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Charybdotoxin Improves Motor Recovery of the Rat After Spinal Cord Injury[1]

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OHNISHI, S. T., J. K. BARR, K. K. SADANAGA AND C. KATAGI. *Charybdotoxin improves motor recovery of the rat after spinal cord injury*. PHARMACOL BIOCHEM BEHAV 31(1) 187–191, 1988.—Charybdotoxin, a highly specific inhibitor of calcium-activated potassium efflux, was found to protect rat spinal cord against dynamic impact injury. In a control (nontreated) group, a weight drop of 10 gram ×5 cm on the T-11 segment of the rat spinal cord paralyzed hindlimbs, and recovery was slow. After 4 weeks, Tarlov scores (a behavioral index) were 1 to 2; the hind legs could not support body weight. In contrast, with animals pretreated 30 minutes prior to the injury by 0.12 mg charybdotoxin/kg (IP), Tarlov scores increased to 3.5–4.5 by three weeks after injury; animals could walk with some deficit. A possible mechanism for the protective effect of this drug is discussed.

Behavioral recovery Calcium-activated potassium efflux Charybdotoxin Paraplegia
Spinal cord injury Tarlov score

IT has been hypothesized that mechanisms of cell damage in spinal cord injury consist of fast and slow processes (3). The fast reaction, which takes place immediately following the contusion, involves rapid ion movements and a loss of action potential (7). It has been shown that after contusion, extracellular calcium ions are absorbed into axonal cells (25,31) while potassium ions leak out of the cells (30). The slow reaction, which includes the water movement causing edema (12,29), calcification (4, 5, 13) as well as degradation of cell membranes, leads to cell death within 24 hours (3, 8, 15). However, the exact mechanisms of these processes are not well understood. Since calcium influx takes place immediately after injury (25,31), the effect of calcium channel antagonists have been studied. However, the efficacy of calcium channel antagonists in protecting the spinal cord may not be remarkable (3). The effect may be related partly to blood vessel dilation enhancing blood circulation.

We have been studying the mechanism of irreversible membrane damage seen in the red blood cells of sickle cell anemia patients (18, 19, 21). It was demonstrated that repeated cycles of sickling and unsickling induced calcium entry, and that entering calcium ions stimulated calcium-activated potassium efflux. This potassium efflux seems to be associated with denaturation of red cell membranes to form irreversibly sickled cells (ISC) (18, 19, 21). We found that inhibitors of calcium-activated potassium efflux could prevent the formation of ISC in a dose-related manner (19,21). In comparing the mechanism of spinal cord injury with that of the formation of ISC, we have noted a striking similarity, i.e., membrane damage causes calcium entry, which stimulates potassium efflux, and finally irreversible membrane damage results within 24 hours.

This prompted us to study the effect of inhibitors of calcium-activated potassium efflux on spinal cord injury. We have used a weight-drop method to produce dynamic injury on the spinal cord (1, 6, 28). We chose rats because of economy, availability of a large number of animals of the same breed, and because rats are known to be suitable animals for the study of spinal cord injury (2, 7, 17, 28).

The first inhibitor tested was quinine, which is known to prevent irreversible denaturation of sickle cell membranes (19,21). We demonstrated that this compound could indeed protect rat spinal cord against injury (20). Since quinine has a variety of pharmacologic actions, the use of a more specific inhibitor is needed if the mechanism producing the effect is to be understood.

In this paper, we tested the efficacy of charybdotoxin [CTX; (16,24)], a newly discovered specific inhibitor for calcium-activated potassium efflux in protecting the spinal cord against injury. We measured changes of extracellular potassium and calcium concentrations as well as recovery of motor performance.

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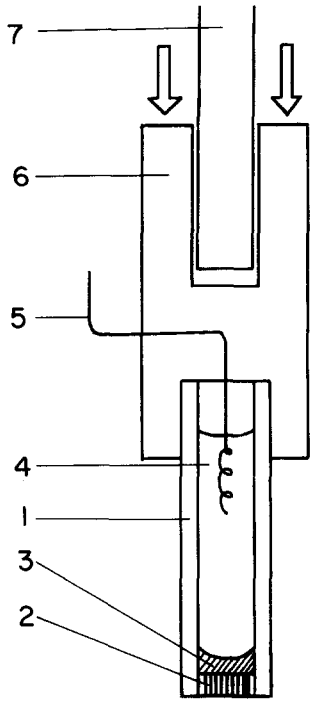

FIG. 1. Schematic illustration of an impounder-electrode assembly. (1) Glass tube (o.d.; 2.4 mm); (2) silane-treated ceramic filter; (3) ion-sensitive resin; (4) salt solution; (5) silver-silver chloride wire; (6) a plastic holder; (7) a guiding rod. Arrows indicate the surface where impounding force is applied (see text for further details).

