ACTIONS OF CRYSTALLINE TETRODOTOXIN, PROCAINE, ESERINE AND NEOSTIGMINE ON THE ELECTRICAL ACTIVITIES OF CRAYFISH MUSCLE FIBERS

YASUMI OGURA, YOSHIO WATANABE AND YOKO MORI

*Department of Pharmacology and Toxicology, Institute of Food Microbiology,
Chiba University, Narashino, Chiba*

Received for publication November 15, 1965

In Japan, it is well known that Fugu-poisoning holds the first rank in the mortality from the food-intoxication. This fact also could be confirmed by Ogura (1) in the statistical survey on the Fugu-poisoning that have happened in the past eighty years. This shows that the cases of this intoxication are over one hundred patients every year, and the mortality amounted to more than sixty per cent. The death was due to central and peripheral respiratory failure. The neurotoxic principle is localized in general in the liver and ovaries of noxious Fugu. In 1909, Tahara (2) succeeded in obtaining fairly pure toxin, and named it "Tetrodotoxin". After that, Yokoo (3) in 1950 first reported the isolation of crystalline toxin, and Tsuda *et al.* (4) in 1952 established the method of mass-production of this crystalline toxin in a different procedure. The chemical structure of this toxin, as illustrated in Fig. 1, was presented by Tsuda *et al.* (5) in 1964, and at the same time supported with Hirata *et al.* (6) and Woodward (7). Recently, accord-

STRUCTURE OF CRYSTALLINE TETRODOTOXIN
(TSUDA)

FIG. 1. Structure of crystalline tetrodotoxin (by Tsuda).

ing to Mosher *et al.* (8), tarichatoxin, isolated from the eggs of the California newt, *Taricha torosa*, has also the same structure as tetrodotoxin.

A series of pharmacological studies of crystalline tetrodotoxin in our laboratory have been carrying out since 1955. In these investigations, we were the first to find that, on the experiments of the isolated guinea pig ileum, tetrodotoxin suppressed completely the contractile response to nicotine and partly that to 5-hydroxytryptamine, but in this concentration no influence to acetylcholine and histamine (9, 10). Thereafter, the experiments have carried forward the inhibitory action on the autonomic ganglion by tetrodotoxin (11-17). It was concluded that tetrodotoxin had an inhibitory action on the process of transmission in the ganglion.

On the other part, Cheymol, Bourillet and Ogura (18) and Ogura (15, 19) have reported that tetrodotoxin decreased the amounts of acetylcholine released from the nerve-endings by tetanic stimulations of the phrenic nerve on the isolated rat diaphragm-nerve preparation. The same action of tetrodotoxin was presented independently also by Fleisher *et al.* (20).

In the previous paper, Ogura and Mori (21) was reported that, in the isolated opener muscle excitatory axon-muscle preparation of crayfish, tetrodotoxin blocked the potentiation of tetraethylammonium bromide on the contractile response and, after tetraethylammonium bromide, inhibitory effect of tetrodotoxin was produced to the maximum even in doses of 10^{-4} or 10^{-3} g/ml, and suggested that the action of tetrodotoxin on the crayfish contractile response may be related to its effect on the motor axon terminals.

However, the question remains on the direct action of tetrodotoxin on the crayfish muscle fibers, because there have been many reports about differences of the electrical properties of crustacean muscle and other ordinarily electrically excitable tissues such as squid giant axon and frog skeletal muscle. All-or-none spikes are rarely produced in the crustacean muscle fiber. Fatt and Katz (22) found that some marine crustacean muscles became all-or-none excitable after treatment with several substances, e.g. tetraethylammonium ions and even procaine. Fatt and Ginsborg (27) suggested that Ca^{++} was the ion carrying inward positive charge during the electrical response of the crayfish muscle. Werman *et al.* (23) showed that lobster muscle pretreated with procaine and some other substances could develop prolonged spikes in an urea or sucrose medium. Barnacle muscle fiber, as reported by Hagiwara and Naka (24), is electrically excitable in all-or-none Ca^{++} spike, especially if inside is worked with EDTA. In short, the electrical properties of crustacean muscle fibers surely differ from those in ordinarily electrically excitable tissue. On the other side, tetrodotoxin in very low concentrations usually blocks impulse conduction in peripheral nerve and skeletal muscle. This phenomenon has been explained by Narahashi *et al.* (25) as a result of selective inhibition of the sodium carrying mechanism in the excitable cell membrane. Consequently it was expected that tetrodotoxin would show different effects on crustacean muscle from that seen in ordinary excitable tissue.

