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Tetrodotoxin Inhibition of a Na^+-Induced Conformation Change in Excitable Membranes

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SUMMARY

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We have observed that tetrodotoxin can inhibit a part of the sodium-dependent increase in 8-anilino-1-naphthalenesulphonate (ANS) fluorescence on guinea pig brain synaptosomes (K_i = 0.5 nM), smooth muscle plasma membrane vesicles (K_i = 100 nM), and erythrocyte ghost membranes (K_i = 20 nM). The potassium-dependent increase of ANS fluorescence in these membrane systems (which resembles that of sodium) was not inhibited by tetrodotoxin. This inhibition by tetrodotoxin was not observed on liposomes prepared from whole lipid extracts of these membranes, or on inside-out erythrocyte ghost vesicles. Concentrations of tetrodotoxin up to 10 μM had no direct effect on ANS fluorescence. Citrate (found in all tetrodotoxin samples) had no direct effect on ANS fluorescence, or on sodium- and potassium-dependent increases in ANS fluorescence.

INTRODUCTION

Tetrodotoxin, the toxin of the Japanese puffer fish, is a useful tool in electrophysiological investigations because of its specific ability to block the early (sodium) conductance, thus preventing the generation of a propagated action potential. It has no effect on the late (potassium) conductance. It has been postulated that TTX[2] blocks the sodium conductance channels stoichiometrically, and recent studies on the binding of radiolabelled TTX to nerve membrane are consistent with this hypothesis (1). [See Hille (2) for a model of the sodium conductance channel.]

The conductance channels may exhibit properties similar to ionophores (ion-carrying molecules). Ionophores generally undergo specific conformation changes when binding the passenger cation. Feinstein (3) has shown, for example, that the ionophore valinomycin undergoes a dramatic conformation change when binding to potassium, monitored using the fluorescent probe ANS. This was not the case when it bound sodium. We were interested, therefore, in determining whether excitable membranes would exhibit conformation changes in response to Na^+ or K^+.

It has been demonstrated that there is an increase in ANS fluorescence in a number of different membrane systems upon titration with increasing concentrations of sodium,

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[2] The abbreviations used are: TTX, tetrodotoxin; ANS, 8-anilino-1-naphthalenesulphonate.

potassium, and other cations, and that this is due to an increase in the number of ANS binding sites. In the bovine erythrocyte Fortes *et al.* (4) have shown that the increased fluorescence observed in the presence of sodium could be totally accounted for by the charge-neutralising effects of the cation. The present experiments were designed to test the effects of Na^+ and K^+ on the number and nature of ANS binding sites and to determine whether Na^+-competing drugs would affect this ion-ANS-membrane interaction.

METHODS

Preparation of membrane vesicles. Human erythrocyte ghost membranes were prepared by the method of Dodge *et al.* (5) from whole human blood obtained from the Toronto General Hospital Blood Transfusion Service. After the final wash in 15 mM Tris-HCl buffer (pH 7.4), they were packed down in 15 mM Tris-HCl buffer (pH 7.4) at 16,000 rpm in a Sorvall RC2B centrifuge for 20 min.

Plasma membrane vesicles from the guinea pig ileum longitudinal smooth muscle were prepared by a method to be described.[3] These microsacs were also packed down in 15 mM Tris-HCl buffer (pH 7.4).

Guinea pig brain synaptosomes were prepared from frozen brains stored at $-5°$, a total of 4–5 g being processed as a batch. The method used was that of Gray and Whittaker (6). The final synaptosome fraction was suspended in 0.32 M sucrose. As in the case of the other membrane fractions, aliquots were resuspended at the time of the experiment, in the final buffer containing various sodium ion concentrations.

Erythrocyte ghost membranes were turned inside out by the technique of Steck *et al.* (7). Right-side-out vesicles were always employed as a control in addition to normal ghosts. The latter were prepared in the same way as inside-out vesicles, except that 0.1 mM $MgCl_2$ was added to stabilize the membrane configuration.

Membrane preparations were standardized on a dry weight basis or by determination of membrane protein, using a combination of an optical density method (8) and that of Lowry *et al.* (9). Stock membrane suspensions contained 10–20 mg of protein per milliliter.

Liposomes were prepared by the technique of Bangham *et al.* (10) from total lipid extracts (11) and suspended in 15 mM Tris-HCl buffer.

