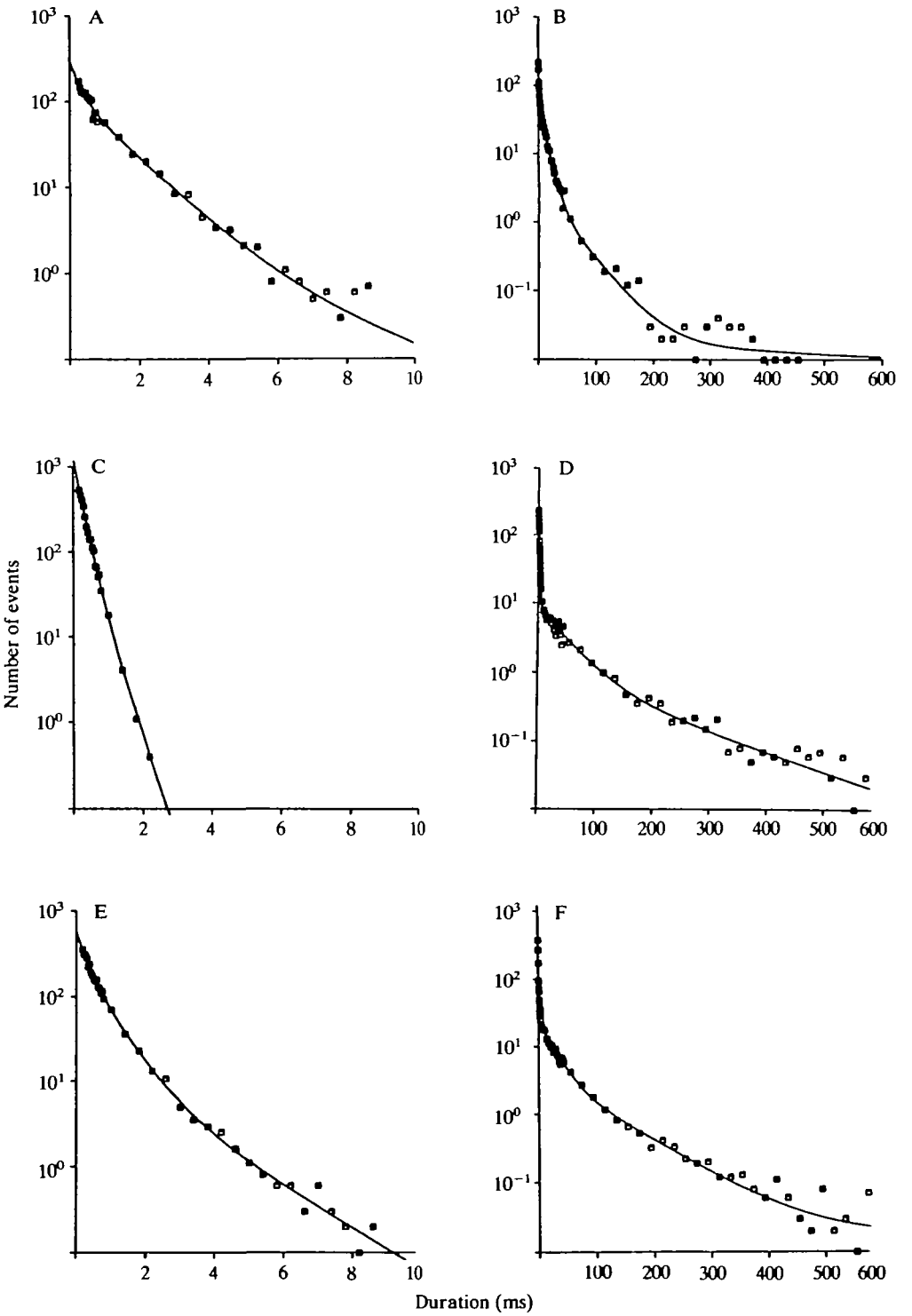


Fig. 10

pathways, i.e. through normal channel closing (with rate constant a) and through channel block (with rate constant k_1). The apparent closing rate (a') of the channel in the presence of drug and at a given membrane potential (V) is given by:

$$a' = a + k_{1(V)}\mathrm{b}\,,$$

where b is the concentration of blocker. It follows that a' should be concentration- and voltage-dependent, i.e. its values should increase exponentially with voltage and linearly with drug concentration (see Neher & Steinbach, 1978). This model proposes that positively charged drugs can enter the mouth of cationic channels, such as the GluR channel, along with the charge-carrying ions, but are unable to pass through the channel. Their presence in the channel prevents ion flow through the channel, such that the channel conductance effectively falls to zero during the block. Subsequent dissociation of the drug from its binding site is necessary before channel closure will occur (but see Lingle, 1983*a*; Neely & Lingle, 1986). It follows from this model that hyperpolarization should increase the blocking rate of the drug and hence the early fast component of decay of biphasically decaying EPSCs, since this component is thought to represent channel block. It should also increase the duration of the slow component of the decay by retarding egress of the drug from the channel. In addition, hyperpolarization should increase the amplitude of the fast decay phase relative to that of the slow phase.