In the present experiments, the actions of tetrodotoxin, procaine and some other

substances on the electrical properties, especially excitability of crayfish muscle were studied in various ionic environments.

MATERIAL AND METHODS

The abductor muscle of the cheliped of the crayfish, *Procambarus clarkii*, was used throughout this study. Preparation was made by the procedure described by Murayama and Yamashita (26).

Two KCl filled microelectrodes for stimulation and recording were inserted into a single muscle fiber under microscopic observation. The current employed for intracellular stimulation or for measurement of the resistance was monitored as voltage drop across a precise series resistor.

The normal bathing solution was van Harreveld's solution; Na^+ : 205.1 mM, K^+ : 5.4 mM, Ca^{++} : 13.5 mM, Mg^{++} : 2.6 mM, Cl^- : 242.7 mM and the pH was kept near 7.5 with 0.2 M Tris maleate buffer. Na^+ -free solution and Ca^{++} -free solution were made by replacing respectively NaCl and CaCl_2 with sucrose. In the test solution, Na^+ -concentration was reduced in the Ca^{++} -free solution and Ca^{++} -concentration was varied in the Na^+ -free solution by substitution with sucrose. The crystalline tetrodotoxin (RTE 3-4, Sankyo Co.) used was kindly provided by Dr. Tsuda of University of Tokyo, Tokyo, Japan. All experiments were carried out at room temperature (20°C–30°C).

RESULTS

1) *Untreated muscles*

The crayfish muscle infrequently shows all-or-none spike responses in normal van Harreveld's solution. When rectangular current pulses were applied through a intracellular microelectrode, the muscle fiber responded with graded depolarizations. Occasionally they were accompanied by rapid small potential changes in the form of a damped oscillation on the initial part of the depolarization, as described by Fatt and Ginsborg (27) and Werman and Grundfest (28). On only a few occasions, all-or-none spikes produced on depolarizing a fiber in van Harreveld's solution.

2) *Development of all-or-none spike potential after treatment with tetrodotoxin and some other substances*

It has already been observed by Fatt and Katz (22) and by Reuben and Grundfest (29) that the normal graded responses of some crustacean muscle fibers could be converted to all-or-none spikes by treating with procaine (Fig. 2, G and H). Adding procaine to a muscle bathed already in nonionic media did not lead to spikes (30). However, on the basis of results which follow, it seems that calcium ions in the surrounding media has no clear causal relation to the development of the spike, since spikes were produced in Ca^{++} -free Harreveld's solution. Similar phenomena resulted from treatment with tetrodotoxin (10^{-5} g/ml), as well as with neostigmine (10^{-5} g/ml) and eserine (10^{-5} g/ml). The high concentrations of these substances were used in our experiments, but in the case of tetrodotoxin, such a phenomenon was recognized rarely in the very low

FIG. 2. A-D: Spike activity in crayfish muscle treated with tetrodotoxin (10^{-5} g/ml). A: Before treatment. B: 10 minutes after treatment. C: 60 minutes after treatment. D: 90 minutes after treatment. E: A spike in muscle treated with eserine (10^{-5} g/ml). F: A spike in muscle treated with neostigmine (10^{-5} g/ml). G and H: Spikes in muscle treated with procaine (10^{-3} g/ml); G: 50 minutes after treatment. H: 70 minutes after treatment. The zero level of the current trace represents zero membrane potential.

concentration (10^{-3} g/ml). Procaine, among the four substances, produced very easily this phenomenon.

Usually it was difficult to determine accurately values for spike amplitude, height of overshoot, threshold depolarization level and spike duration, because these parameters always changed gradually, showing a tendency to increase as shown in Fig. 2, A-D, G and H. Another remarkable effect of treatment with these agents was change of the resting membrane resistance (Fig. 3). When the graded response began to appear, as shown in Fig. 2, B, the resting membrane resistance always decreased to smaller value than that of untreated membrane which had only graded responses. After that, the resistance of the membrane treated with procaine scarcely changed, as illustrated by

FIG. 3. Voltage-current relations of muscle fibers treated with three spike-producing agents; tetrodotoxin (10^{-5} g/ml), eserine (10^{-5} g/ml) and procaine (10^{-3} g/ml) at various time of the treatment, positive ordinates represent depolarization and positive abscissa represent outward current.