Fluorescence was measured in a Zeiss spectrofluorometer with a grating monochromator. The excitation and emission maxima were determined by analysis of the spectrum of ANS bound to membranes and were found to be 380 nm and 486 nm, respectively (uncorrected). All subsequent titrations were carried out at these wavelengths.

8-Anilino-1-naphthalenesulphonic acid was obtained from Eastman Kodak and was recrystallised twice from boiling water before use.

Tetrodotoxin titrations. Equivalent portions (on a dry weight basis) of the various vesicle preparations were resuspended in 15 mM Tris-HCl buffer (pH 7.4) and titrated with TTX in concentrations from 0.1 nM to 10 μM in the presence of 16 μM ANS. The TTX obtained from Sigma Chemical Company contains 5 mg of dry citrate buffer (pH 4.8) per milligram of TTX. We therefore repeated these titrations using 0.1 mM citrate.

Sodium and potassium titrations. Aliquots of the various vesicle preparations were resuspended in 15 mM Tris-HCl buffer (pH 7.4) (except for synaptosomes, which were suspended in 0.32 M sucrose) containing 0, 20, 40, 60, 80, and 180 mM NaCl. The final membrane concentration was approximately 0.07 mg of protein per milliliter, or a dry weight of 0.14 mg/ml for the liposomes. An aliquot of 0.2 mg/ml of ANS stock solution was then added to give a final concentration of 16 μM. Fluorescence was monitored as above. The value of fluorescence intensity in 15 mM Tris-HCl buffer (pH 7.4) was taken as the baseline, and the data were plotted as the reciprocal of the difference in fluorescence intensity between the baseline and the value in a given sodium concentration ($1/\Delta F$) against the reciprocal of the sodium ion concentration ($1/Na^+$). Titrations were repeated in the presence of various TTX and citrate concentrations. Potassium ion was also substituted for sodium ion.

[3] L. Spero, A. J. Siemens, and G. A. Toulis, manuscript in preparation.

RESULTS

Tetrodotoxin titrations. In the absence of Na^+, concentrations of TTX from 0.1 nM to 10 μM had no effect on the fluorescence of the membrane or liposome vesicles examined. TTX is therefore unlike the lipid-soluble anaesthetics, all of which modify ANS binding, as demonstrated by Feinstein *et al.* (12).

Sodium and potassium ion titrations. As has been observed by Vanderkooi and Martonosi (13) and others (14, 15), increasing the concentration of monovalent cations leads to an increased fluorescence (Fig. 1). This has been attributed by Fortes *et al.* (4), using a fluorescent lifetime technique, to an increased number of ANS binding sites. This effect was observed in all the membrane and liposome preparations we examined. The extent of the fluorescence increase in the different preparations produced by 180 mM NaCl was similar.

Biological membranes. In the red cell ghost, smooth muscle microsac, and synaptosome preparations, TTX reduced the fluorescence increase due to increasing Na^+ concentration (Fig. 1). This effect is competitive only over a limited concentration range, in the case of synaptosomes a maximum inhibition being apparent at 0.5 nM TTX. Within the competitive range an "apparent affinity" constant can be calculated for the inhibition by TTX of the Na^+-induced fluorescence change. The values of this constant are given in Table 1.

Increasing the potassium ion concentration also leads to an increase in ANS fluorescence. This increase, however, is not inhibited by TTX in concentrations up to 10 μM (Fig. 2). Concentrations of citrate up to 0.1 mM have no effect on Na^+- or K^+-dependent fluorescence increases.

Liposomes. Despite the observed increase in ANS fluorescence produced by both Na^+ and K^+ on the liposome preparation, no inhibition by TTX could be demonstrated. A similar observation was made on inside-out red blood cell membrane ghosts (Fig. 3). The fluorescence of ANS bound to right-side-out and normal vesicles, however, was inhibited to a similar extent (Table 1).