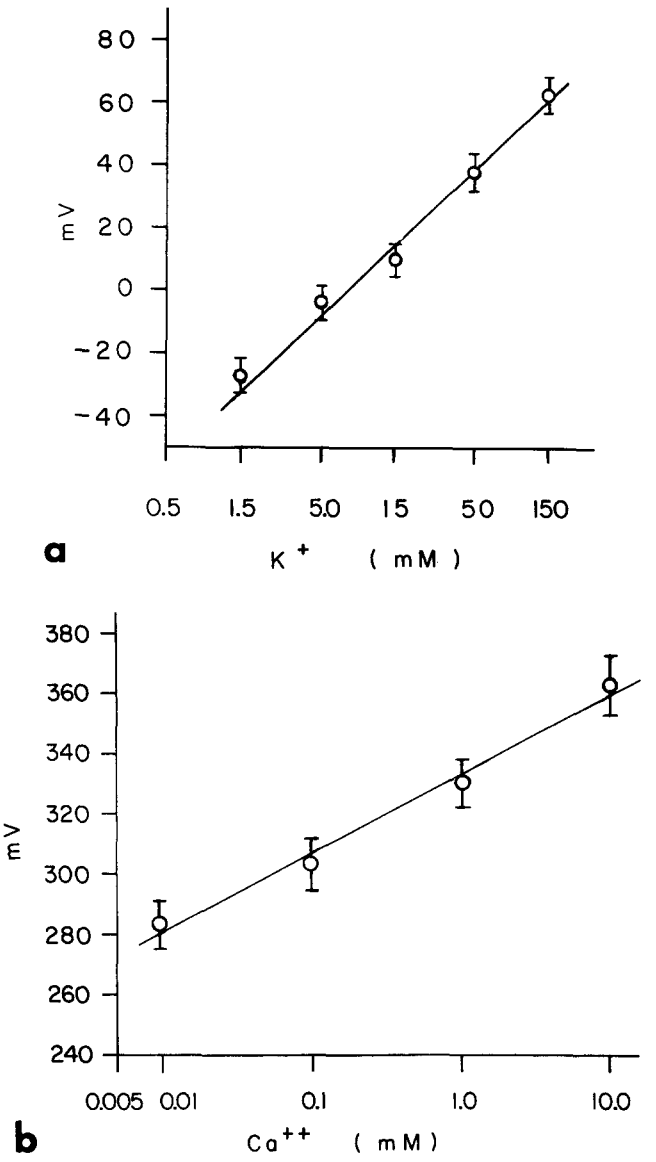

FIG. 2. Calibration curves for surface ion-sensitive electrodes. (a) Potassium electrode and (b) calcium electrode.

METHOD

Drugs

Venom of the scorpion *L. quinquestriatus* was obtained from the Latoxan Scorpion Farm (05150 Rosans, France). Dimethyldichlorosilane was purchased from VWR Scientific (Philadelphia, PA). CTX was purified from the venom according to the method of Smith *et al.* (24) and dissolved in a saline solution (0.36 mg/ml) for the experiments.

Three different methods of application were used. Namely, (1) Preinjury administration: 0.1 ml of 0.36 mg/ml CTX (which is 0.12 mg/kg body weight) administered IP 30 minutes before contusion; (2) Postinjury administration: the same amount of CTX administered IP 3 minutes after contusion injury; and (3) Topical application: 20 μl of 0.036 mg/ml CTX solution (ten times more dilute than the original solution) applied directly to the exposed spinal cord 30 seconds prior to contusion injury. The LD_{50} for CTX in IP injection was 0.6 mg/kg.

Animals

Sprague-Dawley Rats (male; 275–300 g body wt.) were used. Laminectomy was performed on the spinal cord at the eleventh thoracic vertebra (T-11).

