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SAXITOXIN BINDING TO SODIUM CHANNELS IN HEAD EXTRACTS FROM WILD-TYPE AND TETRODOTOXIN-SENSITIVE STRAINS OF *DROSOPHILA MELANOGASTER*

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Summary

Extracts prepared from heads of *Drosophila melanogaster* show high-affinity binding (K_D = 1.9 nM) of [^{3}H]saxitoxin, a compound known to bind to and block voltage-sensitive sodium channels in other organisms. The interaction between saxitoxin and the *Drosophila* saxitoxin receptor is non-cooperative and reversible with a half-life of 18.3 s for binding at 4°C. The saturable binding is specifically inhibited by tetrodotoxin with a K_I = 0.30 nM. The number of saturable binding sites in the extract is 97 fmol/mg protein. Since approx. 50% of the binding activity is recovered in the extract, the number of binding sites in the head is estimated to be 6.4 fmol/mg head. Nerve conduction in *Drosophila* larvae is completely blocked after 20 min in a bathing solution containing 200 nM tetrodotoxin. A comparison between the binding and the electrophysiological studies in *Drosophila* and other organisms suggests that the *Drosophila* saxitoxin receptor is part of the voltage-sensitive sodium channel involved in the propagation of action potentials. A mutant (ttx^s), which is abnormally sensitive to dietary tetrodotoxin, is shown to be indistinguishable from wild type with respect to [^{3}H]saxitoxin-binding properties and physiological sensitivity to tetrodotoxin. These studies provide techniques which can be used to identify mutants with defects in the saxitoxin-binding component of the sodium channel.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; KIU, kallikrein-inhibiting units; Mes, 2-(*N*-morpholino)ethanesulfonic acid; ttx^s, tetrodotoxin-sensitive mutant.

Introduction

A variety of single-gene mutations which affect nervous system function have been isolated in the fruit fly *Drosophila melanogaster* [1]. Electrophysiological techniques have been used to identify mutations which affect various aspects of nerve conduction and synaptic transmission [2—5]. Although these electrophysiological studies provide clues about possible sites of action of the mutations, biochemical experiments are necessary to unambiguously identify the molecular nature of the defects. In addition, with the development of a biochemical assay for a component involved in cell excitability, it will be possible to use a variety of genetic strategies to identify directly genes which affect the structure of such a component [6,7].

As an initial step in the analysis of putative nerve conduction mutants, we describe the use of tritiated saxitoxin in binding studies of the voltage-sensitive sodium channels in *Drosophila*. The two potent neurotoxins used in these studies, tetrodotoxin and saxitoxin, both specifically block the sodium current at low concentrations and thereby abolish the action potential [8]. Specific binding of radioactively labeled toxins has been demonstrated in a variety of organisms, and the good agreement between the biochemically and physiologically measured binding parameters provides convincing evidence that the toxin receptor is associated with the voltage-sensitive sodium channel. (See recent reviews [9—11].) Tritiated saxitoxin has been used as a probe to estimate the density of sodium channels in myelinated [12] and non-myelinated nerve [13] and in innervated and denervated muscle [14]. Labeled toxins can also be used to describe the molecular nature of the binding site [15,16] and to purify the receptor [17].

In this report we characterize the specific binding of [^{3}H]saxitoxin to extracts prepared from heads of a wild-type strain of *D. melanogaster*. In addition, we demonstrate that a mutant, which is abnormally sensitive to orally administered tetrodotoxin, is similar to wild type with respect to [^{3}H]-saxitoxin-binding properties and physiological sensitivity to tetrodotoxin.

Materials and Methods

Drosophila cultures. The wild-type *D. melanogaster* was the Canton-S strain obtained from Dr. J.C. Hall, Brandeis University. The *ttx*^{s} mutant is abnormally sensitive to tetrodotoxin administered by feeding in the culture medium (Hall, L.M. and Osmond, B.C., unpublished results). This mutant was derived from the Oregon-R 369 strain that was made isogenic in 1969 in the laboratory of Dr. D.T. Suzuki, University of British Columbia.

Toxins and buffers. Tetrodotoxin was purchased from Calbiochem and stored at −20°C in 50 mM citrate buffer, pH 4.8. Saxitoxin was the generous gift of Dr. E.J. Schantz, University of Wisconsin, and was stored at −20°C in 50 mM citrate buffer, pH 4.8. Saxitoxin was tritiated and purified by the method of Ritchie et al. [13] and was determined to have a specific activity of 27 dpm/fmol. Tritiated saxitoxin was stored at −76°C in 1 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) buffer, pH 6.5.

