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Inhibition of the receptor for tetrodotoxin in nerve membranes by reagents modifying carboxyl groups

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SUMMARY

Tetrodotoxin binding to crab nerve fibers was measured by the bio-assay technique. Modification of fibers by a carbodiimide and nucleophile at acid pH resulted in a loss of 85% of the normal tetrodotoxin binding. Results of control experiments suggested that the inhibition of tetrodotoxin binding may be due to modification of carboxyl groups at or near the tetrodotoxin receptor.

Tetrodotoxin is a highly specific and potent inhibitor of the transient increase in sodium conductance in excitable membranes[1–4]. The exact structural relationship between the tetrodotoxin receptor and the sodium channel is unknown. However, available evidence suggests that one tetrodotoxin molecule blocks one site, that the receptor is intimately associated with the sodium channel, and that binding of tetrodotoxin does not affect the gating control of conductance[1–8]. The tetrodotoxin molecule includes a guanidinium group[9], and the toxin bears a net positive charge at neutral pH. Since guanidine can substitute for sodium to some extent in nerve[10] it has been postulated that the blocking action of tetrodotoxin results from entry of the guanidinium group in the sodium channel, together with the binding of other portions of the toxin to neighboring membrane groups, possibly *via* hydrogen bonds[5,6].

Studying the pH dependence of $\bar{g}_{Na}$ Hille[11] concluded that an ionized acidic group with a pK of 5.2 constituted a part of the sodium channel and hence of the receptor for tetrodotoxin. A likely candidate for this component would be a carboxyl group and Hille

Abbreviation: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

has included an ionized carboxyl group as an integral part of a recent model for the sodium channel[10]. A phosphate group is suggested as a possible alternative.

If this model is correct, then modification of the nerve membrane by reagents capable of blocking carboxyl or phosphate groups should have strong effects on the binding of tetrodotoxin to the sodium channel. We have tested this idea using the reaction scheme of Hoare and Koshland[12]. In this method carboxyl groups react first (reversibly) with a water soluble carbodiimide, followed by attack by a nucleophile to form a stable derivative. The final result is a quantitative covalent linkage of the carboxyl group to the nucleophile, provided that the latter is present in sufficiently high concentrations (on the order of 0.25–1 M). The method is reasonably specific but some side reactions with other amino acid side chains can occur.

The binding of tetrodotoxin to crab claw nerve fibers was measured using a modification of the bio-assay methods of Moore *et al.*[13] and Keynes *et al.*[7]. When compared to the binding of tritiated tetrodotoxin, the bio-assay has the advantage of freedom from uncertainties due to radioactive impurities[14,15] but does not readily allow an estimation of the relative contribution from non-specific binding[15]. This will be discussed further below. The nerve from a walking leg of the blue crab *Callinectes sapidus* was dissected by the pulling out method[16] and mounted in a plexiglas chamber fitted with platinum electrodes for external recording (Fig.1, left). A small (2 mm) segment of the nerve, at the site of the recording electrode, was isolated by two air gaps. A dose–response curve for tetrodotoxin was then obtained for this nerve by applying 20-μl amounts of various concentrations of toxin in artificial sea water (NaCl 449 mM, KCl 9 mM, $MgCl_2$ 45 mM, $CaCl_2$ 9 mM Tris–HCl, 10 mM, pH 7.5) to the isolated segment (Fig.1, right). Gentle stirring was maintained by insertion of a small, rotating plexiglas paddle. Experiments were done at room temperature. The reduction in the compound action potential was over 90% complete within 10 min and this time was chosen as a constant in all applications of toxin. Following each dose of tetrodotoxin, the toxin was washed off by continuously flowing artificial sea water. Reversal was generally at least 90% complete within 10 min.

Two claw nerves from *Callinectes* were dissected by pulling out, and used for binding of toxin. For the fairly large claw nerves employed in these experiments

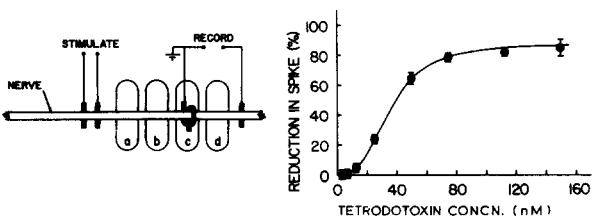

Fig.1. Left, schematic diagram of the recording chamber for the bioassay. Pool a contained artificial sea water. b and d were air gaps, covered to prevent evaporation. 20 μl of each tetrodotoxin concentration were introduced in pool c. The crab leg nerve was sealed everywhere except in regions a, b, c, d with vaseline. Right, dose–response curve for tetrodotoxin on crab leg nerve.

