SIMULTANEOUS MODIFICATIONS OF SODIUM CHANNEL GATING BY TWO SCORPION TOXINS

GING KUO WANG AND GARY STRICHARTZ
Department of Anesthesia Research Laboratories, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT The effects of purified scorpion toxins from two different species on the kinetics of sodium currents were evaluated in amphibian myelinated nerves under voltage clamp. A toxin from *Leiurus quinquestriatus* slowed and prevented sodium channel inactivation, exclusively, and a toxin from *Centruroides sculpturatus* Ewing reduced transient sodium currents during a maintained depolarization, and induced a novel inward current that appeared following repolarization, as previously reported by Cahalan (1975. *J. Physiol. [Lond.]*. 244:511–534) for the crude scorpion venom. Both of these effects were observed in fibers treated with both of these toxins, and the kinetics of the induced current were modified in a way that showed that the same sodium channels were modified simultaneously by both toxins. Although the toxins can act on different sites, the time course of the action of *C. sculpturatus* toxin was accelerated in the presence of the *L. quinquestriatus* toxin, indicating some form of interaction between the two toxin binding sites.

The kinetics of the transiently occurring sodium current passing through an excitable membrane under voltage clamp have been described phenomenologically by two parameters, representing the activation and inactivation processes of the sodium conductance (g_{Na}). Hodgkin and Huxley (1) originally modelled the kinetics of g_{Na} using simultaneous independent activation and inactivation processes to describe the increase and decrease of g_{Na}, respectively. More recent studies of sodium currents and "gating currents" related to the conductance activation process, indicate that inactivation begins with a delay following the initiation of activation (2, 3), and that there may be some type of interaction between sodium activation and inactivation (4, 5). In contrast, experiments on the timing of closing of single sodium channels support a model in which inactivation processes occur independently, whether channels have opened or not (6). We report here that activation and inactivation of sodium channels in the node of Ranvier can be modulated, either separately or together, by separate solutions or mixtures of two purified scorpion toxins, respectively. Simultaneous modification of both activation and inactivation shows that the *Centruroides* toxin, which primarily affects the activation process, binds to a different site on the sodium channel than the *Leiurus* toxin, which affects only inactivation. Even though there are separate binding sites for different toxins, because the onset of action of these toxins differs for simultaneous and separate addition, we conclude that there must be some interactions between the binding sites.

Single myelinated fibers were isolated from sciatic nerves of the toad *Bufo marinus* and voltage clamped as described by Dodge and Frankenhaeuser (7). Neurotoxins were purified from venoms of *C. sculpturatus* and *L. quinquestriatus* scorpions by the method of cation-exchange chromatography (Bio Rex 70 column [Bio-Rad Laboratories, Richmond, CA] followed by a CM-52[Watman, Inc., Clifton, NJ] cellulose column). Multiple neurotoxins affecting sodium currents were found in each species; only the most potent one (toxin IIα) from each species was used in this study. Both purified *Centruroides* and *Leiurus* neurotoxins migrated as a single band in SDS-urea-polyacrylamide (15%) gel, and each had a molecular mass of ~7,000 daltons.[1]

Action potentials were prolonged by treatment with *Leiurus* toxin IIα at 200 nM (Fig. 1*b*), a toxin concentration that saturates almost all of the binding sites of the sodium channels. Increasing the *Leiurus* toxin IIα concentration to 400 and 800 nM produced no further changes in sodium currents measured under voltage clamp. This finding is consistent with a *Leiurus* toxin dissociation constant of 14 nM derived from scorpion toxin binding studies in frog muscle (8). From its pharmacological and chromatographic properties we believe that *Leiurus* toxin IIα is similar or identical to the toxin used by Catterall (8).

[1]Wang, G. K., and G. R. Strichartz. Purification and physiological characterization of neurotoxins from venoms of the scorpions *Centruroides sculpturatus* and *Leiurus quinquestriatus* acting on sodium channels. Submitted for publication.

