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# Block of Glutamatergic Excitatory Synaptic Channels by Chlorisondamine

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SUMMARY

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The effects of chlorisondamine on glutamate-activated channels are described. Chlorisondamine reduced the amplitude of intracellularly recorded excitatory junctional potentials and of responses to iontophoretically applied glutamate. Dose-response curves indicate a noncompetitive interaction. Extracellularly recorded synaptic currents were recorded while holding muscles at different membrane potentials. Chlorisondamine reduced the peak amplitude of synaptic currents and changed the time course of decay. Analysis of these results suggests that chlorisondamine blocks the glutamate-gated channel.

Arthropod neuromuscular preparations have served as models for an excitatory conductance process thought to be mediated by the amino acid, L-glutamate (1–5). One approach to the study of mechanisms by which transmitters produce specific conductance changes is to use pharmacological tools which interfere with particular steps in the process from agonist binding to channel opening, as for cholinergic activation at vertebrate neuromuscular junctions (6–9). Unfortunately, the application of this approach to glutamatergic synapses has been limited by the relative lack of antagonists for the action of glutamate on postsynaptic membranes. Agents that are potent antagonists for the interaction of glutamate with its receptor are entirely lacking (10), but some compounds such as diltiazem (11), fluorazepam (12), pentobarbital (13), and antihistamines (14) have been reported to block glutamatergic excitation in crustacean muscles in a noncompetitive manner at $10^{-4}$ M. As yet, however, the mechanisms by which such noncompetitive antagonists reduce glutamate responses are unclear. In addition, there are some discrepancies in the action of some agents against the nerve-evoked junctional response and the glutamate-evoked response (11, 15). Thus, further study of the mechanisms of action of noncompetitive antagonists at arthropod neuromuscular junctions is required, not only to aid our understanding of mechanisms of transmitter action, but also to clarify further the role of glutamate as an excitatory transmitter.

In this paper we describe the effects of chlorisondamine, a presumed nicotinic ganglionic antagonist (16). We find that chlorisondamine produces a noncompetitive reduction of both glutamate and synaptically activated potentials in a crustacean neuromuscular preparation. Furthermore, we suggest that this effect results from a blockade of channels opened by the synaptic transmitter.

Most experiments were performed on the gm6 muscle of the foregut of the crab, *Cancer irroratus*, or the lobster, *Panulirus interruptus*. This muscle receives a single excitatory innervation mediated by glutamate, although acetylcholine receptors are distributed extrajunctionally on its muscle fibers (17). Muscles were superfused with physiological saline (17, 18) at room temperature or cooled to 12–14°. Standard electrophysiological techniques for recording membrane potential, injection of current, iontophoresis, and nerve stimulation were used. At single junctional sites, ejcs[2] were recorded with a focal extracellular electrode (5–30 μm). They were recorded on tape and averaged with an MINC-11 computer. Chlorisondamine was introduced into the muscle chamber through switches in the perfusion system.

The essential features of the action of chlorisondamine are presented in Fig. 1. Chlorisondamine produced a reduction in the amplitude of intracellularly recorded EJPs evoked by stimulation of the excitatory motor nerve to a gm6 muscle (Fig. 1A). Since the innervation to this muscle is thought to be glutamatergic, the effect of chlorisondamine on responses produced by iontophoretic applications of L-glutamate was examined. As is seen in Fig. 1A, chlorisondamine reduced the amplitude of glutamate responses. The reductions of both the EJPs and

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[2] The abbreviations used are: ejc, extracellular junctional currents; EJP, excitatory junctional potential; ACh, acetylcholine.

