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SODIUM CHANNELS IN VERTEBRATE HEARTS

THREE TYPES OF SAXITOXIN BINDING SITES IN HEART

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The affinity of saxitoxin binding to cardiac sarcolemmal and cytosolic fractions was examined across species. In amphibia (frog) the plasma membrane site demonstrated a high affinity (K_d approx. $5 \cdot 10^{-9}$ M) but the majority of the total sites in the homogenate appeared to be high affinity soluble sites (K_d approx. $2 \cdot 10^{-9}$ M). Chicken and turtle cardiac plasma membrane fractions bound [^{3}H]saxitoxin with 500-fold less affinity (K_d values of approx. $2 \cdot 10^{-6}$ M). No binding was seen in the cytosol. The affinity of cardiac sarcolemmal binding in amphibians correlates quantitatively with the $K_{0.5}$ for the inhibition of sodium currents. Physiological correlation of the low affinity saxitoxin sites in chicken and turtle with toxin concentrations necessary to inhibit the sodium current remains unclear. The hypothesis that frog cytosolic saxitoxin binding sites originated from sarcolemma during homogenization is examined. The presence of three types of saxitoxin binding sites in cardiac preparations supports the existence of sodium channel subtypes.

Introduction

The initial deplorization phase of the cardiac action potential is due to a fast inward sodium current, similar to the sodium currents in nerve and muscle [1–3]. Characterization of the sodium channel, the molecule responsible for the gating of these sodium currents, has been advanced through the use of the selective sodium channel blockers, saxitoxin (STX) and tetrodotoxin (TTX) [4]. With most nerve and muscle membranes the concentrations of these toxins needed to block 50% of the sodium current is 10^{-9}–10^{-8} M [5]. Sodium channels have been purified from rat brain [6], rat skeletal muscle [7] and *Electrophorus* electric organ [8]; the purification uses labelled toxins to follow the high affinity binding. The purified channel has recently been reconstituted into phospholipid vesicles and demonstrates several functional properties of the in situ sodium channel [9–11].

Studies of the cardiac sodium channel, however, have been considerably more difficult than with nerve or muscle. The difficulty with the electrophysiology has been the maintenance of good temporal and spatial control of the membrane during voltage clamp [12–15]. This lack of control is largely due to the multicellular nature of heart preparations. In addition to this problem, most cardiac sodium channels do not bind tetrodotoxin and saxitoxin with a high affinity. The concentration of toxin needed to block cardiac sodium currents is 2–3 orders of magnitude higher than for nerve and muscle (Table I). This low toxin affinity has prevented purification of the cardiac

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TABLE I

ESTIMATES OF APPARENT K_d VALUES FOR TETRODOTOXIN AND SAXITOXIN ON CARDIAC TISSUE AND PARTIALLY PURIFIED MYOCARDIAL MEMBRANES

Reference	Preparation	Toxin	Method used to measure	K_d or $K_{0.5}$
Baer, Best and Reuter [54]	Guinea pig papillary muscle	TTX	Inhibition of Vmax	$1.4 \cdot 10^{-5}$ M
Lombet et al. [47]	Guinea pig homogenates	[^{3}H]en-TTX	Binding on glass filters	$1.15 \cdot 10^{-9}$ M
Catterall and Coppersmith [16]	Guinea pig homogenates	[^{3}H]STX	Binding on glass filters	$0.9 \cdot 10^{-9}$ M
Catterall and Coppersmith [16]	Guinea pig homogenates	TTX	Competition	$10.2 \cdot 10^{-9}$ M
Cohen et al. [55]	Rabbit purkinjie	TTX	Inhibition of I_{Na}	$1 \cdot 10^{-6}$ M
Lombet et al. [47]	Rabbit homogenate	[^{3}H]en-TTX	Binding on glass filters	$1.02 \cdot 10^{-9}$ M
Connor, Barr, Jakobsson [12]	Frog Atrial Trebecula	TTX	Inhibition of I_{Na}	$5 \cdot 10^{-9}$ M
This work	*Rana pipiens* sarcolemma	[^{3}H]STX	Centrifugation binding	$7 \cdot 10^{-9}$ M
Catterall and Coppersmith [16]	*Bufo marinus* homogenates	[^{3}H]STX	Binding on glass filters	$1.4 \cdot 10^{-9}$ M
Iijima and Pappano [45]	4-day chick embryo	TTX	Inhibition of Vmax	$1.1 \cdot 10^{-8}$ M
	6-day chick embryo	TTX	Inhibition of Vmax	$1.3 \cdot 10^{-8}$ M
	18-day chick embryo	TTX	Inhibition of Vmax	$3.0 \cdot 10^{-8}$ M
Marcus and Fozzard [45]	Adult and embryonic chick heart	TTX	Inhibition of Vmax	$5 \cdot 10^{-9}$ M
Lombet et al. [48]	11-day chick embryo homogenates	[^{3}H]en-TTX	Glass filter binding	$1.6 \cdot 10^{-9}$ M
Catterall and Coppersmith [16]	Chicken homogenates	[^{3}H]STX	Glass filter binding	$7.5 \cdot 10^{-9}$ M
This work	Adult chicken heart membranes	[^{3}H]STX	Saxitoxin displacement assay	$1.75 \cdot 10^{-6}$ M
Rogart et al. [21]	Embryonic chick heart homogenate	[^{3}H]STX	Binding assay	$8 \cdot 10^{-9}$ M
This work	Turtle heart membrane	[^{3}H]STX	Saxitoxin displacement assay	$2.3 \cdot 10^{-6}$ M
Lombet et al. [47]	Rat heart homogenates	[^{3}H]en-TTX	Binding on glass filters	$1.1 \cdot 10^{-9}$ M
Catterall and Coppersmith [16]	Rat heart homogenates	[^{3}H]STX	Binding on glass filters	$2.1 \cdot 10^{-9}$ M