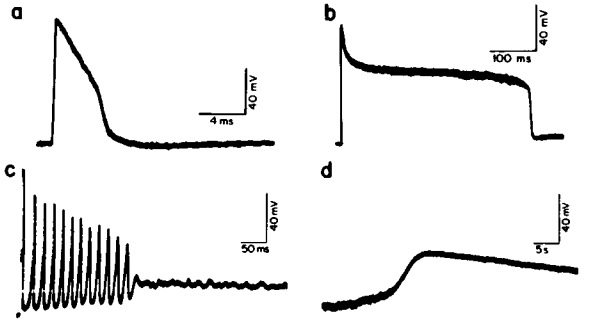

FIGURE 1 Nodal action potentials stimulated by a 300 μs pulse in (*a*) control and (*b*) the same node after expoure to *Leiurus* toxin: 200 nM for 25 min. (*c*) A second node, after *Centruroides* toxin: 20 nM for 10 min. In (*d*), a node was first treated with 200 nM *Centruroides* toxin for 30 min, followed by 200 nM *Leiurus* toxin for 15 min. No pulse was applied during this recording. Spontaneous depolarization of the resting membrane potential reached an amplitude of 45 mV. For all data shown in this paper the ends of fibers were cut in a 120 mM CsCl solution. Toxins were diluted in TEA-frog Ringer containing 105 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 12 mM TEA-Br and 2 mM HEPES buffer (pH 7.2) with 1 mg/ml of bovine serum albumin. These solutions eliminated potassium currents by pharmacological blockage and by replacement of axoplasmic potassium ions. Temperature = 8°C.

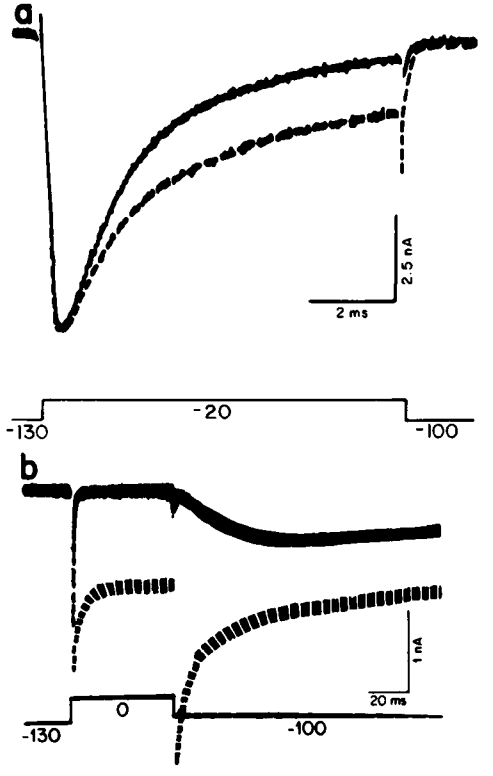

FIGURE 2 Nodal sodium currents under voltage clamp. (*a*) Before (solid line) and after (broken line) treatment with *Leiurus* toxin IIα: 200 nM. Current was measured 2 min after the addition of the toxin. (*b*) a different node after exposure to *Centruroides* toxin IIα: 20 nM for 30 min (solid line), followed by 200 nM *Leiurus* toxin for <1 min (broken line). The membrane potential in mV is shown in the lower trace. Node bathed in 12 mM tetraethylammonium Ringer. Capacitance and leak currents removed by analog subtraction. Temperature = 8°C. I_M is obtained by assuming a value of 40 MΩ for the resistance of the internodal segment over which an ohmic potential drop was measured (see reference 17).