Ion-Sensitive Surface Electrodes

To produce the injury, a weight drop technique developed by Allen (1) and modified by Wrathall *et al.* was used (17,28). In this procedure, a 10 g weight is dropped 5 cm onto an impounder (o.d. 2.4 mm) placed on the exposed spinal cord at T-11. In the original method, this impounder was made with a teflon rod with a tip diameter of 2.4 mm (28). We modified this impounder in order to allow measurement of the ion concentration on the surface of the exposed spinal cord. As shown in Fig. 1, we used a glass tube (o.d. 2.4 mm) with a ceramic filter fused onto the end. The ceramic filter was silane-treated and soaked with an ion sensitive resin manufactured by Fluka (Ronkonkoma, NY). The glass tube was then filled with 100 μM $CaCl_2$ (for calcium measurement) or 100 mM KCl (for potassium measurement). A silver-silver chloride wire was inserted into the salt solution in the glass tube.

The reference electrode was made with the same glass tube assembly except for the omission of both silane treatment and the use of the ion-sensitive resin; the glass tube

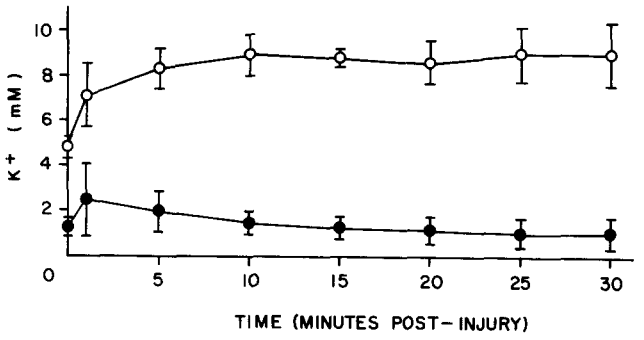

FIG. 3. Changes of surface K^+ concentrations of exposed spinal cord upon dynamic-impact injury (10 g × 5 cm). (○) Injury control and (●) with 0.12 mg/kg CTX (IP) 30 minutes preinjury administration.

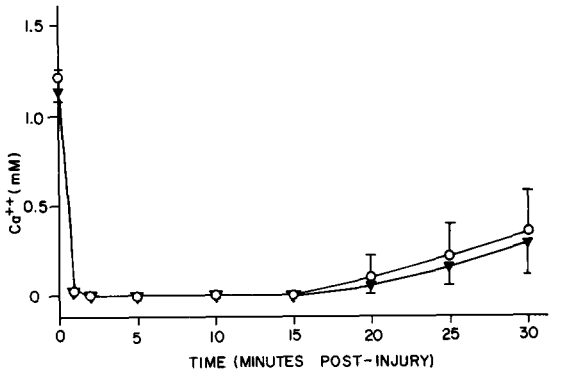

FIG. 4. Changes of surface Ca^2 concentrations of exposed spinal cord upon dynamic-impact injury (10 g × 5 cm). (○) Injury control and (▼) with 0.12 mg/kg CTX (IP) 30 minutes preinjury administration.

was back filled with 4 M NaCl. The reference electrode was placed on the muscle next to the T-11 segment, approximately 5 mm from the exposed cord area. This method enabled us to measure the change in ion concentrations immediately following contusion. Since the impedance of our electrodes was not as high as that of glass microelectrodes, we did not have to use a special high input impedance amplifier. A regular commerical pH meter was used in the mV mode and the output was connected to a strip chart recorder.

Figure 2a shows the calibration for the potassium electrode in which a 47.1 mV change was obtained for a one decade change of potassium concentration. Figure 2b shows the calibration for the calcium electrode; 25.9 mV for each decade of change. Although sensitivities are slightly smaller than the theoretical values (56 mV for KCl and 28 mV for $CaCl_2$), the calibration was reproducible. When electrode was placed on the exposed spinal cord, the reading was stable for 30 minutes if a weight drop was not applied. Sensitivities did not change after impoundment.

