AN EXPLANATION FOR THE APPARENT CHELATION OF CALCIUM BY TETRODOTOXIN*

E. W. Hopkins, III and E. J. Herbst
Department of Biochemistry, University of New Hampshire
Durham, New Hampshire

Received February 1, 1968

The proposal submitted by Ohnishi and Ishida (1967) that tetrodotoxin (TTX), the neurotoxin from the California newt and the Japanese Fugu or puffer fish (Mosher et al., 1964), manifests its effects by a calcium-chelating mechanism was investigated and is questioned on several grounds. Our results indicate that sodium citrate, a preservative present in the commercially available TTX preparation, is responsible for the apparent calcium-binding shown by TTX.

Tetrodotoxin and saxitoxin (STX), the paralytic shellfish poison produced by the dinoflagellate Gonyaulax catenella (Schantz et al., 1966), have been shown to block the early flow of cations responsible for the rising phase of the action potential in nerve and skeletal muscle (Narahashi et al., 1964; Kao and Nishiyama, 1965; Tasaki et al., 1966; Moore et al., 1967; Narahashi et al., 1967b; and Rojas and Atwater, 1967).

A role for calcium ions in the production of action potentials has been postulated by Tasaki and Singer (1966). This theory depicts the action potential as being triggered by the displacement of calcium from the external surface of the membrane by the outward flow of potassium ions. This ion-

* This work supported by a National Science Foundation Traineeship GZ-144. Published with the approval of the Director of New Hampshire Agricultural Experiment Station as Scientific Contribution No. 428.

exchange event is believed to produce phase transitions in the membrane macromolecules resulting in alterations of the permeability properties of the membrane with subsequent generation of the action potential.

Ohnishi and Ishida reported that TTX binds calcium in a ratio of 1:2 (TTX:Ca). They suggested that the formation of a complex involving TTX, calcium ions and membrane phospholipids might act to block the triggering mechanism of the action potential by holding Ca ions more firmly to the membrane, thus precluding their displacement by K ions. Wide acceptance of this interpretation is unwarranted since Kao (1966) has admonished that the commercially available TTX preparation contains 500 μg sodium citrate for each 100 μg TTX. This statement was confirmed by Sankyo Co., LTD (Personal communication). In view of this fact, we deemed it advisable to compare TTX and sodium citrate in the experimental system employed by Ohnishi and Ishida.

MATERIALS AND METHODS

Calcium-chelation was monitored by titrating a solution of calcium with the suspected chelating agent in the presence of Murexide, a calcium-indicator. The difference in absorbance of Murexide at 507 mμ and at 542 mμ is a function of the amount of free Ca in solution (Ohnishi and Ishida, 1967). Absorbances were measured in a Beckman DU-2 spectrophotometer.

Glass-distilled water was employed throughout. Crystalline TTX was obtained from Sankyo Co., LTD through Calbiochem. Saxitoxin was generously supplied by Dr. E. J. Schantz, Ft. Detrick, Md. Murexide was purchased from Matheson Coleman & Bell.

RESULTS

The titration of Ca by TTX illustrated by the graphical procedure employed by Ohnishi and Ishida appears in the top portion of Fig. 1. Extrapolation of the linear portion of this curve to its intersection with the ordinate indicates a binding ratio of 1:2 (TTX:Ca).

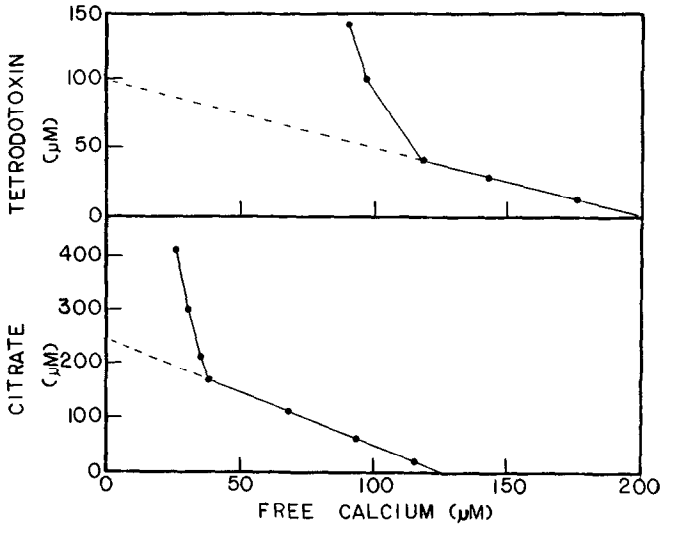


Fig. 1. Titration of a calcium solution by TTX (upper half) and by sodium citrate (lower half). Tris-HCl (pH 8.0), 20 mM; Murexide, 300 μM, 25°C.