sodium channel and has led to consideration of the differences between nerve and muscle sodium channels, which have a high affinity and the cardiac channel. Tetrodotoxin low-affinity sodium channels, sometimes called insensitive sodium channels, have also been described in embryonic cells and muscle cells in culture supporting the notion of two types of sodium channels [16–20]. Rogart et al. [21] further supported the idea of sodium channel subtypes with their measurement of 30-fold differences in the saxitoxin binding constants between chick brain and chick heart.

Electrophysiological observations must be correlated with biochemical properties if we are to understand molecular differences between these two types of sodium channels. This correlation has been difficult in cardiac tissue for the reasons noted. We reported previously the presence of a soluble high affinity saxitoxin binding site from amphibian hearts in ratios greater than 3 : 1 with the plasma membrane site. The existence of this soluble site reinforces the need to understand the relationship between saxitoxin binding and the voltage gated sodium channel. The intent of these studies was to measure the binding of [^{3}H]saxitoxin to plasma membrane fractions from several hearts as a first step in the biochemical characterization of cardiac channels. The question of a physiological role for the amphibian soluble binding site has not been addressed here but the origin and distribution of the site have been investigated.

A preliminary report of these results was communicated to the Biophysical Society [23,24].

Materials and Methods

Adenosine 5′-triphosphate (disodium salt), ouabain, 1-amino-2-naphthol-4-sulfonic acid, phenylmethylsulfonyl fluoride, and *p*-nitrophenylphosphate (disodium salt) were

purchased from Sigma Co. Saxitoxin standard Lot 7 No. 456 was obtained from the Federal Drug Administration, Biofluor scintillation cocktail from New England Nuclear Co. Tetrodotoxin was purchased from Sanyko, Co., Japan. Some of the saxitoxin for labelling was the generous gift of Dr. E. Schantz, University of Wisconsin; other reagents were obtained from the NIH. *Rana pipiens*, *Rana catesbeiana* and *Bufo marinus* were obtained from Lemberger Co., Germantown, WI. Adult white leghorn chickens were obtained from the Poultry farm at the University of Illinois. Turtles, *Chrysemys scripa elegans*, were obtained from Nasco Co., Fort Atkinson, WI. The *Electrophorus electricus* electric organ was the generous gift of Dr. Donald Farquharson.

A. Membrane preparation

Partially purified plasma membranes were isolated from hearts by modifying a procedure used by Bers [25]. Ventricles were dissected free of atria, trimmed of fat and minced with a scalpel. All steps were then carried out at 4°C. The tissue was homogenized in a solution containing 250 mM sucrose, 10 mM Tris-maleate (pH 7.9), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) in a Polytron PT IOST set at 7. Two 4-s bursts were given, separated by 1 min. The volume of homogenizing solution was adjusted to 1 : 15 tissue wet wt./volume and filtered through several layers of cheesecloth. An aliquot of 3 M KCl, 0.25 M sodium pyrophosphate was added to give a final concentration of 0.3 M KCl. This homogenate was centrifuged at 132 000 × *g* for 60 min in an SW 27 rotor in a Beckman L2 75B ultracentrifuge.

