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Effect of *Crotalus Atrox* Venom on Sodium Transport Across the Frog Skin (39885)

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Rattlesnake (Crotalus) venom causes a loss of cortical electrical activity after intravenous injection into anesthetized dog (1) and also induces sudden appearances of high-voltage, slow-wave cortical activity, indicative of cerebral depression, in unanesthetized monkey (2). Crotoxin, a neurotoxin isolated from the venom of *Crotalus durissus terrificus* (3, 4), evokes paralysis in dogs, cats, monkeys, mice, rats, pigeons, guinea pigs, and rabbits (5). Crotamin, a toxic component of *Crotalus durissus terrificus* venom, provokes extension and paralysis of the hindlegs of mice when injected intravenously (6). Crotamin-containing rattlesnake venoms and crotamin itself cause contraction of skeletal muscles, an effect which can be observed on intact and conscious animals as well as on *in situ* and isolated preparations (7). Also, the noncrotaminic rattlesnake venoms elicit a weak, slowly induced paralytic effect. Paralysis by both crotaminic and noncrotaminic venoms has been shown to be due to a direct action on the muscle fiber (7). These results suggested that the primary action of Crotalus venom and its purified fractions might involve changes in the ionic permeability of the cell membrane. Hence, this investigation was undertaken to study the effect of *Crotalus atrox* venom (CAV) on Na^+ and Cl^- transport across the isolated frog skin.

Methods. Bullfrogs, *Rana catesbeiana,* of either sex were kept fasting at room temperature of 25° prior to experimentation. Adult animals were used in these experiments.

The bullfrog was stunned by a blow on the head and its abdominal skin was removed and mounted between two Lucite chambers as a flat sheet having an area of 3.14 cm^2 for the determination of electrical characteristics. The chambers employed were equipped with two sets of electrodes and were similar in design to those described by Ussing and Zerahn (8). Transmural potential difference (PD) and short-circuit current (SCC) were measured by methods similar to those employed by Schultz and Zalusky (9). A voltage clamp device was used to maintain short circuit conditions (10). The skin resistance (R) was calculated from the PD/SCC ratio. To determine whether this was valid, the current–potential relationship of the skin was examined by rapidly reducing the PD in steps with SCC. The preliminary data showed that a linear relationship exists between the current and the clamped transmural PD, the slope of the line being equal to the negative of tissue resistance. A similar current–potential relationship has been reported on the toad-bladder preparation (11). The skin was aerated and mounted between identical phosphate Ringer of the type described by Adrian (12) having a total osmolality of 230 mOsm and a pH of 7.2 at 25°. Unless stated otherwise, two kinds of bathing solution were used: normal chloride Ringer, which contained 105 m*M* Na^+, 106 m*M* Cl^-, and 2.5 m*M* K^+; and sulfate-Ringer, in which chloride was replaced by sulfate, all other ionic constituents being unchanged. Mannitol was used to maintain constant osmolality throughout. When both PD and SCC were stabilized, CAV was added, unless stated otherwise, to the outside bathing medium.

In some experiments, unidirectional fluxes of $^{22}Na^+$ were measured in short-circuited skin, using a deep-well scintillation counter (Nuclear Chicago Model 18). When both SCC and PD were stabilized, fluxes in the absence of CAV were first determined for two to three 15-min periods; CAV was then added and the fluxes were determined for two or three more periods. At least 30 min was allowed for specific activity equilibration between the sodium pool of the skin and the isotope-containing bathing solution

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before the flux measurements were made.

Results. The first series of experiments was designed to find the site of CAV action. Typically, as shown in Fig. 1, CAV added to the outside bathing medium at a concentration of 1 mg% produced an immediate 9.8 ± 3.5% increase in PD and a 11.5 ± 4.6% increase in SCC (N = 18), indicating no significant change in the skin resistance. Moreover, the recovery of the PD and SCC was relatively rapid but was never complete. In contrast, CAV added to the inside bathing medium at a concentration of 1 mg% produced no significant change in PD or SCC.

Because of individual variations in the absolute magnitude of the changes in SCC after addition of CAV, these changes have been expressed as a percentage inhibition or stimulation (α_{SCC}), defined after Curran and Gill (13) as 100 × [1 − (SCC in presence of CAV/control SCC)]. A similar definition has been used for α_{PD} and α_R. The average values for SCC, PD, and R, when CAV was added to the medium bathing the outside surface of the skin at a concentration ranging from 1 to 100 mg/100 ml, are shown in Fig. 2. The PD increase reached an apparent saturable maximum at 100 mg% concentration of CAV. However, SCC peaked at 10 mg% concentration of CAV and tended to decrease at the 50 and 100 mg% dosages. In fact, at 100 mg% concentration of CAV, there was a significant inhibition of SCC. Consequently, the calculated value of R shows a significant increase at the higher CAV concentrations (50 and 100 mg%), while at the lower CAV concentrations there was little or no apparent increase in R.