Although biphasically decaying EPSCs were seen in the presence of chlorisondamine, particularly with low concentrations of drug and at membrane potentials close to the resting potential, the predictions of the open-channel block model were not borne out in our studies. For example, the fast phase decreased rather than increased in magnitude with hyperpolarization and the slow phase decreased rather than

Fig. 10. Dwell-time distributions for data presented in Table 4. A and B are the open- and closed-time distributions, respectively, of single GluR channels recorded with 10^{-4} mol l^{-1} L-glutamate in the patch pipette. These and other distributions in this figure are best fitted according to the equations in Table 4 by means of exponential functions (solid lines through data points). The open-time distribution is fitted by the sum of three exponentials, the closed-time distribution by four. The methods used for determining the number of components required are described in more detail by Kerry *et al.* (1987*a*). C and D are the dwell-time distributions for channels obtained with a patch pipette containing 10^{-4} mol l^{-1} L-glutamate and 5×10^{-5} mol l^{-1} chlorisondamine. Note loss of long open events in open-time distribution, which is best fitted by the sum of two exponentials, and the presence of abnormally long, closed events in the closed-time distribution which is fitted with three exponentials, although a fourth may be present. E and F are the open- and closed-time distributions, respectively, obtained with 10^{-4} mol l^{-1} L-glutamate plus 10^{-4} mol l^{-1} chlorisondamine. The open- and closed-time distributions are best fitted by three and four exponentials, respectively, like the control distributions, although the closed-time distribution of the drug has more long closed times than its equivalent control. All data obtained at a muscle holding potential of -100 mV and filtered at 3 kHz. The ordinates of these probability density functions are given as the number of events per 40 μs time block for the open-time distributions and number of events per 200 μs for the closed-time distributions.

Table 4. *Dwell-time distributions [A, open, $f_o(t)$; B, closed, $f_c(t)$] for GluR channels in the absence (10^{-4} mol l^{-1} sodium L-glutamate alone) and presence of chlorisondamine (with 10^{-4} mol l^{-1} sodium L-glutamate)*

$$\text{A} \qquad f_o(t) = \sum_{i=1}^{N_o} \alpha_j \lambda_j \exp(-\lambda_j t) .$$

Chlorisondamine (mol l^{-1})	N_o	j	α_j	λ_j (ms^{-1})
0	3	1	0·251 (0·44)	3·840 (2·53)
		2	0·696 (0·527)	0·887 (0·869)
		3	0·053 (0·033)	0·323 (0·294)
5×10^{-5}	2	1	0·792	5·330
		2	0·209	2·620
10^{-4}	3	1	0·327	3·980
		2	0·568	1·470
		3	0·105	0·550

$$\text{B} \qquad f_c(t) = \sum_{i=1}^{N_c} \beta_i \delta_i \exp(-\delta_i t) .$$

Chlorisondamine (mol l^{-1})	N_c	i	β_i	δ_i (ms^{-1})
0	4	1	0·178 (0·214)	1·73 (2·48)
		2	0·647 (0·373)	0·100 (0·135)
		3	0·152 (0·353)	0·024 (0·05)
		4	0·023 (0·0604)	0·001 (0·011)
5×10^{-5}	(?)	1	0·369	1·060
		2	0·308	0·032
		3	0·324	0·010
		4	(?)	(?)
10^{-4}	4	1	0·261	1·91
		2	0·391	0·044
		3	0·287	0·012
		4	0·061	0·003

Numbers in brackets are data from Kerry *et al.* (1987*a*).

Data for 10^{-4} mol l^{-1} sodium L-glutamate alone comprised 4000 events pooled from two recording sites; 5×10^{-5} mol l^{-1} chlorisondamine data comprised 4100 events from a single recording site; 10^{-4} mol l^{-1} chlorisondamine data comprised 5300 events pooled from four recording sites.

Single-channel data filtered at 3 kHz. All of the data were obtained at a holding potential of −100 mV.

Distributions are finite mixtures of exponential distributions with N_o (A) and N_c (B) components, where λ_j and δ_i are the apparent closing and opening rates, respectively, for the GluR channel and α_j, β_i are their respective mixture proportions.