(30–40 mg wet wt each) 100 μl of a known tetrodotoxin concentration (usually 75 nM) in artificial sea water was pipetted into a small plexiglas cup. The 2 claw nerves were blotted on hardened filter paper (Whatman No. 541) and added to the cup. After a 10-min incubation, 20 μl were removed from the cup and applied to the leg nerve in the recording chamber. From the resulting percent reduction in spike height and the dose–response curve (established for each leg nerve used) the final concentration of tetrodotoxin in the cup could be found. Binding was not increased by extending the incubation time to 20 min. Correction for dilution by the remaining extracellular fluid was made by incubating the claw nerves in an identical manner in 100 μl of artificial sea water to which had been added [^{14}C] sucrose (0.1 mM, 0.3 μCi/ml). After 10 min two 20-μl aliquots were removed and counted. Finally, the nerves were blotted and weighed.

Modification of carboxyl groups was achieved by the method of Hoare and Koshland[12]. Claw nerves were placed in 125 ml of a solution containing NaCl 449 mM, KCl 9 mM, $MgCl_2$ 45 mM, $CaCl_2$ 9 mM, glycine methyl ester HCl 0.5 M, 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide·HCl (Ott Chemical Co., Muskegon, Mich.) (EDC) 0.1 M, pH 5.0–5.1. A magnetic stir bar on a raised, perforated plexiglas platform provided continuous stirring. pH was maintained at 5.0–5.1 by addition of small amounts of 1 M HCl. Control nerves were treated in an identical manner but in the absence of carbodiimide. After 40 min, nerves were transferred to 125 ml artificial sea water, with stirring, for 30 min. They were then tested for tetrodotoxin binding. Results are shown in Table I. Tetrodotoxin binding in untreated nerves is in agreement with results obtained by Keynes *el al.*[7] on *Maia* walking leg nerve ($53 \cdot 10^{-15} \pm 6 \cdot 10^{-15}$ mole/mg wet wt). Treatment with glycine methyl ester at pH 5 alone did not significantly affect binding of tetrodotoxin. However, when the complete reaction scheme was present, binding was reduced by 85%. To control against the possibility that traces of reagents were carried through the washing and incubation steps, ultimately causing a reduction in the spike height of the leg nerve, claw nerves were treated as above, but were incubated in 100 μl of artificial sea water, with no tetrodotoxin. No reduc-·

TABLE I

TETRODOTOXIN BINDING BY NORMAL AND MODIFIED NERVE

Tetrodotoxin bound by crab claw nerves: untreated (artificial sea water only); control in artificial sea water + 0.5 M glycine methyl ester, pH 5, 40 min; and modified in artificial sea water + 0.5 M glycine methyl ester + 0.1 M EDC, pH 5, 40 min, as described in text. Values are means ± S.E.; number of experiments in parentheses.

	Tetrodotoxin bound (10^{-15} moles/mg wet wt of nerve)
Untreated	43 ± 7 (7)
Artificial sea water + glycine methyl ester	41 ± 13 (3)
Artificial sea water + glycine methyl ester + EDC	7 ± 4 (3)

tion in spike height was observed when a 20-μl aliquot was applied to the leg nerve. In some experiments an additional 100 mM NaCl was added to the glycine methyl ester control solution to substitute for 100 mM EDC (added as the hydrochloride) in order to guard against effects due to changes in osmotic strength. Results were identical to those with glycine methyl ester and normal salt concentrations.

Claw nerves were also tested for excitability following treatment with EDC–glycine methyl ester. Untreated nerves in stirred artificial sea water for 70 min retained full electrical activity, as measured by external recording. However, nerves exposed to glycine methyl ester, at pH 5, with or without addition of EDC, followed by washing in artificial sea water failed to conduct action potentials. Invertebrate axons often fail to conduct impulses below pH 5.5 at room temperature although our results indicate that the tetrodotoxin receptor survived at pH values below that resulting in conduction block.

The carbodiimide reaction is not entirely specific for carboxyl groups and some side reactions have been shown to occur.