In order to find out the effect of CAV on Na^+ transport in the presence of a relatively impermeable anion such as sulfate (14) in the medium, NaCl in the usual Ringer solution was replaced with Na_2SO_4, mannitol making up the osmolal difference. The addition of 1 or 100 mg% CAV to the outside medium induced parallel increases in both PD and SCC. Those skins (N = 3) exposed to 1 mg% CAV gave a mean percentage increase in SCC of 8%, while the mean percentage increase in PD was 9%. The skins (N = 3) exposed to 100 mg% CAV gave a mean percentage increase in SCC of 23%, while the mean percentage increase in PD was 21%.

In order to gain additional insight on the site of action, the interaction between CAV and antidiuretic hormone (ADH) was studied. As seen in Fig. 3, addition of 250 milliunits/ml of ADH to the inside bathing solution in the presence of 10 mg% CAV in the outside bathing solution elicited a stimulation of both PD and SCC. In the converse experimental design where 10 mg% CAV was added to the outside bathing solution after 250 milliunits/ml of ADH had stimulated PD and SCC, both PD and SCC increased (N = 3). These results suggest that the site of action of CAV is different from that of ADH.

The presence of high Ca^{2+} in the outside bathing medium interferes with Na^+ transport across the frog skin through a mechanism different from that of ADH (15). The

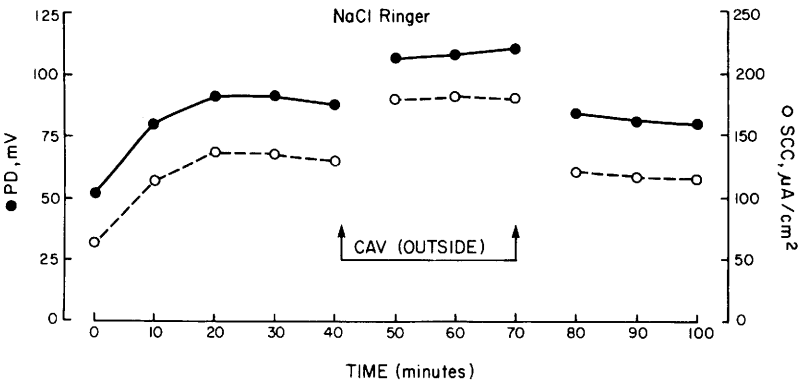

FIG. 1. A typical experiment showing the stimulation of PD and SCC by the addition of 1 mg% CAV to the outside bathing medium.

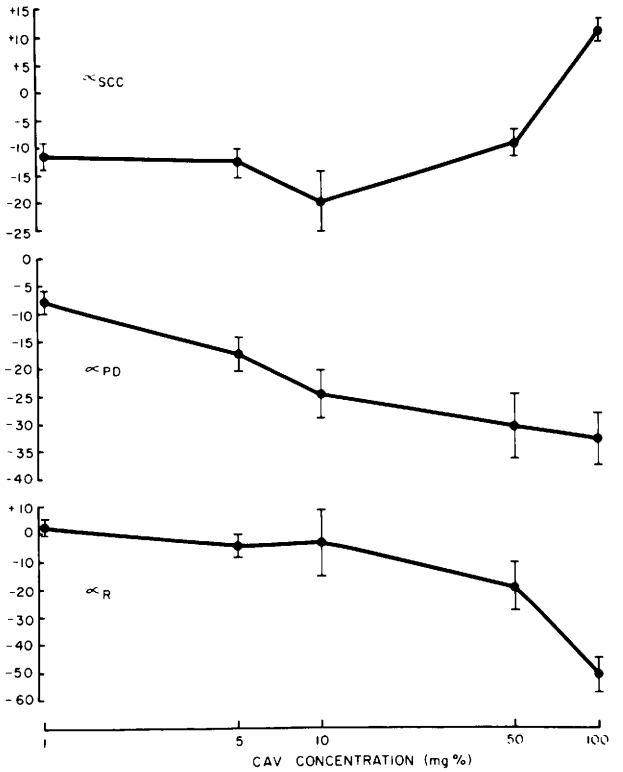

FIG. 2. Percentage inhibition (positive inflection) or stimulation (negative inflection) of SCC (α_{SCC}), PD (α_{PD}), and R (α_R) as a function of CAV added to the medium bathing the outside surface of the skin. Each point is the mean of 11 paired experiments.