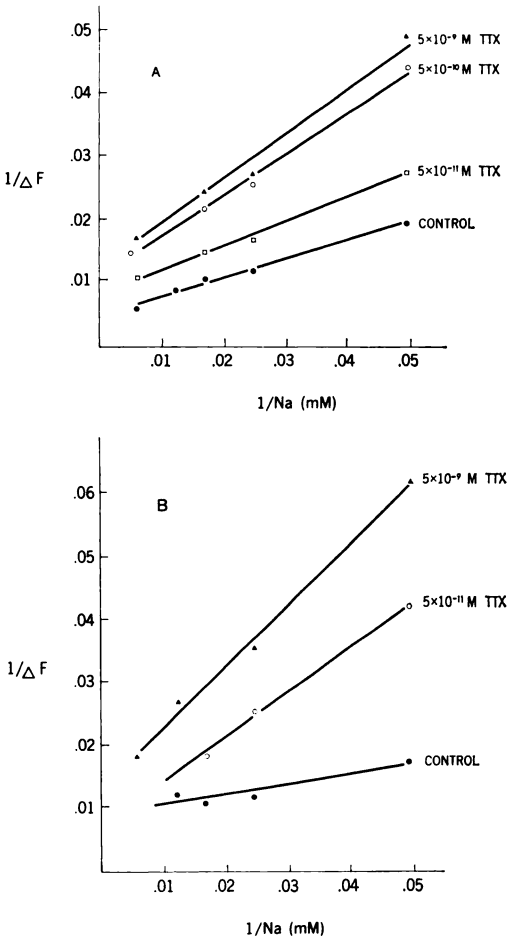

FIG. 1. *Effect of sodium ion concentration on fluorescence of ANS bound to guinea pig brain synaptosomes (A) and ileum plasma membrane vesicles (B) and inhibition by TTX*

Units of fluorescence are explained in the text.

DISCUSSION

Tetrodotoxin is a monoacidic base (pK_a 8.5) with very low lipid solubility and a specific ability to block the inward sodium current during an action potential, a property it shares with the less specific, lipid-soluble, local anesthetics (16). Since it has been usual to consider changes in ANS binding to be due either to charge interactions or to changes in membrane hydrophobicity (4), it is not surprising that TTX has no effect on ANS binding.

TABLE 1

"Apparent affinity" constants determined for tetrodotoxin on interaction between sodium and various membrane fractions, as determined by ANS fluorescence changes

The ANS concentration was 16.7 μM. Excitation was at 380 nm; emission, at 486 nm.

Membrane	K_i of TTX
	nM
Guinea pig brain synaptosomes	0.5
Guinea pig ileum smooth muscle, plasma membrane vesicles	100.0
Human erythrocyte ghosts	20.0

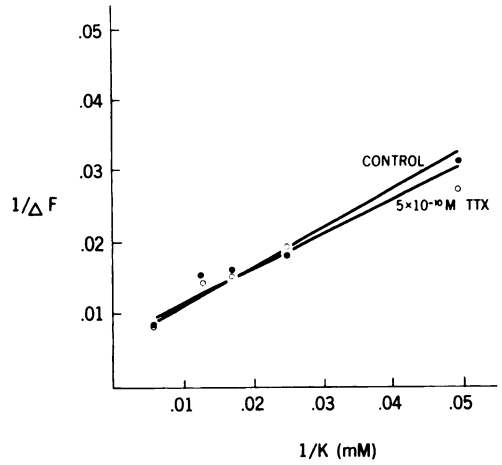

FIG. 2. *Effect of potassium ion concentration on fluorescence of ANS bound to guinea pig brain synaptosomes and lack of inhibition by TTX*

Units of fluorescence are explained in the text.

Both Na^+ and K^+ increased the binding of ANS to membrane vesicles and liposomes. As has been noted, this has been considered to be due to charge effects only (4). However, TTX is able to inhibit a part of the Na^+-mediated increase in ANS binding, but not the K^+-mediated changes. This suggests that a conformation change may occur in some component of the membrane, which leads to an increase in ANS binding and which is specific for Na^+.

The membrane site of this conformation change appears to be a protein or lipoprotein, since liposomes prepared from total lipid extracts of the membranes are not sensitive to

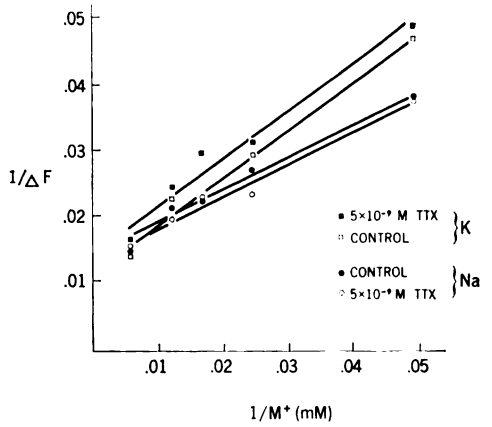

FIG. 3. *Effect of potassium and sodium on fluorescence of ANS bound to inside-out erythrocyte ghost vesicles and lack of effect of TTX*

□, potassium without TTX; ■, with 5 nM TTX: ●, sodium without TTX; ○, with 5 nM TTX. Units of fluorescence are explained in the text.