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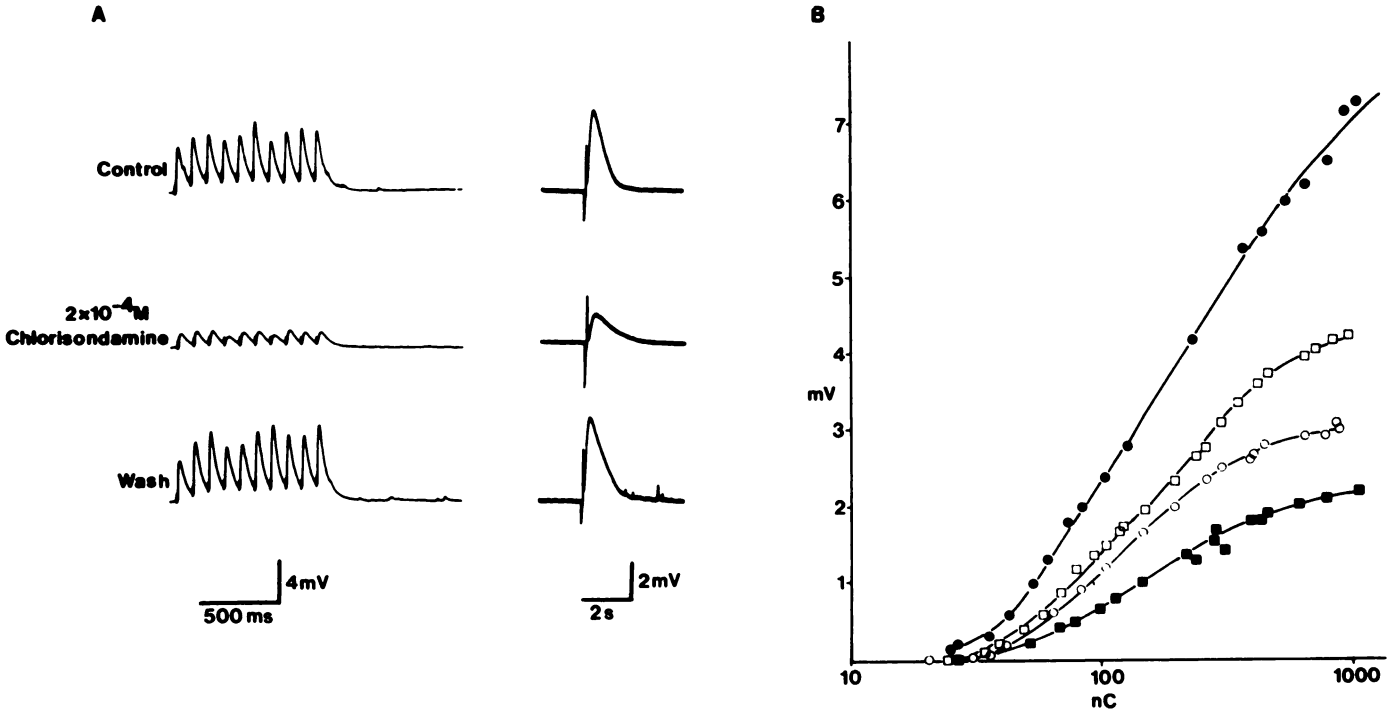


FIG. 1. *Effect of chlorisondamine on neurally evoked EJPs and glutamate iontophoretic responses (A) and on glutamate dose-response curves (B)*

A. In the *left* panel, the nerve to muscle gm6 of *Cancer* was stimulated with trains of pulses through a suction electrode resulting in EJPs. In the *right* panel, 50-msec pulses were passed through a 1 M sodium glutamate iontophoretic electrode. In both panels the *top* traces are controls, the *middle* traces in the presence of $2 \times 10^{-4}$ M chlorisondamine, and the *bottom* traces following wash in physiological saline. Temperature was 20°.

B. The abscissa corresponds to the total current passed through the iontophoretic electrode, and the ordinate is the amplitude of the response. ●, Dose-response relationship in control saline; □, in $2 \times 10^{-4}$ M chlorisondamine; ○, in $5 \times 10^{-4}$ M chlorisondamine; ■, in $10^{-3}$ M chlorisondamine. The glutamate responses on this preparation were somewhat less sensitive to chlorisondamine than were most preparations. Temperature was 12°.

the glutamate responses were dose-dependent, with 50% block at approximately $1.5 \times 10^{-4}$ M. Iontophoretic responses to quisqualic acid, a glutamate receptor agonist, were similarly reduced. In addition to its effects on the amplitude of the intracellular potentials, chlorisondamine prolonged the time courses of all three potentials. No effect of chlorisondamine on membrane potential or membrane resistance was observed.