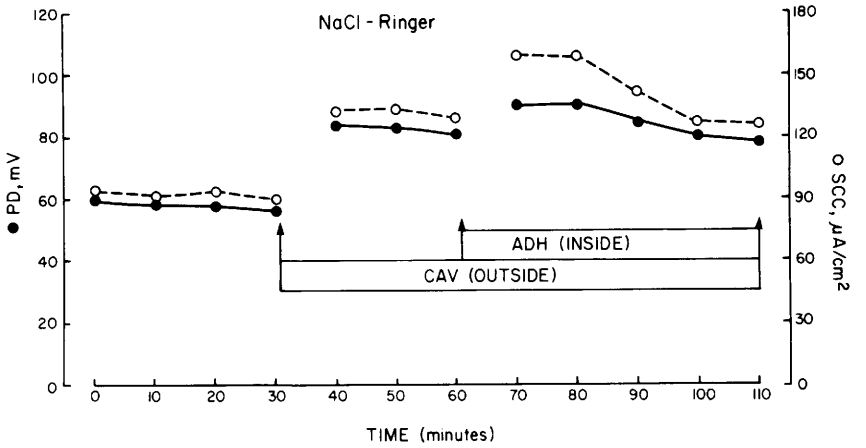

FIG. 3. Effect of ADH added to the inside bathing medium on outside 10 mg% CAV-stimulated PD and SCC.

possible interaction between CAV and high Ca^{2+} was thus studied. The increase of Ca^{2+} concentration from 1.8 to 7.2 m*M* in the outside bathing medium in the presence of 1 or 100 mg% CAV in the same compartment abolished the effects elicited by CAV (N = 4). In the converse experimental design where 7.2 m*M* Ca^{2+} was present in the outside bathing medium, the addition of 1 or 100 mg% CAV to the same compartment

caused no change in the measured electrical parameters, PD and SCC ($N = 4$).

In order to discern the ionic nature of the CAV-induced SCC, determinations of the unidirectional outside to inside (J_{OI}) and inside to outside (J_{IO}) Na^+ fluxes, using $^{22}Na^+$ in paired preparations when their short-circuit currents matched, were performed.

As shown in Table I, the mean J_{OI} of Na^+ before CAV addition is significantly less ($P < 0.005$) than the mean J_{OI} of Na^+ after the addition of CAV. However, there is no significant difference in the mean J_{IO} of Na^+ before and after the addition of CAV. Also, there is no significant difference between the net mean outside to inside flux of Na^+ (J_{OI}^{NET}) and the mean SCC before the addition of CAV. This strongly implicates active Na^+ transport, in the absence of CAV, as being identical to the SCC. Since there is no significant difference between the J_{OI}^{NET} of Na^+ and mean SCC after CAV has stimulated SCC, J_{OI}^{NET} of Na^+ must be identical to the SCC under these conditions.

Discussion. Although the clinical manifestations of Crotalus poisoning may be quite variable, previous work indicates that Crotalus venom induces a widespread direct action on excitable membranes (7). The mechanism underlying these actions of Crotalus venom on excitable membranes is not yet clear, but it seems probable that Crotalus venom induces changes in the ionic permeability of the excitable membrane.

The present investigation reveals that, at least in the frog skin, CAV induces definite changes in the Na^+ transport system. Although the exact mode of action of CAV is difficult to assess, primarily because of the complicated nature of the Na^+ transport system across frog skin, data obtained in this work shed light on CAV's possible mode of action. In describing site of action, it is important to note that 1 mg% CAV is consistently more effective when it is added to the medium bathing the outside surface of the skin (Fig. 1). Characteristically, CAV induced a great increase in R at both 50 and 100 mg% concentrations, but it had very little effect on R at the lower concentrations of 1, 5, and 10 mg% (Fig. 2). This observation together with the fact that CAV failed to induce a change in R of the skin bathed with sulfate-Ringer led us to speculate that, at 50 and 100 mg% CAV concentrations, a primary action of CAV is to impede the passive permeability of the skin to chloride. At all concentrations, with the exception of 100 mg%, CAV consistently induced increases in SCC. This can be explained either by CAV inducing an increase in the passive permeability of the outside membrane to Na^+ or by a direct action of CAV on the Na^+ pump or by CAV reducing the passive inside to outside flux of Na^+. Since 1 mg% CAV added to the inside bathing medium had no effect on PD and SCC, coupled with the fact that the electrical effects were seen within a few seconds after addition of the venom to the outside bathing medium, one can rule out CAV exerting a direct action of the Na^+ pump which is located on the inside membrane (16). Our $^{22}Na^+$ flux data indicate that the evoked increase in SCC by CAV is totally a Na^+-carrying current, for before CAV addition there was no significant difference in mean J_{OI}^{NET} of Na^+ and mean SCC (Table I). After CAV addition there was a significant ($P < 0.005$) increase in both J_{OI}^{NET} of Na^+ and SCC in an equivalent fashion, resulting in no significant difference between these stimulated characteristics. The CAV-induced increase in SCC was not due to a decrease in the unidirectional J_{IO} of Na^+ because there was no significant change in that flux after the addition of CAV (Table I). On the basis of these flux data we would like to suggest that the most feasible explanation of the CAV-induced increase of SCC involves an increase in the permeability of