FIG. 4. Effects of varying calcium-concentration in a sodium-free medium. A-C : Spikes in a muscle treated with procaine (10^{-3} g/ml). A : A spike in the 'normal van Harreveld's solution. Note the prolongation of the spike duration (compare with the spikes in Fig. 2, G and H). B : A spike produced in a van Harreveld's solution where all sodium ions were replaced with equiosmotic sucrose. C : A spike in the sodium-free solution containing fourfold calcium ions. D-F : Spikes in another muscle treated with procaine (10^{-3} g/ml). D : A spike in solution containing normal calcium concentration. E : A spike in fourfold calcium. F : A response in presence of eightfold calcium.

Grundfest (30), (Fig. 3, C), while the resistance of the membrane treated with tetrodotoxin and eserine always increased gradually again during several ten minutes. However the all-or-none spike was not abolished even though membrane resistance increased more than the value before treating with agents.

Effects of Ca^{++} -free and Na^{+} -reduction: Complete removal of calcium ions caused disappearance of the overshoot of the spike (Fig. 6, G and H), although such effects were usually incomplete unless some calcium binding substance, e.g. EDTA, was used. Open marks in Fig. 5 show the effects of Na^{+} -reduction in the Ca^{++} -free Harreveld's solution. The membrane potential and the spike height did not remarkably change in Na^{+} -reduction from 200 to 10 mM. The spike response immediately stopped in the Na^{+} - and Ca^{++} -free media, which was an isotonic solution containing KCl and MgCl_2 of normal concentration and sucrose. While in the isotonic Tris solution the spike response did not cease (Fig. 6, C and D).

Effects of Na^{+} -free and varying Ca^{++} -concentration: When the muscle was exposed to the Na^{+} -free Harreveld's solution, in which all NaCl was replaced with isotonic sucrose, the spike response did not show any change in either the spike height, the overshoot or the threshold (Fig. 4, B and D); although the spike duration always shortened when it had been prolonged for long treatment with these agents, as shown in Fig. 4, A and B. Fig.

FIG. 5. Effects of varying sodium concentration (open marks) on the resting membrane potential (large circles), the threshold depolarization (small circles) and the active membrane potential (triangles), in the muscles treated with procaine (10^{-8} g/ml). Ordinate: Voltage difference from the surrounding medium. Abscissa: Concentrations of sodium and calcium ions in the surrounding medium. Broken line in the figure represents the slope of 29 mV for a tenfold increase in Ca^{++} -concentration.

FIG. 6. Effects of calcium-free and excess calcium. A: A spike in calcium-free van Harreveld's solution. B: A spike in a solution of 40 mM calcium added to van Harreveld's solution in the same muscle as A. C: A spike in isotonic Tris solution. D: A spike in a solution of 28 mM-calcium added to Tris solution in the same muscle as C. E-H: Change of configuration of spike potential in a calcium solution containing potassium and magnesium of normal concentration, after 2 minutes (E), 5 minutes (F), 10 minutes (G) and 15 minutes (H) from exposure to the test solution.

5 shows the membrane potential, the threshold depolarization level and the spike amplitude as functions of external Ca^{++} -concentration (solid marks) varied from 0.5 to 100 mM. It must be noticed that these are not simple monotonic functions of external Ca^{++} -concentration. Decreasing of the external Ca^{++} -concentration less than about 5 mM always caused decrement of the spike overshoot, while the resting potential remained at a constant level. The changing rate of overshoot fitting in with the line of 29 mV for a tenfold increase in Ca^{++} -concentration as indicated by the broken line in Fig. 5. On the other hand, increasing the external Ca^{++} -concentration above about 5 mM produced a consistent increment of the resting membrane potential and a slight decrease of the overshoot (Fig. 4, D, E and F), although there were some cases in which the overshoot increased (Fig. 4, B and C).

The increase of the overshoot in crustacean muscle spike by excess external calcium has been already described by Fatt and Ginsborg (27), and by Hagiwara and Naka (24). But there was a remarkable difference of the effective Ca^{++} -concentration between the results obtained from barnacle muscle by Hagiwara and Naka (24) and the shift of the observed overshoot from the expected one on the line of 29 mV for a tenfold Ca^{++} -increase in the crayfish muscle occurred at much lower Ca^{++} -concentrations than the barnacle. Consequently, in excess calcium, the two muscles considerably differed from each other in changes of the overshoot and membrane potential with increase of the external Ca^{++} -concentration; increment of the overshoot was not always effected in excess calcium in the crayfish muscle (Fig. 4, D, E and F), although the threshold membrane potential always shifted in a positive direction. Such decrease of the overshoot was clearly observed when all sodium ions in the van Harreveld's solution were replaced by

FIG. 7. Potentiating effect of sodium on the spike depressed in the calcium solution. A : A spike in van Harreveld's solution. B and C : Depression of the spike in high calcium solution. D-F : Recovery of the spike by addition of sodium (D : 8 mM- Na^+ , E and F : 28 mM- Na^+) to the surrounding calcium solution.

calcium ions (Fig. 6, E-H). Because the overshoot decreased in spite of increase of the threshold, configuration of the response was very similar to one before treatment with the agent; small spikes were left in the form of damped oscillation.