Homogenization buffer consisted of 0.2 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 μg/ml pepstatin, 20 KIU/ml aprotinin, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.0. Physiological buffer was Jans' buffer A [18] consisting of 2 mM KCl, 128 mM NaCl, 1.8 mM $CaCl_2$, 4 mM $MgCl_2$, 36 mM sucrose and 5 mM Hepes buffer, pH 7.0. Assay buffer was a modified physiological buffer with reduced levels of divalent cations and consisted of 2 mM KCl, 128 mM NaCl, 0.2 mM $CaCl_2$, 36 mM sucrose and 5 mM Hepes buffer, pH 7.0.

Preparation of head extracts. Flies were grown at 25°C and stored frozen (−76°C) until use. Fly heads were collected as described previously [19] and homogenized in ice-cold homogenization buffer (100 mg heads/ml) in a glass tissue grinder (Arthur H. Thomas, Co.) with thirty strokes of a Teflon pestle at 1700 rev./min. To remove debris, the homogenate was centrifuged at 2000 × *g* for 10 min at 4°C. The pellet was resuspended in homogenization buffer and the centrifugation was repeated. The pooled supernatant fluids were centrifuged at 33 000 × *g* for 30 min at 4°C. The resulting pellet was usually resuspended in assay buffer to an equivalent of 200 mg heads/ml and designated as 'head extract'. One head weighs about 0.1 mg and represents about 10% of the total weight of a fly. Head extracts which had been frozen at −76°C for less than one week showed no detectable loss of activity when compared with freshly prepared extracts.

Saxitoxin-binding assay. For determination of total [^{3}H]saxitoxin binding, extracts equivalent to 20 mg heads were incubated with a known concentration of [^{3}H]saxitoxin (0.5—60 nM, specific activity 27 dpm/fmol) in 150 μl of assay buffer. To determine nonspecific binding, parallel samples were incubated in an identical solution containing in addition 10 μM unlabeled saxitoxin. Following a 2-h incubation at 4°C (equilibrium conditions), the sample was filtered on a vacuum manifold through a pre-wetted glass fiber filter (Whatman GF/C) and immediately washed with two 5-ml aliquots of ice-cold assay buffer. The initial filtration and the two washes took a total of 3 s. The filters were placed in glass vials containing 6 ml scintillation cocktail (4 g PPO, 0.05 g POPOP, 333 ml Triton X-100, and 667 ml toluene) and held overnight before counting to insure stability of the counts. The counting efficiency was determined by the external standard method and was between 34% and 40% in most experiments. Except where noted experiments were conducted at 4°C to prevent back exchange of tritium from [^{3}H]saxitoxin. Specific binding of [^{3}H]saxitoxin was determined by subtracting nonspecific binding from the total binding.

Protein determination. Protein content was measured by the method of Lowry et al. [20] using bovine serum albumin as the standard. In general, 100-mg heads resulted in a final extract containing a total of 3.0—3.5 mg protein.

Electrophysiology. Late third instar *Drosophila* larvae were split down the dorsal mid-line and the internal organs were removed. The preparation was pinned to a Sylgard-coated, 35-mm tissue culture dish to expose the array of ventral muscle fibers and their innervating nerves which emanate from the ventral ganglion. The preparation was bathed in 2 ml physiological buffer and all experiments were conducted at 22°C. A nerve bundle was stimulated near the ventral ganglion by a suction electrode of 10 μm diameter carrying a 10 V,

0.1 ms pulse. Excitatory junctional potentials from the corresponding muscle fibers were recorded intracellularly with 30—100 MΩ glass microelectrodes filled with 3 M KCl.

For iontophoretic stimulation of the muscle fiber, 1 M L-glutamate or D-glutamate at pH 8 was applied from separate chambers of a 70 MΩ double-barreled glass micropipette onto the neuromuscular synapse of the ventral lateral longitudinal fibers as described by Jan and Jan [21]. Negative current pulses of 2—5 ms duration and 50 nA amplitude were used. To prevent muscle twitching during iontophoresis, the preparation was bathed in calcium-free physiological buffer (solution H of Jan and Jan [18]).

In all experiments a chlorided silver wire in the bath was the ground electrode. Pulses were generated by a Tektronix series 160 pulse generator and muscle responses were recorded differentially with a Meta-Metrics AK 47 differential amplifier and a Tektronix dual-beam oscilloscope.