Carraway and Triplett[17] have demonstrated an irreversible binding of EDC to protein sulfhydryl groups. Control experiments were designed to test whether block of sulfhydryl groups inhibited the tetrodotoxin receptor. *N*-Ethylmaleimide, which reacts with sulfhydryl groups with high specificity at moderate concentrations, blocks excitation in nerve, while other sulfhydryl reagents have little or no effect[18]. Conduction block with external application of 2 mM *N*-ethylmaleimide occurs within 30–40 min. Crab claw nerves treated with artificial sea water containing 20 mM *N*-ethylmaleimide for 40 min retained 60% of their tetrodotoxin-binding ability. These results suggest that the observed effects of EDC–glycine methyl ester on tetrodotoxin binding are not due primarily to block of sulfhydryl groups, although some contribution from this reaction is possible. Edwards *et al.*[19] have demonstrated that EDC–glycine methyl ester is capable of reaction with phosphodiester groups of free phospholipids (see also Khorana[20]). The reaction product with phospholipids, unlike that with carboxyl groups, is acid labile, and Edwards *et al.*[19] and Stuesse[21,22] have used this reversibility as a means of further resolving the effects of EDC–glycine methyl ester at the neuromuscular junction. We treated crab claw nerves as above with EDC–glycine methyl ester at pH 5.0–5.1, then placed them in artificial sea water brought to pH 5 with $NaHCO_3$–HCl buffer for 40 min before washing in normal artificial sea water. Tetrodotoxin binding by these nerves was $12 \cdot 10^{-15} \pm 5 \cdot 10^{-15}$ (4) mole/mg wet weight, not significantly higher than earlier results (Table I). Using [^{14}C]glycine methyl ester Godin and Schrier[23] found that virtually all of this nucleophile bound to red cell membranes in the presence of EDC was linked to the protein component of the membrane. Phospholipids in red cell membranes thus seem not to be susceptible to attack by EDC–glycine methyl ester. These results do not rule out the possibility that phosphate groups may play a role at the tetrodotoxin receptor, but suggest that the observed blocking action of this carbodiimide and nucleophile is more likely due to reaction with carboxyl groups. Finally, EDC reacts slowly with tyrosine phenolic groups to form an *O*-aryl isourea (1.3 of 4 tryosines in unfolded chymotrypsin were modified after 1 h in 0.1 M EDC, 1 M glycinamide)[24]. The reaction may be reversed by exposure to hydroxylamine

at pH 7 and this reversal serves as a test for tyrosine modification. In a single experiment no reversal was noted on treatment with artificial sea water containing 0.5 M hydroxylamine HCl, pH 7, but this result was not considered conclusive since excessive gas release appeared to mechanically disrupt the fibers. Tetrodotoxin binding by control (no EDC) fibers was only 60% of normal.

The results reported here are consistent with the idea that the tetrodotoxin receptor may include an anionic group at the binding site. These data and those of Hille[10] suggest that a carboxyl group may be involved, but other possibilities, phosphate groups in particular, are not entirely ruled out. Alternatively, the modified group might be close to, but not actually part of the receptor. Modification of this neighboring group might then inhibit binding either sterically or through an induced conformational change in the receptor. After activation of a reactive group by the carbodiimide, condensation might occur with a neighboring membrane nucleophile leading to altered membrane structure in the region of the receptor. Present experiments do not allow further distinction among these possible modes of action. Finally, an unknown amount of the measured binding may be non-specific, *i.e.* to sites other than sodium channels. Colquhoun *et al.*[15] found significant non-specific binding in rabbit vagus and garfish olfactory nerves (using [^{3}H]tetrodotoxin), but an indeterminably small amount in lobster leg nerve, a preparation somewhat similar to that used here. Their data suggest that at 75 nM tetrodotoxin we should expect fully to saturate the specific sites, but there might be some contribution from non-specific sites as well.

Edwards *et al.*[19] have described similar findings regarding the acetylcholine receptor at the frog neuromuscular junction. The post synaptic depolarizing action of acetylcholine was depressed by EDC–glycine methyl ester and was not reversed at low pH. Using the modifying reagents employed here as well as others, Stuesse[21,22] and Stuesse and Katz[25] have found presynaptic as well as postsynaptic effects of carboxyl group modification of the neuromuscular junction. Several ATPase systems in erythrocyte membranes[23], mitochondria[26] and bacteria[27] have also proved susceptible to carbodiimide modification.

Modification by a carbodiimide and nucleophile is potentially valuable for other studies since a wide variety of substances may be used as the latter reagent. The reaction might therefore serve as a means of introducing a fluorescent, electron dense or spin label at the receptor for tetrodotoxin in excitable membranes.

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