(?) Insufficient data.

increased in duration as the membrane potential was increased. Perhaps these inconsistencies are not entirely unexpected since Lingle *et al.* (1981) have previously found that the time constant of the slow, late phase of biphasically decaying EPSCs of crab and lobster muscle fibres, which occur during chlorisondamine application, also declines with hyperpolarization. They suggested that the unblocking rate for the drug may increase with hyperpolarization.

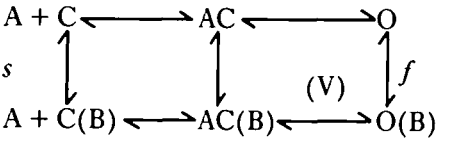
The appearance of bursts of closings during channel openings when chlorisondamine was present in the patch pipette along with L-glutamate and when the muscle fibre was clamped at a membrane potential close to its resting potential gives further support to the conclusion that this drug is an open-channel blocker of locust muscle GluR. However, the increased frequency of these blocking events which would be expected to follow an increase in drug concentration (and hyperpolarization) would be insufficient alone to cause the apparent reduction in conductance of the single channel seen in our studies with 10^{-4} mol l^{-1} chlorisondamine. There would also have to be an increase in unblocking rate for this to happen. It is generally considered unlikely that the unblocking rate of an open-channel blocker will be dependent upon drug concentration and it would be unusual to have an unblocking rate that is increased with hyperpolarization for a cationic drug. However, it is difficult to explain the apparent reduction in channel conductance and the concomitant loss of brief blocking events unless these conditions hold. Perhaps chlorisondamine passes through the GluR channel when the drug concentration and transmembrane potential are high (Ashford *et al.* 1984*a*). Although chlorisondamine is a large molecule, and as such might not be expected to pass through the GluR channel, decamethonium, which is also a large molecule, seemingly passes through the open AChR channel of vertebrate skeletal muscle (Creese & England, 1970; Milne & Byrne, 1981), and Lingle (1983*b*) invokes a similar mechanism to explain the anomalous voltage-dependence of hexamethonium on crustacean acetylcholine-induced currents.

The reduction in amplitude of twitch, EPSP, miniature EPSP and EPSC, and the long closed periods recorded in the single-channel studies when chlorisondamine was present in the patch pipette, support the idea that this drug blocks the closed GluR channel. A simple closed-channel block scheme would be:

$$\begin{array}{ccc} A + C & \rightleftharpoons & O \\ \downarrow & & \\ C(B) & & \end{array},$$

where chlorisondamine binds to the closed receptor channel to form a complex C(B), which does not allow channel gating by agonist. However, many of the data reported herein do not fit this simple scheme. For example, the increase in EPSC amplitude with hyperpolarization is one major inconsistency. Also, when the concentration of chlorisondamine was raised five-fold in the single-channel experiments %OT did not decrease as would be expected from this model. Lambert *et al.* (1983) have previously alluded to the possibility that current ideas on channel blocking may be

oversimplified. A more complex scheme, but one which qualitatively fits the data better is:



where C, O, C(B) and O(B) are the closed, open, closed-blocked and open-blocked forms of the receptor channels, respectively, and f and s are fast and slow reaction rates, respectively. According to this scheme an increase in drug concentration in the absence of agonist would increase the probability of closed-channel block, but with agonist present, especially at high membrane potentials, the equilibrium would favour open-channel block. During the EPSC there would be two routes to the open channel, one of which, that from blocked but closed channels, would exhibit a voltage-dependence, with the transition from closed- to open-channel block being favoured by high transmembrane potentials. This could account for the complex decays of the EPCSs seen in chlorisondamine and for the unusual voltage-dependent properties of these synaptic events. Unfortunately, until more information is available on the concentration- and voltage-dependence of %OT and the long closed times seen in the single-channel studies when chlorisondamine was present in the patch pipette it is not possible to comment on the likely transition rates between the proposed closed-blocked and open-blocked channel states when agonist is bound to GluR. However, to explain the reversal of the effectiveness of chlorisondamine on hyperpolarization, one would still need to invoke the possibility that the off-rate of the drug from the channel occurs *via* inward unblocking.

In conclusion, we propose that chlorisondamine blocks the open channel of the GluR of locust muscle but with an unblocking rate that is unusually voltage-dependent. Furthermore, we propose that this drug also binds to the GluR when its channel is closed, but that when agonist subsequently binds to the receptor the blocked GluR channel may convert to an open but blocked channel state in which the affinity of chlorisondamine for the receptor channel is lower.

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