Results

Physiological sensitivity of Drosophila larvae to tetrodotoxin and saxitoxin

To provide a basis for interpretation of tritiated saxitoxin-binding studies, it is necessary to demonstrate the ability of tetrodotoxin and saxitoxin to block nerve conduction in *Drosophila*. A dissected third instar larval preparation was used for this analysis because the nerves and muscle fibers are readily accessible to experimental manipulation and can be bathed directly in toxin-containing solution. Since it is difficult to record nerve impulses directly in *Drosophila*, nerve conduction was monitored by intracellularly recording the excitatory junctional potential in a muscle fiber in response to electrical stimulation of the corresponding motor nerve. A loss in the evoked response of the muscle is indicative of a block in nerve conduction by the toxins, since the muscle itself is insensitive to tetrodotoxin and saxitoxin. When bathed in a solution of 1 μM tetrodotoxin or saxitoxin, muscle response to nerve stimulation was blocked; however, the muscles still responded with normal excitatory junctional potentials to iontophoretically applied L-glutamate, the putative excitatory neuromuscular transmitter [21]. The same muscles did not respond to D-glutamate.

Experiments summarized in Fig. 1 illustrate the ability of various concentrations of tetrodotoxin and saxitoxin to block nerve conduction in *Drosophila*. Within 5 min following toxin addition, nerve conduction is affected by toxin concentrations greater than or equal to 300 nM, but not by lower concentrations. After 20 min, nerve conduction is routinely blocked by greater than or equal to 200 nM toxin and conduction is impaired at even lower concentrations. No difference between saxitoxin (+) and tetrodotoxin (all other symbols) could be detected in these electrophysiological experiments. Experiments involving the first and second muscle fibers from the ventral mid-line (triangles and squares) did not differ from those in which the position of the fiber was not controlled (circles).

Saturability of saxitoxin binding

After it was established that *Drosophila* are physiologically sensitive to

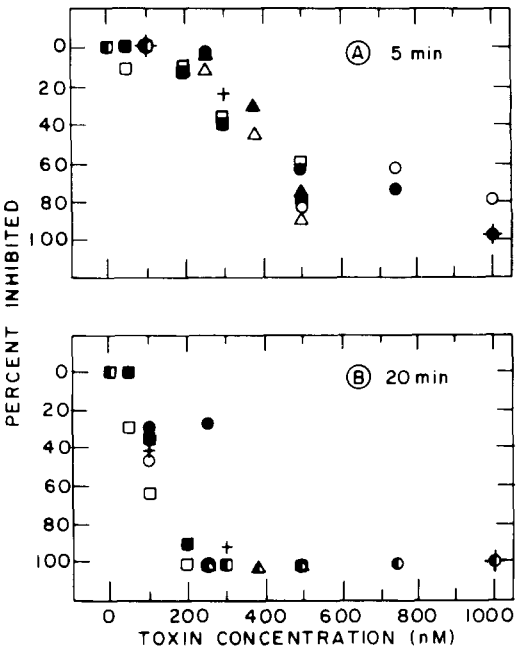

Fig. 1. Sensitivity of *Drosophila* larvae to tetrodotoxin and saxitoxin as determined electrophysiologically. 20 μl of tetrodotoxin or saxitoxin in 50 mM citrate buffer was mixed into the bath at time zero to yield the indicated final toxin concentration. Following toxin addition, nerves were simulated at 15—30 s intervals to test for the presence of an evoked response (excitatory junctional potential) in the corresponding muscle fiber. As discussed in Results, loss of the evoked response is indicative of a block in nerve conduction. The ordinate indicates the percent of muscle fibers which lost the evoked response within 5 min (A) or 20 min (B) following toxin addition. Each point represents results from at least ten different animals. The effects of tetrodotoxin were studied on the wild-type Canton-S (●, ▲, ■) and the tetrodotoxin-sensitive mutant *ttx*^{s} (○, △, □) and the effects of saxitoxin were studied on Canton-S alone (+). The different symbols represent variations in experimental design described in Results.