Under voltage clamp the sodium current (I_{Na}) is directly proportional to the sodium conductance, g_{Na}; during a depolarizing membrane potential step the initial, rapid increase of I_{Na} represents the opening of sodium channels during activation and the subsequent, slower decrease of this current represents a closing of channels during the inactivation process (Fig. 2*a*, solid line). *Leiurus* toxin IIα specifically slows the inactivation process, which has two components in frog and toad nodes, both of which are slowed by *Leiurus* toxin IIα (reference 9; Fig. 2*a*, broken line). On average the peak current was not changed [I_{Na} (+ toxin)/I_{Na} (control) = 1.03 ± 0.05, n = 9], unlike the decrease in sodium current produced by crude *Leiurus* venom (10, 11). In addition, in about half of the experiments, exposure to *Leiurus* toxin IIα produced a persistent sodium current that did not inactivate during maintained depolarizations ($t_{1/2} > 1$ s), similar to the effect produced by a toxin from *Buthus eupeus* scorpion venom (12). The relationship between membrane depolarization potential and the peak I_{Na} during that depolarization was identical in control and toxin-treated nodes, and the time courses of I_{Na} up to the peak current were indistinguishable between these two conditions when measured with an expanded time scale (data not shown). These observations showed that sodium activation was unchanged by *Leiurus* toxin and thus, g_{Na} kinetics of the toxin-treated fibers were very similar to those previously reported using crude venom of *L. quinquestriatus* (10, 11).

The specific effect of *Leiurus* toxin IIα on the sodium inactivation processes does not in itself prove that inactivation is independent of the activation of g_{Na}. In fact, it has been suggested that *Leiurus* toxin binds to a site that controls both the activation and the inactivation processes (8), based on the observation that radiolabeled *Leiurus* toxin binding has a voltage-dependence that exactly overlaps the voltage dependence of the activation parameter. In muscle membranes depolarized by KCl, the affinity of radiolabeled *Leiurus* toxin is correspondingly reduced (8). Furthermore, the fraction of sodium channels able to close directly from the conducting state to the resting states is modified by *Leiurus* venom, as measured by the "off" component of gating current that flows during membrane repolarization (11). Both of these observations suggest that bound *Leiurus* toxin may interact with structures involved in sodium channel activation as well as inactivation. Therefore, we have used different scorpion toxin, which modifies sodium activation, to detect interactions between activation and inactivation processes.

The venom from the scorpion *C. sculpturatus* has only a small effect on the kinetics of sodium current during a maintained depolarization, but induces an unusual current after membrane repolarization (13). Purified *Centruroides* toxin IIα (20 nM) produced repetitive action potentials and a long, noisy plateau in single nerve fibers (Fig. 1*c*).

Under voltage clamp the sodium current was changed slightly during the initial depolarization (see Fig. 3*b*); the peak conductance was reduced by ~35% due to a selective reduction in amplitude of the rapidly inactivating component, but the inactivation time constants were unchanged. More apparent, a "toxin-induced" current appeared after repolarization of the membrane potential, with an activation time constant of 24 ms (Fig. 2*b*, solid line), and a time constant of decline of 850 ms (not shown), values comparable to a previously reported current induced by crude *Centruroides* venom in frog myelinated fibers (13). In that report it was proposed that during depolarization the activation process is modified by *Centruroides* venom, and that upon repolarization its voltage dependence is shifted in the negative direction by 40–50 mV. Such a shift will result in a persistent activation of sodium channels at −100 mV. According to that proposal, the activation of the induced current is due to the removal of sodium inactivation at negative potentials and the decline of the current due to the unusually slow decay of the toxin-modified activation process. Our results agree with this analysis, and we also observed that the size of the peak I_{Na} measured during a depolarization rapidly declined while the size of the induced current slowly increased during 5–10 min of continuous exposure to *Centruroides* toxin IIα.