Experimental Procedures

Animals were anesthetized with Nembutal (50 mg/kg, IP). The spinal cord was exposed at the T-11 level by laminectomy. The skin wound was temporarily closed and the animal allowed to recover from the effects of laminectomy. Several hours later, the animal was anesthetized with 1.5% halothane in air and CTX was administered IP (the combination of Nembutal and CTX seemed to cause complications). Thirty minutes later, the T-11 segment was again exposed and the impounder was placed on the exposed dura. A 10 gram weight was then dropped 5 cm to hit the impounder. The weight was then removed after 5 seconds, but the impounder remained on the surface of the spinal cord from which the change of either calcium or potassium concentration was recorded. After 30 minutes, the impounder was removed and the skin was sutured closed. The animals were returned to the animal facility after regaining consciousness, and received necessary care for the paralysis resulting from this type of injury (compress bladder to expel urine twice daily).

Behavioral Tests (Tarlov Score)

This test is a modified version of the Tarlov's scoring method for rats (11). The animal is placed in a large open field and is observed and rated for the use of hindlimbs in locomotion. Each hindlimb is observed individually and graded as follows: 0, no movement in hindlimb, no weight bearing; 1, barely perceptible movement in hindlimb, no weight bearing; 2, frequent and/or vigorous movement of hindlimb, no weight bearing; 3, hindlimb can support weight, may take a few steps; 4, walk with only mild deficit; 5, normal walking. In order to minimize subjectivity, a "blind" scoring procedure was employed (experimental treatments unknown to the observer).

Statistical Significance

A data point was calculated from the results from 8 rats, and shown in the figures as the mean±standard deviation (shown by vertical bars). The statistical significance of the difference between two groups was assessed by Wilcoxon's nonparametric test.

RESULTS

Measurements of Surface Ion Concentrations

Figure 3 demonstrates the change of potassium concentration on the surface of the exposed spinal cord after contusion. In control (nontreated) animals, there was an immediate potassium concentration increase, which leveled off after about 10 minutes and was maintained for 30 minutes. In experimental animals which received CTX IP 30 minutes prior to injury, the initial surface potassium concentration was already decreased, and the rise of potassium concentration upon injury was significantly inhibited ($p<0.001$).

Figure 4 shows an example of calcium concentration change in control animals following injury. It indicates that calcium ions quickly fell below the sensitivity of the electrode (10 μM) after the contusion. After 15 minutes, the calcium concentration slowly recovered. CTX did not change movements of calcium ions in a statistically significant way ($p>0.2$).

Recovery of Motor Activity

Figure 5 shows the recovery of motor performance of control and CTX-treated animals after contusion as expressed by the Tarlov scores. When CTX was not applied, the Tarlov score slowly recovered to a level of 1 to 2 after 3 weeks. After 4 weeks, the Tarlov score was 1.88±0.78 (n=8). The animals could not support their body weight.

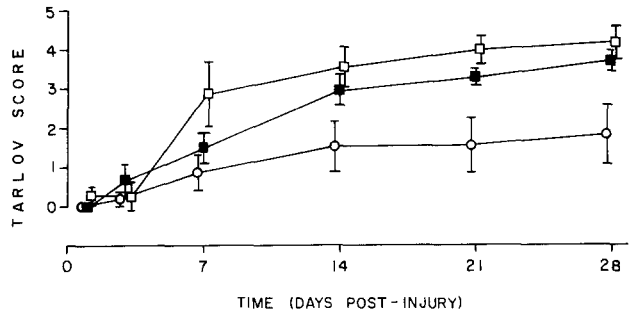

FIG. 5. Individual Tarlov scores for animals evaluated for 4 weeks postinjury. (○) No drug treatment; (□) 30 minutes preinjury administration; and (■) 3 minutes postinjury administration of 0.12 mg/kg IP of CTX.

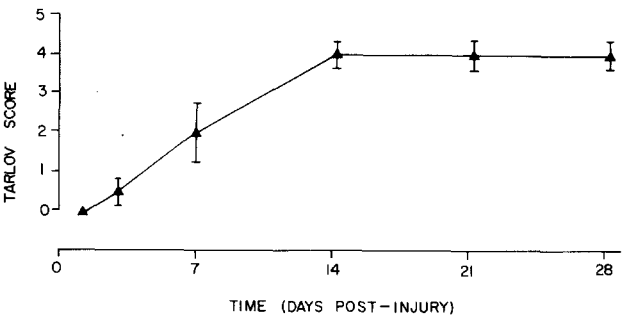

FIG. 6. The motor recovery with topical application of CTX (20 μl of 0.036 mg/ml; 30 seconds before injury).