saxitoxin and tetrodotoxin, biochemical experiments were undertaken to determine whether specific binding of radioactive saxitoxin to *Drosophila* head extracts could be detected. The binding of [^{3}H]saxitoxin to *Drosophila* extracts was measured using a rapid filtration assay to separate the extract-bound [^{3}H]saxitoxin which is retained on the filter, from the free [^{3}H]saxitoxin which passes through. Fig. 2 shows the binding of [^{3}H]saxitoxin to a fixed amount of wild-type fly head extract assayed over the concentration range of 0.5— 51 nM [^{3}H]saxitoxin. The total binding appears to be composed of two parts: a low-affinity, linear binding and a high-affinity, saturable binding. Excess (greater than or equal to 200-fold) unlabeled saxitoxin completely abolishes the saturable binding component and allows the linear, nonspecific component to be determined directly. Fig. 2 demonstrates that the net binding, as determined by total minus nonspecific binding, satisfies the following relationship for saturable, non-cooperative binding:

$$\mathrm{STX}_{\mathrm{bound}} = \frac{M[\mathrm{STX}]}{K_{\mathrm{D}} + [\mathrm{STX}]}$$

where $\mathrm{STX}_{\mathrm{bound}}$ = bound [^{3}H]saxitoxin, [STX] = the concentration of free

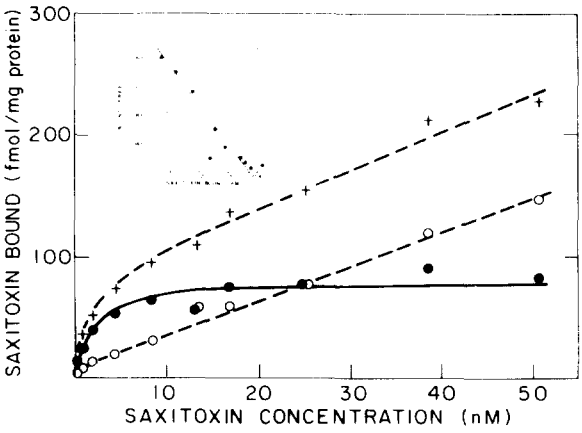

Fig. 2. Binding of [^{3}H]saxitoxin to *Drosophila* extracts. Canton-S wild-type extract equivalent to 20 mg heads was incubated with increasing concentrations of [^{3}H]saxitoxin in a total volume of 150 μl for 2 h at 4°C (equilibrium conditions). Total binding (+) and nonspecific binding in the presence of 10 μM saxitoxin (○) were determined by the rapid filtration assay as described in Materials and Methods. Specific binding (●) was calculated by subtracing nonspecific binding from the total. Each point represents the average of duplicate samples. - - - - - -, the least-squares fits to the total binding and nonspecific binding data. The saturable binding (———) was calculated from the relationship described in Results: $STX_{bound} = M[STX]/(K_D + [STX])$, where STX_{bound} is given in fmol binding sites per mg protein. The concentration of free [^{3}H]saxitoxin is essentially the same as the concentration of added [^{3}H]-saxitoxin. The inset shows the Scatchard replot of the data from this experiment. ———, the best fit to the data by linear regression analysis. The slope of the line is $-1/K_D$ and the intercept of the abscissa is the concentration of saturable binding sites.

[^{3}H]saxitoxin, M = the number of saturable binding sites, and K_D = the equilibrium dissociation constant for saxitoxin binding.

To determine the equilibrium dissociation constant (K_D) and the number of saxitoxin-binding sites (M), these data were replotted as shown in the inset in Fig. 2 in the form described by Scatchard [22]:

$$\frac{STX_{bound}}{[STX]} = \frac{1}{K_D}(M - STX_{bound})$$

The data from this experiment together with that from three other independent experiments on wild-type extracts (shown in Fig. 3A) indicate that the K_D is 1.9 nM. This dissociation constant is indicative of high-affinity binding of saxitoxin to *Drosophila* extracts and is similar to K_D values determined using excitable tissues from other organisms. (See Table I for comparison.) In this same group of experiments the number of saxitoxin-binding sites (M) was found to be 97 fmol/mg protein. During the preparation of the extract, approximately one-half of the binding material is lost. Thus, in these experiments the number of binding sites in the starting material is approximately 6.4 fmol/mg wet weight heads. The number of saxitoxin-binding sites in *Drosophila* is therefore lower than that reported for other preparations (Table I).