On the other hand, a more striking increase of the overshoot was found rather clearly in the solution which contained sodium with calcium than in the case of the isotonic calcium solution (Fig. 6, A-D). When isotonic sodium solution was added to the external isotonic calcium solution after abolishing of the spike, the spike always recovered and the overshoot increased in spite of a net decrease of the external calcium concentration (Fig. 7). Such a phenomenon never occurred with increasing of sodium in Ca^{++} -free solution. Therefore it seems that the increase of the overshoot is not due to Na^+ alone, but to simultaneous presence of both ions.

DISCUSSION

Effects of environmental ionic changes on the spike activity in the present experiments were remarkably similar to those of the smooth muscle of taenia coli of guinea-pig (31). The smooth muscle generates a spike in sodium deficient or in sodium-free solution. The size and the overshoot of the spike does not depend on the external Na^+ -concentration over wide range. However when calcium was absent in the surrounding solution (containing only sucrose), the spike activity quickly stopped. Bülbring and Kuriyama (31) proposed two possible explanations for action of calcium on the smooth muscle: 1) the external calcium may prevent the leakage of sodium through the resting membrane so that triggering of sodium remaining in the tissue, and 2) the calcium ions themselves may carry the change and generate the action potential in the absence of sodium.

The results shown in Fig. 5 indicate that the above mentioned second explanation is suitable for the spike generation in the crayfish muscles treated with those agents, because they had an initial slope close to that expected for a Ca^{++} -electrode, just as in the barnacle muscle (24). However, there is a difference between the both muscles; in the normal size spike can be initiated in lower Ca^{++} -concentration than the case of the latter. Furthermore, in the crayfish muscle treated with the spike inducing agents and also in the smooth muscle of the taenia coli, it seems that the sodium ions also can be the carrier of the positive charge for the spike activity in Ca^{++} -free solution.

Tetrodotoxin is the inhibitor of the Na^+ -carrying mechanism in the ordinary excitable tissue. In spite of such an action, in relatively high concentration, it led to spikes in the crayfish muscle and had no effect on the spontaneous spikes in the smooth muscle of taenia coli (31-33). Therefore the Na^+ -carrying mechanism in those muscles may differ from that in ordinary excitable tissue.

SUMMARY

Electrical properties were studied in the muscle fiber membrane treated with tetrodotoxin and some other substances in the crayfish, *Procambarus clarkii*.

1. All-or-none spike potentials were produced in some muscle fibers treated with tetrodotoxin, neostigmine, eserine and procaine (10^{-5} – 10^{-3} g/ml); while the normal muscle fibers exhibited graded depolarization. Such a phenomenon was produced very easily with procaine, and three other substances were in the weak activity in comparison with procaine.

2. Na^+ -reduction in the Ca^{++} -free Harreveld's solution had no effect on the resting membrane potential and the amplitude of the spike, which was induced by the agents, in the range of Na^+ -concentration from 10 to 200 mM. The overshoot of the spike tended to disappear on removal of external calcium.

3. The overshoot of the spike potential increased with increasing external Ca^{++} -concentration in the range from 0.5 to 5 mM along a slope of 29 mV for a tenfold Ca^{++} -increase in the solution, in which all sodium ions were replaced with sucrose. Removal of Na^+ from the Harreveld's solution had no effect on the resting membrane potential, the overshoot and the threshold depolarization. In the excess calcium and sodium-free solution, the resting membrane potential and the threshold depolarization level remarkably increased. The overshoot showed a slight decrease at most, but in a few cases it actually showed a slight increase.

4. The spike potential was suppressed when all external sodium ions were replaced with calcium ions. This suppressed potential recovered after addition of external sodium. Then the overshoot remarkably increased in spite of decrease of external net Ca^{++} -concentration. It is supposed that simultaneous presence of both the ions caused the striking increase of the spike height.