Recently it has been shown that glutamate depolarizes some crustacean excitatory axons (19). It seemed possible that part of the reduction of the glutamate iontophoretic response might be mediated by the inhibition by chlorisondamine of the release of the normal excitatory transmitter from the presynaptic terminal. This was excluded by the finding that the abolition of synaptic transmission by the addition of 20 mM $Mn^{2+}$ to physiological saline produced no substantial change in the iontophoretic glutamate responses or on the block produced by chlorisondamine. Thus, these effects of chlorisondamine on the glutamate-activated response are postsynaptic.

The effect of chlorisondamine on dose-response curves produced by varying the amplitude of the current through the glutamate iontophoretic electrode was examined. Chlorisondamine produced a similar percentage block at all glutamate doses with a reduction in the maximal response to glutamate (Fig. 1B). This suggests a noncompetitive interaction. Such dose-response curves may be difficult to interpret for two reasons. First, the transport of glutamate from the iontophoretic electrode may vary in a nonlinear fashion with the current passed through the electrode. Second, there may be appreciable desensitization at higher glutamate doses. To circumvent the first problem, dose-response curves were constructed with double-barrel glutamate electrodes to generate multiples of an arbitrary dose of glutamate (a modification of ref. 20). Chlorisondamine had an identical effect on curves obtained with both the single- and double-barrel electrode methods. Furthermore, chlorisondamine had no effect on the rate of desensitization of sequential glutamate iontophoretic responses. Thus, the reduction of the maximal responses to glutamate in the presence of chlorisondamine cannot be explained by either nonlinearities in the movement of glutamate out of the electrodes or by an enhancement of desensitization at large glutamate doses. The noncompetitive nature of the chlorisondamine block of the glutamate response indicates that chlorisondamine does not act by directly interfering with the binding of glutamate to the receptor.

The depolarizing response to glutamate shown by these and other crustacean muscles is only one of several different glutamate responses in these animals. Glutamate can also elicit increases in $K^+$ conductance and in $Cl^-$ conductance in neurons of the stomatogastric ganglion (18), and we have recently discovered a glutamate-

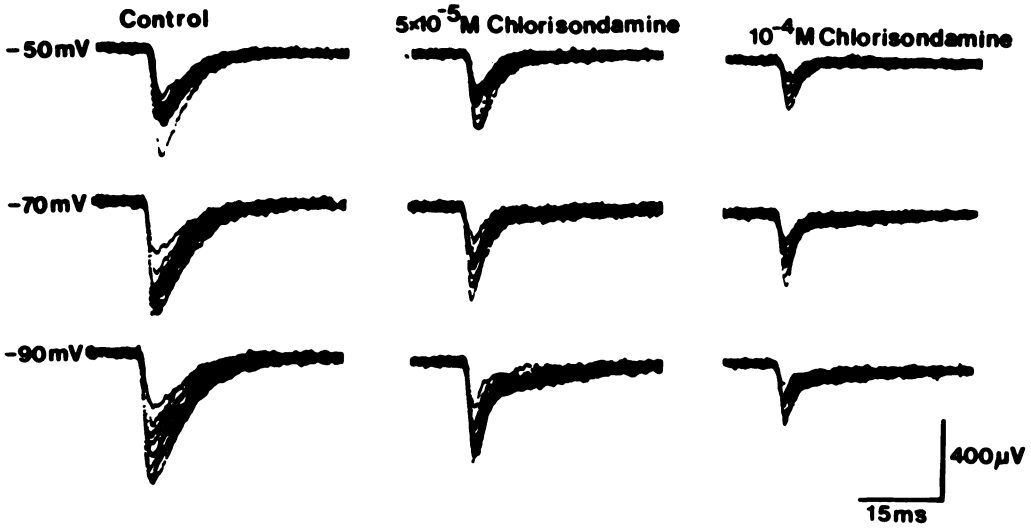


FIG. 2. *Effects of chlorisondamine on ejcs recorded at different membrane potentials*