Because the dissociation constant and the number of binding sites are important parameters in the saxitoxin-binding analysis of putative sodium channel mutants in *Drosophila*, it is important to determine the amount of variability in these measurements. Although there is some scatter in the data

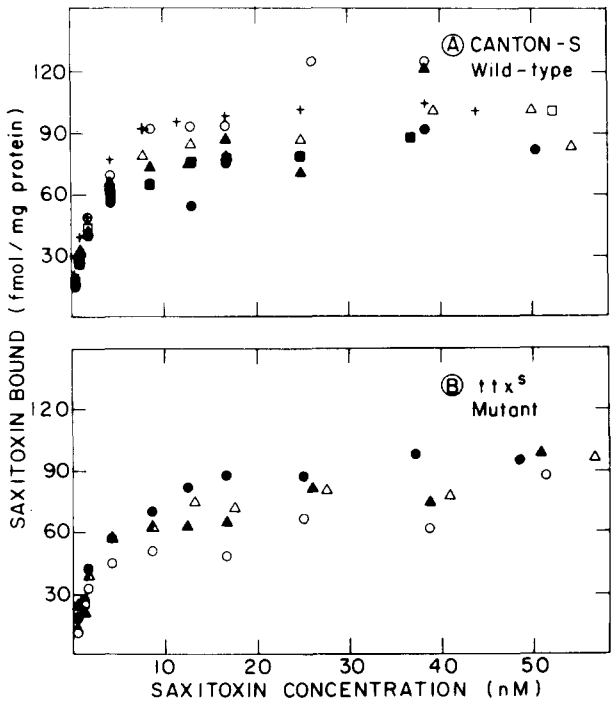

Fig. 3. Saturable saxitoxin binding to extracts from Canton-S wild-type (A) and the tetrodotoxin-sensitive mutant ttx^s (B) strains. Specific binding was determined in a series of independent experiments conducted as described in Fig. 2. 2-h incubations and the subsequent washes were conducted at either 4°C (●, ▲, ■, +) or 22°C (○, △). Each point represents the average of duplicate samples.

TABLE I

COMPARISON OF SAXITOXIN-BINDING STUDIES IN *DROSOPHILA* AND OTHER SPECIES

Data in brackets are the reference numbers.

Tissue	Preparation	K_D (nM)	M	
			fmol/mg wet weight	pmol/mg protein
Insect brain	*Drosophila* head extracts	1.9 *	6.4 *	0.097 *
Non-myelinated nerve	Rabbit vagus [13]	1.8	110	—
	Lobster walking leg [13]	8.5	94	—
	Garfish olfactory [13]	9.8	377	—
	Squid giant axon [29]	4.3	—	—
Myelinated nerve	Rabbit sciatic homogenate [12]	1.3	17.1 **	—
	Frog sciatic [10]	5.3	3.1	—
Muscle	Rat diaphragm [14]	3.8	24	—
	Frog sartorius (Southern) [14]	4.3	25.6	—
	Frog sartorius (Northern) [14]	3.8	14.6	—
Mammalian brain	Rat brain synaptosomes (0°C) [24]	0.5	—	1.9

* These values are the averages from four experiments on wild type at 4°C.

** Corrected for uptake by non-myelinated fibers.

in the four experiments summarized in Fig. 3A, the K_D can be determined within a range of plus or minus 11%, while the number of binding sites (M) shows a variation of plus or minus 16%. Therefore, it should be possible to identify mutants in which these parameters fall outside this range of variation.

The specific binding of 10 nM [^{3}H]saxitoxin to *Drosophila* extracts is proportional to extract concentration up to at least 9.8 mg protein/ml which is equivalent to 300 mg heads/ml. An amount of extract equivalent to as little as 10 mg heads (0.33 mg protein) shows specific binding of 150 cpm which is sufficient for accurate analysis.

Reversibility of saxitoxin binding

To determine the rate of dissociation for saxitoxin binding to *Drosophila* extracts, excess unlabeled saxitoxin was added to extracts which had been equilibrated with 5 nM [^{3}H]saxitoxin for 2 h at 4°C. The amount of [^{3}H]-saxitoxin bound at various times following the addition of unlabeled toxin satisfies the relationship for exponential decay:

$$\ln(\text{toxin bound}) = \ln(\text{toxin bound at time zero}) - k_{-1}t$$

where t = time after addition of excess unlabeled toxin. Therefore, the dissociation constant k_{-1} was determined from the slope of the line to be 0.038 s^{-1} which is equivalent to a half-life ($t_{1/2}$) of 18.3 s. The reversible binding has kinetics comparable to those measured in other tissues [23,24].