The Sankyo preparation of TTX (m.w. 319) contains 500 μg sodium citrate (m.w. 294.11) for every 100 μg TTX, or 100 μmoles TTX contains 540 μmoles sodium citrate. The titration of Ca ions by sodium citrate is shown in the lower half of Fig. 1. Extrapolation of this curve reveals a binding ratio of 2:1 (citrate:Ca).

The TTX concentration range employed was 0-200 μM. On the basis of 1.0 μmole TTX containing 5.4 μmoles sodium citrate, the range of concentration of sodium citrate was 0-1080 μM.

In sharp contrast, saxitoxin (0-90 μM), which is chemically and pharmacologically quite similar to TTX (Kao and Fuhrman, 1967; Narahashi et al., 1967b) does not bind calcium under the same experimental conditions used for TTX.

Ohnishi and Ishida stated that calcium-binding by TTX was reduced when the pH was lowered below 8.0. Fig. 2 shows the effect of pH on Ca-binding by sodium citrate. A binding ratio of 10:7 (citrate:Ca) is indicated at pH 8.4; however, at pH 7.0 a ratio of 45:10 is obtained.

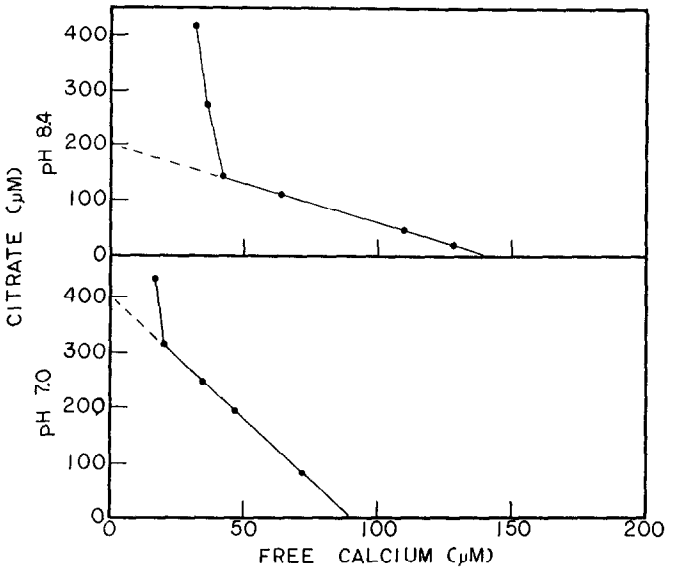
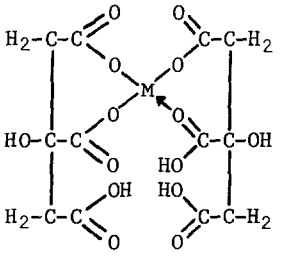


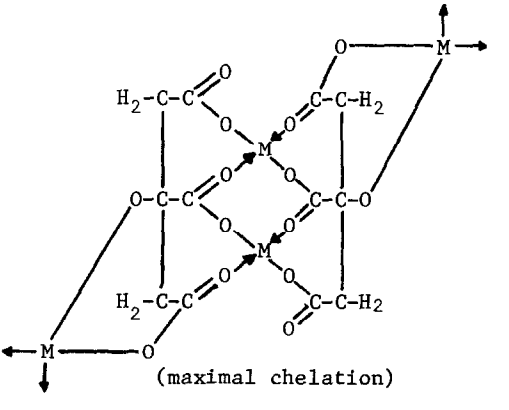
Fig. 2. Effect of pH on Ca-binding by sodium citrate. Experimental conditions are those of Fig. 1. except the pH is 8.4 (upper half) and 7.0 (lower half).