When the *Leiurus* toxin IIα was added to a nerve fiber that had been pretreated with *Centruroides* toxin IIα, not only was inactivation slowed during the maintained depolarization, but the magnitude and the kinetics of the current induced by *Centruroides* toxin IIα were also changed. The toxin-induced current became larger, and its turn-on kinetics much faster, with a rise time of <2 ms (Fig. 2*b*). All the currents could be blocked by 100 nM saxitoxin, which specifically blocks sodium channels (14). Neither of the effects of the two scorpion toxins, whether added individually or together, could be reversed by extensive washing with Ringer's solution. It was also found that in the presence of *Leiurus* plus *Centruroides* toxins the unclamped membrane showed transient, spontaneous depolarizations (Fig. 1*d*), and within 10 min the fiber was no longer excitable.

The sequence of toxin additions did not effect the final current kinetics (compare Figs. 2*b* and 3*b*). When *Centruroides* toxin was added after exposure of the node to *Leiurus* toxin (Fig. 3*b*) the immediate effects were a reduction of peak sodium current during the initial depolarization and the appearance of a modified toxin-induced current that had a pattern similar to that resulting from the opposite order of addition of the toxins (Fig. 2*b*).

The changes in the sodium currents when both toxins are present provide direct evidence for separate binding sites for *Centruroides* and *Leiurus* toxins. Firstly, during the depolarization the *Leiurus* toxin IIα exerted its effect even in the presence of *Centruroides* toxin IIα (Fig. 2*b*). Both fast and slow inactivation processes were slowed, as occurred with *Leiurus* toxin alone, and the non-inactivating component of g_{Na} became apparent during long depolarizations. Also, the effect of *Centruroides* toxin IIα in reducing the peak current during the initial depolarization still occurred in the presence of saturating concentrations of *Leiurus* toxin IIα. The relative amplitudes of g_{Na} inactivating with different time constants varied from node to node (e.g., compare Figs. 2*b* and 3*b*), but the separate effects of *Leiurus* toxin IIα in slowing inactivation and of *Centruroides* toxin IIα in reducing peak g_{Na} were always distinguishable during this depolarization.

Secondly, the addition of *Leiurus* toxin IIα enlarges the current induced by *Centruroides* toxin IIα upon repolarization, and modifies its turn-on kinetics. The turn-on of the toxin-induced current with both toxins present becomes much faster, as it no longer follows the recovery of the normal inactivation process, and is often difficult to separate from the decay of the sodium current "tails" that correspond to the closing of open sodium channels as they return to their resting states.

If the two toxins could not modify the same sodium channels simultaneously, then sodium current kinetics upon repolarization to −100 mV would consist of two clearly separable components, one from *Leiurus* toxin-modified channels with a large, rapidly decaying tail that reduced currents to near zero in <1 ms (as in Fig. 2*a*, broken line) plus a second slowly developing component from *Centruroides* toxin-modified channels that would

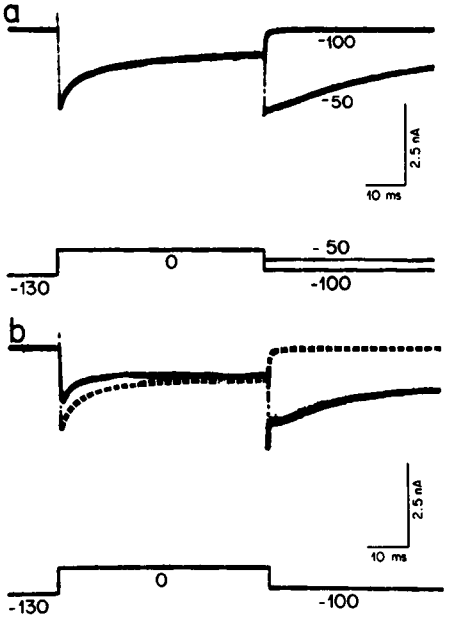

FIGURE 3 Sodium currents under voltage clamp in (*a*) node treated with 200 nM *Leiurus* toxin for 15 min. The membrane potentials are shown in the lower trace. Slowly decaying current is apparent upon repolarization to −50 mV. (*b*) The same node subsequently exposed to 200 nM *Centruroides* toxin (solid line, measured within 1 min after the *Centruroides* toxin was added; the current trace in *a* repolarized to −100 mV is shown as a broken line in *b*). Note the similarity of the toxin induced current upon repolarization to −100 mV in *b* to the current at −50 mV in *a*.

produce an inward current that slowly increased with a time constant of ~25 ms (as in Fig. 2*b*, solid line). The current kinetics that are actually observed are distinctly different and can be accounted for by assuming that each toxin acts characteristically and simultaneously on activation and inactivation processes in the same population of sodium channels. This is shown experimentally in Fig. 3.