Tetrodotoxin inhibition of saxitoxin binding

Tetrodotoxin has been shown to interfere with [^{3}H]saxitoxin binding in a variety of organisms [14,23]. Fig. 4 summarizes a series of independent experiments which demonstrate that tetrodotoxin also inhibits [^{3}H]saxitoxin binding to *Drosophila* extracts. The binding satisfies the relationship for competitive inhibition:

$$\text{STX}_{\text{bound}} = \frac{M[\text{STX}]}{K_D(1 + I/K_I) + [\text{STX}]}$$

where M = the number of saturable saxitoxin-binding sites; K_D = the equilibrium dissociation constant for saxitoxin binding; K_I = the equilibrium dissociation constant for tetrodotoxin binding, and I = the concentration of tetrodotoxin. Since the concentration of [^{3}H]saxitoxin used in these experiments was equal to the K_D of 2 nM, K_I can be directly determined from the slope when the data are plotted in the simplified form:

$$\frac{M}{\text{STX}_{\text{bound}}} = 2 + I/K_I$$

The K_I of 0.30 nM indicates that tetrodotoxin is a very effective inhibitor of saxitoxin binding in *Drosophila*.

Most experiments in this study were conducted at 4°C to prevent the back exchange of tritium from [^{3}H]saxitoxin. However, to demonstrate that [^{3}H]-saxitoxin binding occurs at physiological temperatures, several saxitoxin-binding and tetrodotoxin inhibition experiments were conducted at 22°C in a manner completely analogous with those at 4°C (compare open and closed

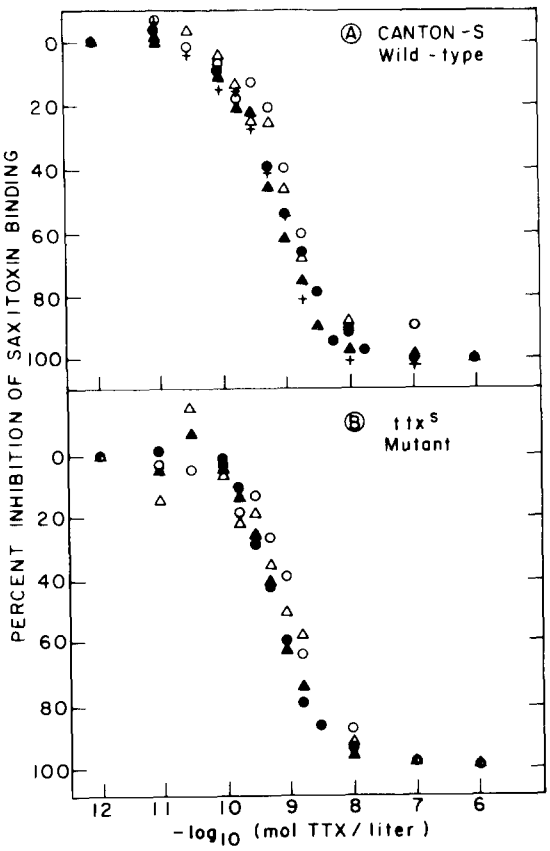

Fig. 4. Inhibition of saxitoxin binding by tetrodotoxin. Canton-S wild-type (A) and *ttx^s* (B) extracts equivalent to 20 mg heads were incubated with 2 nM [^{3}H]saxitoxin and varying concentrations of terodotoxin in a total volume of 150 μl for 2 h. Incubations and the subsequent washes were conducted at either 4°C (●, ▲, +) or 22°C (○, △). 100% binding is defined as [^{3}H]saxitoxin binding in the presence of 10^{-12} M tetrodotoxin. Other experiments have shown that this is the same as binding in the absence of tetrodotoxin. Background binding in the presence of 10 μM unlabeled saxitoxin has been subtracted. Each point represents the average of duplicate samples.

symbols in Figs. 3 and 4). At 22°C [^{3}H]saxitoxin binding to *Drosophila* extracts is saturable (M = 108 fmol/mg protein), shows high affinity (K_D = 2.44 nM) and is inhibited by low concentrations of tetrodotoxin (K_I = 0.5 nM). As summarized in Table II, saxitoxin binding to *Drosophila* extracts may be only slightly affected by temperature changes in the range of 4—22°C whereas tetrodotoxin binding displays a stronger temperature dependence.