The pellet was rinsed with homogenizing buffer to reduce the concentration of K^+ and raised in homogenizing solution to which 200 mM choline chloride was added. This pellet fraction was layered over a 34% sucrose step and centrifuged at 132 000 × *g* for an hour. The interface was pelleted in buffered choline media and raised in 42% sucrose. This dense fraction was layered under a step gradient of 7 ml aliquots of 2%, 34% and 37% sucrose in 200 mM choline chloride and centrifuged 3 h at 100 000 × *g*. Fraction A had a density of 1.12–1.14 g/ml (28–32%). Fraction B was 1.15–1.17 g/ml (35–39%) and fraction C included all material more dense than 1.17 g/ml. Fractions were assayed for protein, $(Na^+ + K^+)$-ATPase activity, K^+-stimulated *p*-nitrophenylphosphatase activity and [^{3}H]saxitoxin binding as outlined below.

The distribution of [^{3}H]saxitoxin binding sites in *Rana pipiens* heart fractions was determined using a preparation previously described [26]. Briefly, the ventricles were homogenized in a solution containing 84 mM K_2SO_4, 1 mM calcium gluconate, and 10 mM Tris (pH 7.4) and centrifuged at 5000 × *g* for 10 min. The supernatant (S_1) was centrifuged for 2 h over a 30% sucrose solution at 130 000 × *g*. The supernatant (S_2), interface (I_2) and pellet (P_2) were collected. The interface was diluted and centrifuged for an hour at 130 000 × *g*.

B. Enzyme and [^{3}H]saxitoxin assays

$(Na^+ + K^+)$-ATPase was assayed as described by Jones et al. [27]. Basal Mg^{2+}-ATPase activity was measured in a medium containing 5 mM $MgCl_2$, 5 mM NaN_3, 50 mM histidine at pH 7.0 and 2 mM ATP. Total activity was measured with 100 mM NaCl and 20 mM KCl in the presence of saturating concentrations of ouabain. Inorganic phosphorus was measured with the procedure described by Stanton [28]. Approximately 40 μg of protein was used for each measurement. Ouabain-inhibited potassium-stimulated *p*-nitrophenylphosphatase activity was measured according to Barchi et al. [29]. Potassium stimulation was measured in the presence of 20 mM KCl. Protein was determined by a modification of the method of Lowry et al. [30] as reported by Barr et al. [26].

The saxitoxin was labelled by New England Nuclear Co. by incubation in ^3H_2O at 50°C [31]. The exchange labelling of ^{3}H at the 12 carbon position of saxitoxin is temperature dependent; thus, in order to minimize back exchange of the tritium from [^{3}H]saxitoxin, the toxin was stored at −70°C and all assays were carried out at 0–4°C. Purification of labelled saxitoxin was done at 4°C following the procedure described by Ritchie et al. [32]. The radiochemical purity of the labelled toxin was determined by bioassay using a desheathed frog sciatic nerve. The specific activity of the labelled saxitoxin varied from 3 to 9 Ci/mmol and the purity from 50 to 90%.

Assays for saxitoxin binding to tissue homo-

genates and sarcolemma fractions were performed with equilibrium dialysis or with a centrifugation assay. For the centrifugation assay samples containing protein and toxin were incubated on ice for a least an hour in a total volume of 0.4 ml. Aliquots of 0.1 ml were transferred to three microfuge tubes. Six microfuge tubes were placed in an SW-27 centrifuge tube partially filled with water to prevent the tubes from collapsing during centrifugation. The samples were centrifuged at $45\,000 \times g$ for 30 min to separate free toxin from that bound to the sarcolemma. The supernatant was aspirated and two rinses of cold buffer were quickly aspirated. The microfuge tube was then cut and the pellet placed in a glass scintillation vial with 0.3 ml of 30% H_2O_2. Digestion of the pellet was usually completed within 10 min at 100 °C. The vials were cooled and 10 ml of Biofluor scintillation cocktail was added. Internal standards were used to determine the counting efficiency. All binding assay points as well as the enzyme assays were done in triplicate.