Two KCl-filled electrodes were inserted into a muscle fiber, and extracellular ejcs were recorded with a 10-μm fire-polished pipette filled with physiological saline. The nerve was continuously stimulated at 5.5 Hz with a suction electrode. The *top* traces in each panel were taken at −50 mV, the *middle* traces at −70 mV, and the *bottom* traces at −90 mV. Experiments represented in the *left* panel were conducted with physiological saline (control); in the *middle* panel, in the presence of $5 \times 10^{-5}$ M chlorisondamine; and in the *right* panel, in the presence of $10^{-4}$ M chlorisondamine. In this experiment, at −50 mV the intracellularly recorded EJPs were reduced 38% in $5 \times 10^{-5}$ M chlorisondamine and approximately 53% in $10^{-4}$ M chlorisondamine. Temperature was 13.5°

evoked increase in $Cl^-$ conductance on several stomach muscles in *Cancer* and *Homarus* (19a). Chlorisondamine ($10^{-3}$ M) was without effect on these other glutamate-evoked responses.

To clarify further the mechanism of action of chlorisondamine, we studied its effects on extracellularly recorded synaptic currents (ejcs) while holding the membrane potential at different levels. The raw data resulting from one such experiment are shown in Fig. 2, and illustrate two features of the action of chlorisondamine. First, chlorisondamine reduced the amplitude of the peak currents. Second, chlorisondamine changed the time course of the decay of the ejc. In order to quantitate these effects, ejcs were evoked at frequencies less than 10 Hz and computer-averaged. Figure 3 is a plot of the amplitude of the computer-averaged peak currents as a function of membrane potential in the presence and absence of $10^{-4}$ M chlorisondamine. Chlorisondamine reduced the amplitude of the peak currents in a voltage-dependent manner. This reduction was more prominent at hyperpolarized membrane potentials. The voltage-dependent nature of this block suggests that the site of action of chlorisondamine is within the electric field of the membrane, similar to the action of putative channel blockers on cholinergic currents (21).

The rate of decay of synaptically evoked currents is thought to reflect the average channel-closing time (3, 22, 23). Figure 4 shows computer-averaged records in the presence and absence of $5 \times 10^{-5}$ M chlorisondamine with the computer-drawn regression analyses. In physiological saline the decays of the ejcs were well fit with a single exponential (regressions were calculated between approximately 70% and 15% of the peak currents), and in the experiment shown in Fig. 4 were 6.46 msec at −50 mV and 7.73 msec at −90 mV. Control ejcs also showed a late non-exponential tail. In the presence of chlorisondamine the ejc decay was frequently best fit by a double exponential, with a fast component faster than the control value and a slow component slower than the control value. In the experiment shown in Fig. 4, the fast time

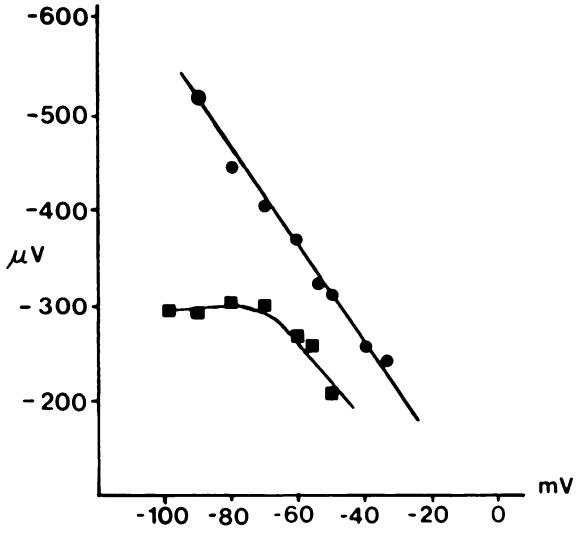


FIG. 3. *Voltage dependence of the reduction in peak amplitude of ejcs by chlorisondamine*