Analysis of a tetrodotoxin-sensitive mutant (ttx^s)

Adults and larvae from the tetrodotoxin-sensitive mutant strain (*ttx^s*) are 50-fold and 6-fold, respectively, more sensitive than wild type to tetrodotoxin administered by feeding in the culture medium (Osmond, B.C. and Hall, L.M., unpublished results). The *ttx^s* mutation maps as a single genetic locus to the right of *scarlet* on the third chromosome. To determine whether the altered sensitivity is due to an alteration in the toxin receptor of the sodium channel,

TABLE II

SUMMARY OF SAXITOXIN-BINDING PARAMETERS FOR *D. MELANOGASTER*

Results shown are an average of 2–4 independent experiments. The range of values obtained is enclosed in parenthesis.

Binding parameters	Temperature (°C)	Canton-S wild type	ttx^s mutant
K_D (nM)	4	1.88 (1.77–2.09)	2.03 (2.02–2.04)
	22	2.44 (2.28–2.60)	2.03 (1.91–2.15)
M (fmol/mg protein)	4	97 (81–111)	90 (84–95)
	22	108 (96–119)	78 (68–87)
k_{-1} (s^{-1})	4	0.038	0.041
K_I (nM) for tetrodotoxin	4	0.30 (0.20–0.42)	0.26 (0.22–0.29)
	22	0.50 (0.41–0.58)	0.59 (0.52–0.65)

ttx^s flies were tested for differences from wild type with respect to their physiological and biochemical responses to tetrodotoxin and [^{3}H]saxitoxin. All experiments were conducted in a manner analogous to those previously described for wild type.

Electrophysiological experiments summarized in Fig. 1 compare the tetrodotoxin sensitivity of the ttx^s mutant with that of wild type. A 5-min exposure to 400 nM tetrodotoxin is required to block nerve conduction in 50% of the cases. After a 20-min exposure, nerve conduction is blocked in 50% of the cases by 100 nM tetrodotoxin. Within the variability of these experiments, no difference in tetrodotoxin sensitivity was found between the mutant and wild type.

Saxitoxin-binding studies were also conducted to determine whether the ttx^s mutation shows alterations with respect to saxitoxin and tetrodotoxin dissociation constants, number of saturable binding sites, and rate of dissociation. The saturable binding of [^{3}H]saxitoxin to ttx^s extracts is illustrated in Fig. 3B. Analysis of these data shows that at 4° C, the equilibrium dissociation constant is 2.03 nM and the number of saturable binding sites is 90 fmol/mg protein. There are no significant differences between these parameters in mutant and wild type (Fig. 3, Table II). Similarly, no difference was detected with respect to rate of dissociation (Table II). A comparison of Fig. 4A and B indicates that tetrodotoxin is equally effective in the inhibition of saxitoxin binding to mutant and wild type extracts. At 4° C, the K_I for tetrodotoxin inhibition in the mutant is 0.26 nM, while that for wild type is 0.30 nM. With respect to all of the parameters tested, there was no difference between the mutant and wild type at either 4° C or 22° C.

Discussion

Our results indicate that the rapid filtration assay can be used to describe a specific saxitoxin receptor in extracts of *D. melanogaster* heads. Under the conditions used in this assay at saxitoxin concentrations equal to the K_D, the specifically bound counts are 78% of the total providing a very good signal-to-noise ratio. The receptor reversibly binds saxitoxin with high affinity. The K_D of 1.9 nM determined for the receptor in *Drosophila* is within the range of K_D

values (0.5—9.8 nM) reported for saxitoxin binding to a variety of tissues in other organisms (see Table I). In *Drosophila* extracts the half-life of the receptor-saxitoxin complex at 4°C is 18.3 s. This is similar to the half-lives reported in other systems at 20°C: i.e. in solubilized garfish nerve $t_{1/2}$ = 13 s [23] and in rat brain synaptosomes $t_{1/2}$ = 18 s [24].

The saxitoxin receptor in *Drosophila* is also similar to that in other tissues in that [^{3}H]saxitoxin binding is inhibited by tetrodotoxin. However, in *Drosophila* the K_I for receptor-tetrodotoxin interaction (0.5 nM at 22°C) is lower than the K_I of 12.7 nM found at 4°C in rat extensor digitorum longus muscle (Hansen Bay, C.M. and Strichartz, G.R., unpublished results). It is also lower than the K_D values determined directly from [^{3}H]tetrodotoxin binding at 20—21°C in the following other non-myelinated nerves: rabbit (K_D = 3 nM), lobster (10 nM), and garfish (10 nM) [25]. In addition, the receptor-toxin interaction in *Drosophila* is similar to that in other organisms in that the tetrodotoxin and saxitoxin-binding affinities are weaker at higher temperatures (Ref. 24; Hansen Bay, C.M. and Strichartz, G.R., unpublished results).