TTX. Moreover, the loss of TTX sensitivity in the inside-out ghost membrane vesicles implies that this protein is only accessible from the outer face of the membrane. Similar conclusions had been drawn by Moore (17) in his work on perfused squid axon.

The value obtained for the "apparent" affinity constant of TTX for the synaptosome vesicles is consistent with the values found for electrophysiological blockade in squid axon and the more direct measurement of TTX binding by Colquhoun and Ritchie (1). Thus it is reasonable to postulate that these observed conformation changes may be related to the actions of TTX *in vivo*.

The observation of TTX sensitivity in red blood cell membrane and smooth muscle membrane raises some questions, as neither membrane has previously been shown to be sensitive to TTX.

The electrophysiological parameters of smooth muscle are not modified by TTX, as demonstrated by Gershon (18), in agreement with other electrophysiological evidence obtained by Tomita (19) that Ca^{2+}, not Na^+, carries the inward current. However, Bülbring and Kuriyama (20) have shown that the Na^+ concentration can modify the electrophysiological parameters. It is possible,

therefore, that Na^+ channels do exist in smooth muscle but that under physiological conditions they do not contribute to the generation or propagation of action potentials.

The red blood cell membrane is not considered to be electrically excitable. This is probably due to its high resting chloride permeability (21). Seeman (22) has shown that some red cell membrane properties can be correlated with "local anesthetic effects." It may not be unreasonable, therefore, to postulate the presence of sodium channels (or an ionophore). The apparent affinity of TTX for both the red cell and smooth muscle membrane is lower than for the synaptosomes. This may reflect the vestigial nature of the sodium ionophores in these two membranes. Moreover, Redfern *et al.* (23) have recently demonstrated that the action potentials generated in denervated skeletal muscle are not TTX-sensitive but are mediated by Na^+. Inability to demonstrate a physiological effect of TTX may not be a good criterion, therefore, for absence of sodium ionophores.

The possibility that these effects are due to a Na^+-mediated conformation change of $(Na^+ + K^+)$-ATPase has been considered. TTX does not inhibit the activity of this membrane enzyme.[4] In addition, the effect of Na^+ on the fluorescence of ANS bound to partially purified soluble $(Na^+ + K^+)$-ATPase has been shown to be very small;[5] these changes, moreover, were not inhibited by TTX.

We must conclude, therefore, that Na^+ can induce a conformation change in some outward-facing membrane component which is specifically blocked by TTX. If this component were involved in sodium cross-membrane translocation, one might predict that TTX could block the Na^+-induced conformation change and thus prevent sodium movement across the membrane.

It is relevant to this discussion that Conti *et al.* (24) have demonstrated that at least part of the fluorescence increase in ANS-stained squid giant axons during voltage clamp may be due to conformation changes in the membrane and not arise as an artifact of electrical stimulation. Moreover, Tasaki *et al.* (25) have reported that TTX can suppress the early phase of the fluorescence increase under conditions in which the early sodium current is blocked. They also reported that even on increasing the TTX concentration they could inhibit only part of the fluorescence increase.

In preliminary experiments local anaesthetics have been shown to block a part of the Na^+-mediated increase in ANS binding and, at 10-fold higher concentrations, part of the K^+-mediated increase. This selectivity is reversed in the inside-out ghost preparation. In view of the data of Seeman *et al.* (26), who have shown that there are 10^6 molecules of anaesthetic per square micron of membrane, we feel that these local anaesthetics influence the sodium and potassium ionophores secondarily to changing the over-all properties of the membrane matrix in which the ionophores are supported.

Tetrodotoxin has a very high apparent affinity constant for a specific membrane component and is, in addition, highly specific in its actions. This suggests that the TTX binding site and the sodium ionophore are intimately related. The calculations of Colquhoun and Ritchie (1) indicate that the stoichiometry is 1 tetrodotoxin molecule per sodium channel (or ionophore). At this time no data are available on tetrodotoxin binding to smooth muscle membrane. Hafemann (27), using a displacement method, has so far been unable to find specific binding of tetrodotoxin to red blood cells. Colquhoun *et al.* (28), however, have demonstrated a nonsaturable tetrodotoxin compartment in red cells.

[4] A. K. Sen, unpublished observations.

[5] L. Spero and A. K. Sen, unpublished observations.

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