The nerve was continuously stimulated at 5.5 Hz with a suction electrode, and 40 sweeps were averaged for each point. The abscissa is the membrane potential from which the synaptic potentials originated. The ordinate corresponds to the average ejc amplitude. ■, In the presence of $10^{-4}$ M chlorisondamine; ●, in the presence of physiological saline (control). These data extrapolate to an estimated reversal potential of +1 mV. Note that the ordinate axis originates from 100 μV. Intracellular EJPs recorded during this experiment were 5.5 mV at −90 mV and 2.6 mV at −50 mV. These small membrane potential changes during the recording of the ejcs thus introduce a small but inconsequential error in these plots. Temperature was 13°.

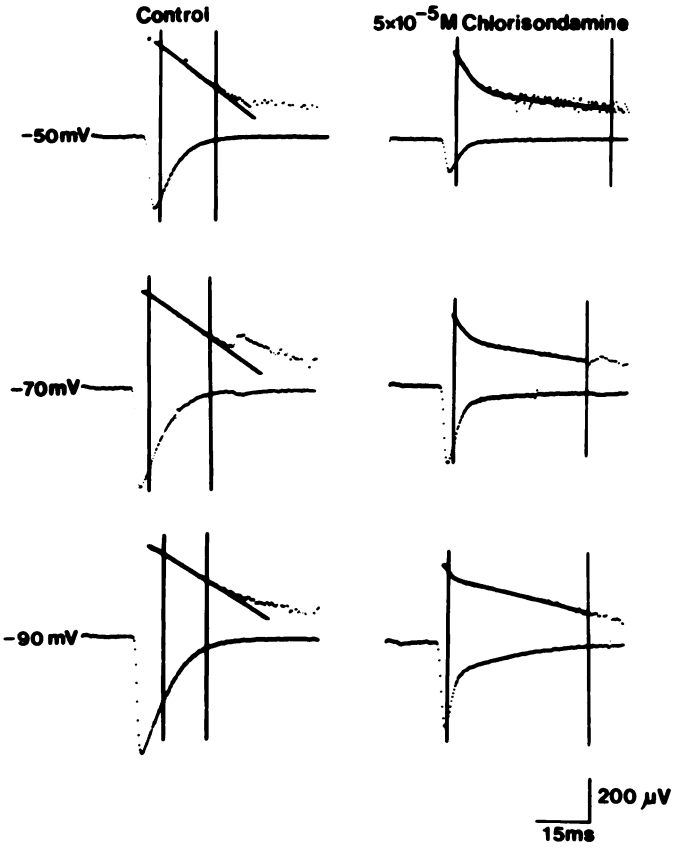


FIG. 4. *Computer-averaged ejcs and exponential plots of ejc decays*
Data are from the same experiment as those shown in Fig. 2. An MINC 11 computer was used to average 40 sweeps at each membrane potential and to generate plots. The *top* traces in each pair correspond to the semilogarithmic plot of current decay with the computer-generated fit over the range indicated. *Left* traces are ejcs in physiological saline; the *right* traces are in the presence of $5 \times 10^{-5}$ M chlorisondamine. Experiments depicted in the *top* panel were conducted at −50 mV; the *middle* panel at −70 mV; and the *bottom* panel at −90 mV. The time constants of the control ejcs were (from *top* to *bottom*) 6.46 msec, 7.01 msec, and 7.73 msec. The time constants in $5 \times 10^{-5}$ M chlorisondamine were 2.55 msec and 43.87 msec at −50 mV, 2.12 msec and 31.87 msec at −70 mV, and 1.00 msec and 20.13 msec at −90 mV. Temperature was 13.5°.