With IP preinjury administration of CTX, the recovery was remarkable: a week after the contusion, animals were able to support weight on their hindlimbs (Tarlov score 3). After 4 weeks, the Tarlov score was 4.13±0.41 (n=8). The animals could walk with a deficit. The difference between nontreated control and preinjury treated was statistically significant ($p<0.001$).

With postinjury administration, it took approximately two weeks until the Tarlov score reached 3. In 4 weeks, the score reached 3.75±0.25 (n=8). The animals could either support body weight or walk with some deficit. The difference between nontreated and postinjury treated was also significant ($p<0.001$).

With topical application (preinjury administration), recovery was relatively fast (Fig. 6). The Tarlov scores reached 3–4 in two weeks, and 4.0±0.35 (n=8) in 4 weeks. The animals could walk with a deficit. The differences between nontreated and topically-treated was statistically significant ($p<0.001$).

DISCUSSION

Our results clearly demonstrate that CTX is remarkably effective in protecting spinal cord from injury. When injected IP in a single dose thirty minutes before contusion, animals made a significant recovery within 3 weeks. When CTX was applied locally on the injury site 30 seconds before injury, it also provided a high level of protection. A more modest recovery was made following postinjury administration of CTX. These results suggest that the site of action of CTX may be on the neural cell membranes at the injury site.

Since we did not use glass microelectrodes such as were used by other investigators (25, 30, 31), our measurements did not indicate the exact changes in the interstitial ion concentrations. We were measuring the surface concentrations of either calcium or potassium ions on the epidural surface. Preliminary results suggest that the dura and arachnoid mater in the rat may be permeable to ions. We perfused artificial spinal fluid into the subarachnoid space and measured ion concentration changes on the epidural surface using our surface electrode. The electrode responded to changes of ion concentrations of the perfusate (unpublished results). For example, in nontreated animals, an increase of potassium concentration after injury was about 4 mM with our electrode, while the other investigators have obtained a change of about 50 mM with a glass microelectrode (30). Therefore, we used our electrodes to obtain qualitative data on ion movements. However, our electrode has several advantages over glass microelectrodes. Namely, (a) it is easy to prepare, (b) it does not require a high input impedance amplifier (a commericial pH meter can be used in the mV mode, and the output of the pH meter can be directly connected to a strip chart recorder, and (c) the diameter of the electrode was fabricated to 2.4 mm so that the electrode itself could be used as an impounder. This enabled us to measure the changes of ion concentrations immediately after impounding.

It has been suggested that entering calcium ions activate enzymatic disintegration of the cell membranes and eventually cause cell necrosis (4, 10, 23, 27). As shown in Fig. 4. CTX did not inhibit calcium entry, but did prevent potassium efflux. Remarkable recovery of motor activity as evidenced by the Tarlov scores indicated that the inhibition of potassium efflux has a beneficial effect in protecting the neural cells. We also tried postinjury administration of CTX (IP administration 3 minutes after injury). As shown in Fig. 5, the recovery of motor performance was less remarkable than that in preinjury administration.

Quinine, another inhibitor for calcium-induced potassium efflux (19), has also been found to protect the spinal cord against injury (20). Preliminary histological analysis suggests that the section of spinal cord at the epicenter in CTX-treated rats sustains less damage (unpublished results). At least two possible mechanisms of protection could be postulated for CTX and quinine: (1) since an increase of extracellular potassium concentration depolarizes neural cell membranes and inhibits nervous conduction, the inhibition of potassium efflux would prevent conduction failures; (2) an inhibition of potassium efflux may reduce the amount of sodium influx, and therefore prevent edema formation. Since CTX is a newly discovered compound, its pharmacologic action has not been extensively studied. Therefore, we cannot rule out the possibility that CTX might have still some other effect through which it provides protection to the spinal cord. Further studies are needed to elucidate the protective mechanism of CTX.

Many investigators have demonstrated that both calcium influx and potassium efflux take place upon spinal cord injury as well as in cerebral ischemia (3, 14, 25, 30–32). However, the relationship between calcium and potassium movements has not been well studied. Our results suggest that calcium-activated potassium efflux may play an important role in the mechanism of spinal cell necrosis and paraplegia in animals. We also have preliminary evidence that CTX provides protection against brain ischemia in the rat (22,26). Studies to assess the efficacy of CTX in other ischemia models are in progress in our laboratory.

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