After exposure to *Leiurus* toxin IIα alone, repolarization to -100 mV produces a rapidly decaying current tail, but repolarization to -50 mV yields an inward current that decays much more slowly, reflecting the activated sodium channels which normally close slowly at -50 mV and which, due to *Leiurus* toxin IIα, have not become inactivated during the depolarizing pulse (Fig. 3*a*). When the same node is then exposed to *Centruroides* toxin IIα, the current kinetics after repolarization to -100 mV (Fig. 3*b*) become very similar to those following repolarization to -50 mV with *Leiurus* toxin IIα alone. This would be predicted by assuming that a population of the sodium channels is both prevented from inactivating, by *Leiurus* toxin IIα, and has its activation parameters shifted by about -50 mV, by *Centruroides* toxin IIα, so that the non-inactivating channels now close very slowly upon repolarization to -100 mV. The observed current kinetics can only be explained by requiring that the two scorpion toxins act on the same population of channels, and therefore, during repolarization as during depolarization, activation and inactivation can be modified simultaneously. Furthermore, the synergistic effect of these two toxins in depolarizing the unclamped nodal membrane is apparent (Fig. 1*d*). We conclude that there are separate binding sites for different classes of scorpion toxins on the extracellular surface of individual sodium channels, and that these sites can be occupied simultaneously.

These electrophysiological results are consistent with the biochemical results of Jover et al. (15) showing that the binding of an iodinated neurotoxin from *Centruroides suffusus* is unaffected by *Leiurus*-like toxins. However, both Meves and Rubly (16) and we have shown that the multiple neurotoxins in *C. sculpturatus* fall into two classes, one of which gives effects like that of *Leiurus* toxins. Without knowing the physiological effects of the labeled *Centruroides suffusus* toxin, it is impossible to draw mechanistic conclusions concerning such effects from the binding data alone. Our results are also consistent with the observations of Meves et al. (18) that mixtures of different scorpion toxins isolated from *C. sculpturatus* venom can simultaneously modify the kinetics of sodium currents.

There is also evidence from our experiments for interactions between the scorpion toxin sites that modify activation and inactivation. The induced current upon repolarization in the presence of both toxins decayed more slowly than the current induced by *Centruroides* toxin IIα alone: a time constant of decline of 5 s. compared with 850 ms. This slowing could not be due to the direct effects of *Leiurus* toxin IIα on the removal of sodium inactivation at -100 mV, because the time constant (14–15 ms) for that process was not significantly changed by *Leiurus* toxin. We also observed that the onset of the induced current after the addition of *Centruroides* toxin alone (20–200 nM) usually took at least 5 min, but occurred within 1 min if preceded by exposure of the node to *Leiurus* toxin IIα. The rate of binding or the action of one toxin appears to be affected by the presence of the other. It remains for further experiments to clarify this point and to reconcile our results with the gating current data and the voltage-dependent binding of *Leiurus* toxin.

We thank Dr. Stephen Raymond for helpful comments on this paper. This research was conducted while Dr. Wang was a Postdoctoral Fellow of the Muscular Dystrophy Association. Supported by U.S. Public Health Service grant NS12828 (NS18467) and a Muscular Dystrophy Association Research Award to Dr. Strichartz. An abstract presenting some of these results has previously appeared (19).

Received for publication 1 April 1982 and in revised form 22 June 1982.