DISCUSSION

Our data indicate that the results of Ohnishi and Ishida must be reinterpreted. Since 100 μmoles of TTX also contains 540 μmoles of sodium citrate it is clear that the binding ratio of 1:2 (TTX:Ca) is artifactual and merely reflects the citrate:Ca ratio of 2:1 as illustrated in Fig. 1. This binding ratio is in agreement with the theoretical minimal potential of citrate for metal-chelation (Parry and Dubois, 1952):



(minimal chelation)



(maximal chelation)

That saxitoxin (as the HCl-salt) does not bind calcium is further evidence against a TTX-Ca chelate. Although it is possible that this could be one of the few clear distinctions between the two toxins, it is highly unlikely.

The finding that pH and Ca-chelation are related in a direct manner is in sharp contrast to the known pharmacological behavior of TTX. Camougis et al. (1967) found that over the pH range 7.05 to 8.90 action potential inhibition by TTX was greater at lower pH levels. This apparent contradiction may be ascribed to sodium citrate (Fig. 2). Ionization and hence chelate formation of the carboxyl and hydroxyl groups of the citrate molecule is clearly related in an inverse manner to the pH. The importance of the hydroxyl groups in the TTX molecule is probably related to their great influence on the positive charge of the guanidinium group (Narahashi et al., 1967a). The bulky nature of the TTX molecule also argues against its being a satisfactory ligand.

Finally, investigations of excitation processes driven by a calcium or other divalent cation mechanism have revealed that neither TTX nor STX has any blocking effect (Hagiwara and Nakajima, 1965; Nonomura et al., 1966; Hagiwara and Nakajima, 1966; Koketsu and Nishi, 1966; Reuben et al., 1967; and Coraboeuf and Vassort, 1967). Tetrodotoxin has no effect on miniature end-plate potentials (MEPP's) or on the release of transmitter substances at neuromuscular junctions (Gershon, 1967). These are phenomena believed to be driven by a calcium mechanism (Del Castillo and Stark, 1952).

REFERENCES

Camougis, G., Takman, B.H., and Tasse, J.R.P., Science 156, 1625 (1967).
Del Castillo, F.F. and Stark, W.Y., J. Physiol. 116, 507 (1952).
Coraboeuf, E. and Vassort, G., Compt. Rend. Acad. Sci. (Paris), 264, 1072 (1967).
Gershon, M.D., Brit. J. Pharm. Chemother. 29, 259 (1967).
Hagiwara, S. and Nakajima, S., Science 149, 1254 (1965).
Hagiwara, S. and Nakajima, S., J. Gen. Physiol. 49, 793 (1966).
Kao, C.Y. and Nishiyama, A., J. Physiol. 180, 50 (1965).
Kao, C.Y., Pharmacol. Rev. 18, 997 (1966).
Kao, C.Y. and Fuhrman, F.A., Toxicon 5, 25 (1967).

Koketsu, K. and Nishi, S., Life Sciences 5, 2341 (1966).
Moore, J.W., Blaustein, M.P., Anderson, N.C., and Narahashi, T., J. Gen. Physiol. 50, 1401 (1967).
Mosher, H.S., Fuhrman; F.A., Buchwald, H.D., and Fischer, H.G., Science 144, 1100 (1964).
Narahashi, T., Moore, J.W., and Scott, W.R., J. Gen. Physiol. 47, 965 (1964).
Narahashi, T., Moore, J.W., and Poston, R.N., Science 156, 976 (1967a).
Narahashi, T., Haas, H.G., and Therrien, E.F., Science 157, 1441 (1967b).
Nonomura, Y., Hotta, Y., and Ohashi, H., Science 152, 97 (1966).
Ohnishi, T. and Ishida, A., Biochem. Biophys. Res. Commun. 27, 552 (1967).
Parry, R.W. and Dubois, F.W., J. Amer. Chem. Soc. 74, 3749 (1952).
Reuben, J.P., Brandt, P.W., Girardier, L., and Grundfest, H., Science 155, 1263 (1967).
Rojas, E. and Atwater, I., Proc. Nat. Acad. Sci. U.S., 57, 1350 (1967).
Schantz, E.J., Lynch, J.M., Vayvada, G., Matsumoto, K., and Rapoport, H., Biochemistry 5, 1191 (1966).
Tasaki, I., Singer, I., and Watanabe, A., Amer. J. Physiol. 211, 746 (1966).
Tasaki, I. and Singer, I., Ann. N. Y. Acad. Sci., 137, 792 (1966).