constant was 1.0 msec and the slow component was 20.13 msec. The alteration of the ejc decay by chlorisondamine is consistent with the notion that the site of action of chlorisondamine is the channel opened by the natural transmitter. The double exponential shown in the presence of the drug might be explained by a model in which the drug first blocks the open channel and subsequently dissociates from the blocked open channel, before it eventually closes (6). Hyperpolarization favors the presence of the cationic chlorisondamine in a blocking site, thus producing a greater reduction in peak currents at hyperpolarized potentials. By analogy with the block of acetylcholine-gated channels at the neuromuscular junction, the slow component of the ejc decay would be thought to represent primarily the rate of dissociation of blocker from the channel. Surprisingly, the slow component of the ejc decay in chlorisondamine is accelerated by hyperpolarization, suggesting the unexpected possibility that dissociation of chlorisondamine from its blocking site is favored by hyperpolarization. Thus, some other explanation for the double-exponential decay of ejcs in chlorisondamine may be necessary.

These experiments showed an apparent prolongation by hyperpolarization of the ejc in 0.9% physiological saline in 14 fibers from 4 muscles. For six sites at 12–13°, an *e*-fold change in the time constant of ejc decay occurred every 147 ± 46 mV (mean ± standard deviation), determined over membrane potentials from approximately −50 mV to −120 mV. At both crayfish and locust glutamatergic synapses the rate of decay of evoked currents is shortened by hyperpolarization (2, 23, 24), and at least in the locust this was verified by using noise analysis (4). There are several possible explanations for the difference between our results on the crab muscle fibers and those on crayfish and locust. First, it is possible that for some reason (e.g., transmitter uptake) the decay of the focally recorded extracellular synaptic current is not indicative of the channel-closing time. Second, there are reports that channel properties may be largely influenced by ionic milieu (25, 26), and therefore a marine crustacean might differ substantially in this respect from crayfish or locust. We hope to determine this directly with noise analysis. Third, it is possible that the natural transmitter at this crab stomach muscle may not be glutamate, although the evidence strongly suggests that it is (17). To attempt to rule out this possibility we demonstrated that chlorisondamine is equally effective in blocking glutamate iontophoretic responses on the walking leg-opener muscle in the same crab, indicating that the same types of channels are most likely involved at both muscles. Furthermore, the action of chlorisondamine is specific for the glutamate-evoked excitatory response due largely to an increase in $Na^+$ conductance, and the drug has no effect on glutamate-activated conductances to other ions. Thus, despite the possibility that the ejc decay may not accurately reflect channel-closing time at this synapse, the most parsimonious explanation of the present data is that chlorisondamine blocks the excitatory channels activated by glutamate and the natural transmitter.

Since chlorisondamine is best known as a nicotinic ganglionic blocking agent (16), the effects of other nicotinic ganglionic blocking agents on the glutamate- and synaptically evoked potentials on the gm6 muscle were examined. None of these (including curare, hexamethonium, trimethapan, mecamylamine, atropine, pempidine and dihydro-β-erythroidine) affected the glutamate or synaptic responses on gm6 (17). However, these agents were quite effective at low concentrations in reducing the amplitude of cholinergic responses on the foregut gml muscle (27) and in reducing the amplitude of the extrajunctional ACh response on gm6 (17). Chlorisondamine produced approximately a 50% block of these ACh responses at $2 \times 10^{-6}$ M (17, 27). Preliminary experiments indicate that these effects may also be channel interactions. These data, taken with fluctuation analyses of current noise produced by either ACh or glutamate on the same gm6 muscle fibers (28), suggest that channels activated by ACh and glutamate on the same cell are different.

Numerous studies with a variety of receptor agonists, antagonists, and channel blockers have provided insights into the kinetics and states of receptor-channel activation at cholinergic receptors (29, 30). Descriptions of glutamate-activated channels indicate that similar mechanisms exist in noncholinergic systems (10). Through the use of agents such as chlorisondamine, which interfere with the glutamatergic excitatory process, further understanding of the kinetics of non-cholinergic channel activation may be gained.

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