TABLE I. SODIUM FLUXES IN AMPHIBIAN RINGER.[a]

	J_{OI} (nEq/cm²/min)	J_{IO} (nEq/cm²/min)	J_{OI}^{NET} (nEq/cm²/min)	SCC (nEq/cm²/min)
Before CAV addition	21.76 ± 4.53 (5)	6.31 ± 3.12 (5)	15.45 ± 4.93	18.27 ± 4.12
After CAV addition	63.28 ± 8.09 (5)	11.44 ± 5.27 (5)	51.84 ± 9.50	52.09 ± 8.89

[a] Average values ± SEM are given for the number of experiments shown in parentheses.

the outer-facing membrane to Na^+. This is borne out by the flux data which show that CAV affects only the J_{OI} unidirectional Na^+ flux by significantly ($P < 0.005$) increasing it.

Although the receptors involved in the effect of CAV on the skin cannot be clearly elucidated on the basis of the present study, it appears that they are different from the receptors for the action of ADH on Na^+ transport. On the other hand, the action of CAV is prevented when the skin was treated with high Ca^{2+} (7.2 m*M*), which is known to decrease the passive permeability of the outer membrane to Na^+ (15). Also, the effect of CAV is abolished when 5.4 m*M* Ca^{2+} is added to the outside bathing medium which contains 1.8 m*M* Ca^{2+}. These results suggest that both CAV and Ca^{2+} may interact at the same receptor site on the outer membrane. The fact that the treatment of skin with CAV did not interfere with the usual effect of high Ca^{2+} further suggests that the affinity of Ca^{2+} for this common site of action is far greater than that of CAV.

Summary. The present investigation involving CAV on the ion transport systems in isolated skin has shown that (i) PD and SCC are stimulated in an equal fashion at low concentrations, (ii) R drastically increases at high concentrations, (iii) the mechanism for stimulating SCC is independent of that produced by ADH, (iv) Ca^{2+} abolished any effect on PD or SCC produced by CAV, and (v) active sodium transport is stimulated at low CAV concentrations.

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1. Vick, J. A., Ciuchta, H. P., and Polley, E. H. Nature **203,** 1387 (1964).
2. Vick, J. A., "Neuropoisons," Vol. 1, p. 77. Plenum Press, New York (1971).
3. Slotta, K. H., and Fraenkel-Conrat, H. L., Nature **142,** 213 (1938a).
4. Slotta, K. H., and Fraenkel-Conrat, H. L., Ber. Deut. Chem. Ges. **71,** 1076 (1938b).
5. Brazil, O. V., Mem. Inst. Butantan **33,** 981 (1966).
6. Cheymol, J., Bourillet, F., and Roch-Areiller, M., Biochem. Pharmacol. **12,** 126 (1963).
7. Tu, A. T., "Neuropoisons," Vol. 1, p. 93. Plenum Press, New York (1971).
8. Ussing, H. H., and Zerahn, K., Acta Physiol. Scand. **23,** 110 (1951).
9. Schultz, S. G., and Zalusky, R. J., Gen Physiol. **47,** 567 (1964).
10. Rothe, C. F., Quay, J. F., and Armstrong, W. McD., IEEE Trans. Biomed. Eng. **16,** 160 (1969).
11. Pendleton, R. G., Sullivan, L. P., Tucker, J. M., and Stevenson, R. E., J. Pharmacol. Exp. Ther. **164,** 348 (1968).
12. Adrian, R. H., J. Physiol. London **151,** 154 (1960).
13. Curran, P. F., and Gill, J. R., J. Gen. Physiol. **45,** 625 (1962).
14. Klahr, S., and Bricker, N. S., J. Clin. Invest. **43,** 922 (1964).
15. Curran, P. F., Herrera, F. C., and Flanigan, W. J., J. Gen. Physiol., **46,** 1011 (1963).
16. Koefoed-Johnson, V., and Ussing, H. H., Acta. Physiol. Scand. **42,** 298 (1958).

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