The number of saturable saxitoxin-binding sites in *Drosophila* head extracts (6.4 fmol/mg wet weight of heads) is significantly lower than that reported for many other nerve and muscle preparations (see Table I). Although the *Drosophila* head extract is the least homogeneous preparation with regard to cell type, this discrepancy is somewhat surprising since the number of saturable α-bungarotoxin-binding sites in *Drosophila* heads is comparable to that found in electric eel and is an order of magnitude higher than that found in rat brain [19,26]. In our saxitoxin-binding experiments, the assay conditions have not been systematically optimized. Thus, our estimate of the number of saturable binding sites may underestimate the total.

To demonstrate that radioactive saxitoxin or tetrodoxin binding to excitable tissues reflects binding to sodium channels, it is important to correlate binding studies with electrophysiological studies. For nerve preparations which can be voltage-clamped, the physiological K_D for toxin binding to the sodium channel can be defined as the concentration of toxin that blocks maximum sodium conductance by 50%. The K_D values for saxitoxin and tetrodotoxin determined in this manner for frog node of Ranvier and squid giant axon lie in the range of 0.6—4.8 nM [27—29] and are in good agreement with K_D values determined in [^{3}H]saxitoxin-binding studies which lie in the range of 0.5—9.8 nM as summarized in Table I. As stated previously, the K_D for [^{3}H]saxitoxin binding in *Drosophila* is comparable to that determined in other systems; but electrophysiological measurements of the K_D using the voltage-clamp method have not been made due to the small size of the neurons. However, our results show that 200 nM tetrodotoxin completely abolishes nerve conduction in *Drosophila* larvae within 20 min following bath application of the toxin. Although this is a high concentration compared to the K_D from binding studies, it is to be expected for this experimental design. For example, in rabbit vagus nerve where the K_D for [^{3}H]saxitoxin binding is 1.8 nM [13] and the electrophysiologically determined K_D for tetrodotoxin is 3—5 nM [30], a high concentration of at least 100 nM was required to completely block nerve conduction [30]. Thus, our electrophysiological and toxin-binding results in *Drosophila* are similar to findings in other organisms and are consistent with the suggestion

that the saxitoxin receptor in *Drosophila* is part of the voltage-sensitive sodium channel.

We were interested in determining whether the abnormal sensitivity to tetrodotoxin exhibited by the ttx^s mutation was due to an alteration in sodium channel numbers or in its toxin-binding properties. In our experiments there were no significant differences between the mutant and wild type with respect to numbers of receptors or other [^{3}H]saxitoxin-binding parameters which would account for the 50-fold difference in adult sensitivity to dietary tetrodotoxin. There also was no difference in electrophysiologically determined sensitivity to tetrodotoxin between mutant and wild type. Although the difference in larval sensitivity to dietary tetrodotoxin is less dramatic than that in adults, a 6-fold difference should have been detectable in our electrophysiological studies. On the basis of these experiments, we conclude that the ttx^s mutation does not directly affect the toxin-binding component of sodium channels. The molecular defect in ttx^s most likely lies in some other area such as detoxification enzymes, gut permeability, or feeding behavior. Although this mutation does not appear to affect the toxin-binding properties of the sodium channel, it can be used to find mutations in the toxin receptor by screening mutagenized ttx^s flies for resistance to tetrodotoxin. Use of the ttx^s strain will allow low concentrations of tetrodotoxin to be used in the screen.

Three temperature-sensitive paralytic mutants have been described which affect conduction of action potentials. In the mutants nap^{ts} and $para^{ts}$ nerve conduction is blocked at a high temperature where conduction in the wild type is still normal [3,5]. In another mutant, *comatose*, adults show either a decrease in nerve conduction velocity or a delay in transmitter release [3]. The [^{3}H]saxitoxin-binding studies presented here can be extended to the analysis of these and other potential sodium channel mutants. These temperature-sensitive mutants might display, in vitro, a temperature-induced difference in saxitoxin-binding parameters. Such experiments are in progress in our laboratory.

Note added in proof (Received November 13th, 1979)

Kauvar [31] has also demonstrated saturable binding to *Drosophila* homogenates using [^{3}H]tetrodotoxin.

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