REFERENCES

1. Hodgkin, A. L., and A. F. Huxley. 1952. The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol. (Lond.)*. 116:497–506.
2. Bezanilla, F., and C. M. Armstrong. 1977. Inactivation of the sodium channel. I. Sodium current experiments. *J. Gen. Physiol.* 70:549–566.
3. Goldman, L., and C. L. Schauf. 1972. Inactivation of the sodium current in *Myxicola*. *J. Gen. Physiol.* 59:659–675.
4. Armstrong, C. M., and F. Bezanilla. 1977. Inactivation of the sodium channel. II. Gating current experiments. *J. Gen. Physiol.* 70:567–590.
5. Goldman, L. 1975. Quantitative description of the sodium conductance of the giant axon of *Myxicola* in terms of a generalized second-order variable. *Biophys. J.* 15:119–136.
6. Horn, R., J. Patlack, and C. F. Stevens. 1981. Sodium channels need not open before they inactivate. *Nature (Lond.)*. 291:426–427.
7. Dodge, F. A., and B. Frankenhaeuser. 1959. Sodium currents in the myelinated nerve fibre of *Xenopus laevis* investigated by the voltage clamp technique. *J. Physiol. (Lond.)*. 148:188–200.
8. Catterall, W. A. 1979. binding of scorpion toxin to receptor sites associated with sodium channels in frog muscle. Correlation of voltage-dependent binding with activation. *J. Gen. Physiol.* 74:375–392.
9. Chiu, S. Y. 1977. Inactivation of sodium channels: second order kinetics in myelinated nerve. *J. Physiol. (Lond.)*. 273:573–596.
10. Koppenhofer, E., and H. Schmidt. 1968. Die wirkung von skorpiongift auf die ionenstrome des Ranvierschen schnurrings. II. Unvollstandige Natrium-inaktivierung. *Pflugers Arch. Eur. J. Physiol.* 303:150–161.
11. Nonner, W. 1979. Effects of *Leiurus* scorpion venom on the "gating" current in myelinated nerve. *In* Advances in Cytopharmacology. B. Ceccarelli and F. Clementi, editors. Raven Press, New York. 3:345–352.
12. Mozhayeva, G. N., A. P. Naumov, E. D. Nosyreva, and E. V. Grishin. 1980. Potential-dependent interaction of toxin from venom of the scorpion *Buthus eupeus* with sodium channels in myelinated fibre. Voltage clamp experiments. *Biochim. Biophys. Acta*. 597:587–602.
13. Cahalan, M. V. 1975. Modification of sodium channel gating in frog myelinated nerve fibres by *Centruroides sculpturatus* scorpion venom. *J. Physiol. (Lond.)* 244:511–534.

14. Hille, B., 1968. Pharmacological modifications of the sodium channel of frog nerve. *J. Gen. Physiol.* 51:199–219.
15. Jover, E., F. Couraud, and H. Rochat. 1980. Two types of scorpion neurotoxins characterized by their binding to two separate receptor sites on rat brain synaptosomes. *Biochem. Biophys. Res. Commun.* 95:1607–1614.
16. Meves, H., and N. Rubly. 1981. Effect of various fractions of *Centruroides sculpturatus* venom on the node of Ranvier. *J. Physiol. (Lond.).* 320:143 P.
17. Sigworth, F. J. 1980. The variance of sodium current fluctuations at the node of Ranvier. *J. Physiol. (Lond.).* 307:97–129.
18. Meves, H., N. Rubly, and D. D. Watt. 1982. Effect of toxins isolated from the venom of the scorpion *Centruroides sculpturatus* on the Na currents of the node of Ranvier. *Pflugers Arch. Eur. J. Physiol.* 393:56–62.
19. Wang, G. K., and G. R. Strichartz. 1981. Isolation of three neurotoxins from *Centruroides* scorpion venom and their action on sodium channels. *Soc. Neurosci. Abstr.* 7:206.7.