REFERENCES

- 1) OGURA, Y. : *Ann. Rept. Inst. Food Microbiol. (Chiba Univ.)* **11**, 79 (1958)
- 2) TAHARA, R. : *J. Pharm. Japon.* **29**, 587 (1909)
- 3) YOKOO, A. : *J. Chem. Soc. Japan.* **71**, 590 (1950)
- 4) TSUDA, K. AND KAWAMURA, M. : *J. Pharm. Soc. Japan.* **71**, 282 (1952)
- 5) TSUDA, K., TACHIKAWA, R., SOKAI, K., TAMURA, C., AMAKATSU, O., KAWAMURA, M. AND IKUMA, S. : *Chem. Pharm. Bull.* **12**, 642 (1964)
- 6) GOTO, T., KISHI, Y., TAKAHASHI, S. AND HIRATA, Y. : *Tetrahedron letters* **14**, 779 (1964)
- 7) WOODWARD, R.B. : *Pure Appl. Chem.* **9**, 49 (1964)
- 8) MOSHER, H.S., FUHRMAN, F.A., BUCHWALD, H.D. AND FISHER, H.G. : *Science* **144**, 1100 (1964)
- 9) OGURA, Y. : *Seitai no Kagaku* **9**, 281 (1958)
- 10) HAMADA, J. : *Ann. Rept. Inst. Food Microbiol. (Chiba Univ.)* **10**, 73 (1957)
- 11) TSUKADA, O. : *J. Chiba Med. Soc.* **36**, 1369 (1960)
- 12) OGURA, Y. AND HORI, H. : *Folia pharmacol. japon.* **57**, 274 (1961)
- 13) OGURA, Y. AND NAKAJIMA, S. : *Ann. Rept. Inst. Food Microbiol. (Chiba Univ.)* **16**, 62 (1963)
- 14) OGURA, Y. : *Fugu-poisoning, in Food Hygien (Shokuhin Eiseigaku)* P. 186, Asakura Co., Tokyo (1963)
- 15) OGURA, Y. : *Folia pharmacol. japon.* **59**, 61§ (1963)
- 16) OGURA, Y., HAMADA, J. AND TSUKADA, O. : *Ibid.* **56**, 35 (1960)
- 17) OGURA, Y. : *Ann. Rept. Inst. Food Microbiol. (Chiba Univ.)* **15**, 93 (1963)
- 18) CHEYMOL, J., BOURILLET, F. AND OGURA, Y. : *Arch. int. Pharmacodyn.* **139**, 187 (1962)

- 19) OGURA, Y. : *Contribution à l'étude du mécanisme d'action de la tétrodotoxine au niveau de la jonction neuromusculaire, Thèse de l'université de Paris (France)* (1962)
- 20) FLEISHER, J.H., KILLOS, P.J. AND HARRISON, C.S. : *J. Pharmacol.* **133**, 98 (1961)
- 21) OGURA, Y. AND MORI, Y. : *Ann. Rept. Inst. Food Microbiol. (Chiba Univ.)* **16**, 64 (1963)
- 22) FATT, P. AND KAZT, B. : *J. Physiol.* **120**, 171 (1953)
- 23) WERMAN, R., REUBEN, J.P. AND GRUNDFEST, H. : *Biol. Bull.* **199**, 347 (1960)
- 24) HAGIWARA, S. AND NAKA, K. : *J. Gen. Physiol.* **48**, 141 (1964)
- 25) NARAHASHI, T., MOORE, J.W. AND SCOTT, W.R. : *J. Gen. Physiol.* **47**, 965 (1964)
- 26) MURAYAMA, K. AND YAMASHITA, Y. : *J. Fac. Sci. Hokkaido Univ. Ser. VI, Zool.* **15**, 123 (1962)
- 27) FATT, P. AND GINSBORG, B.L. : *J. Physiol.* **142**, 516 (1958)
- 28) WERMAN, R. AND GRUNDFEST, H. : *J. Gen. Physiol.* **44**, 997 (1961)
- 29) REUBEN, J.P. AND GRUNDFEST, H. : *Biol. Bull.* **119**, 335 (1960)
- 30) GRUNDFEST, H. : *Ann. N.Y. Acad. Sci.* **94**, 409 (1961)
- 31) BÜLBRING, E. AND KURIYAMA, H. : *J. Physiol.* **166**, 29 (1963)
- 32) NONOMURA, Y. AND OHASHI, H. : *Folia pharmacol. japon.* **61**, 81§ (1965)
- 33) OGURA, Y., MORI, Y. AND WATANABE, Y. : to be published