Binding assays on the cytosolic fractions were carried out using either equilibrium dialysis or gel filtration assays [33,34]. Equilibrium dialysis was carried out in Plexiglass chambers with two 120-μl compartments separated by a dialysis membrane. Labelled saxitoxin, with and without an excess of unlabelled saxitoxin, was placed in one half of the chamber and the protein sample was placed in the other half. Equilibrium of the toxin in both sides of the chamber occurred within 18 h at 4 °C. The contents from both sides of the chamber were counted in Biofluor scintillation fluid. The gel filtration assay was modified to use 2.5 ml of Sephadex G-50–80, equilibrated in appropriate buffer, in a plugged 3.0 ml disposable syringe. Aliquots of equilibrated toxin and protein were layered onto the Sephadex. After a 30-s soaking time, the centrifuge tubes were spun for 90 s at top speed in a desk top centrifuge. The eluant was counted in Biofluor. Less than one percent of the total counts applied to the gel were eluted in the absence of protein. The gel filtration assay was calibrated against equilibrium dialysis; the results of both assays agreed within ten percent.

A linear component of binding was measured in the presence of $4 \cdot 10^{-5}$ M saxitoxin and subtracted from the total binding to give the saturable binding component. The saturable component was fitted with a Langmuir hyperbolic function [35] using a weighted non-linear iterative curve fitting routine based on the Levenberg-Marquardt algorithm [36,37]. The equation used:

$$\text{Saxitoxin bound} = B_m\,([\text{Saxitoxin}]/([\text{Saxitoxin}] + K_d)) \quad (1)$$

where B_m is the binding capacity in pmol/(mg protein) and K_d is the apparent dissociation constant. Data were linearized following the method of Rosenthal [38] and a linear least-squares routine was used to fit the data. Unless otherwise specified, binding measurements were done in a medium containing 200 mM choline chloride, 1 mM EDTA, 10 mM Tris at pH 7.4. This medium was chosen to reduce aggregation and to minimize sodium competition with [^{3}H]saxitoxin [39].

Results

A. Membrane enrichment

Membrane fractions were separated using high speed and density gradient centrifugation. In order to evaluate the purity of the membrane fraction, ouabain inhibited ($Na^+ + K^+$)-ATPase was chosen as a sarcolemmal marker, following work by Jones et al. [40] characterizing the separation of sarcoplasmic reticulum from sarcolemma in dog heart. Results of ($Na^+ + K^+$)-ATPase assays are shown in Table II. The Mg^{2+}-ATP levels were very high in the crude fractions and without the addition of sodium azide to the assay medium, ouabain-inhibitable ($Na^+ + K^+$)-ATPase activity could not be measured. Very little stimulation of the basal Mg^{2+} activity was seen with the addition of Na^+ and K^+; additionally the presence of ouabain decreased the activity well below the apparent basal levels.

Ouabain-inhibited ($Na^+ + K^+$)-ATPase activities showed an average enrichment of 6-fold in the membrane fraction. To test our assumption that measurement of ouabain inhibited ($Na^+ + K^+$)-ATPase activity was a reliable marker for the plasma membrane we also measured ouabain-inhibited K^+ *p*-nitrophenylphosphatase. The enrichment of this marker, see Table II, correlated well with ouabain-inhibited ($Na^+ + K^+$)-ATPase enrichments as expected since both assays are

TABLE II

INHIBITION OF ACTIVITIES BY OUABAIN

$(Na^+ + K^+)$-ATPase assays were carried out in a medium containing 50 mM histidine, pH 7.0, 2 mM ATP, 5 mM $MgCl_2$, 0.5 mM EGTA, 5 mM NaN_3. Final concentrations of NaCl, KCl and ouabain were 120 mM, 20 mM and 1 mM, respectively. Samples were incubated at 37 °C for 10 min before the addition of ATP. The reaction was terminated after 10 min by the addition of cold 40% trichloroacetic acid. Phosphorus was measured as described in the text. Potassium-stimulated *p*-nitrophenylphosphatase was measured in a solution containing 3 mM $MgCl_2$, 0.1 mM EGTA, 0.005% deoxycholate, 50 mM Tris (pH 7.8) and 3 mM *p*-nitrophenyl phosphate. The reaction was incubated for 10 min before adding the substrate. The reaction was stopped by adding 0.1 M NaOH after 15 min.

Animal	Fraction	$(Na^+ + K^+)$-ATPase activities (μmol/mg per h) (mean ± S.D.)			
		Mg^{2+}	Mg^{2+}, Na^+, K^+	Mg^{2+}, Na^+, K^+ + ouabain	Ouabain-inhibited
Chicken	Homogenate	2.73 ± 0.44	2.51 ± 0.11	0.57 ± 0.24	1.94
	FractionA	15.82 ± 0.36	17.89 ± 0.49	6.03 ± 0.16	11.85
	Fraction B	10.84 ± 0.64	12.98 ± 0.43	4.84 ± 0.25	8.14
Rana. pipiens	Homogenate	7.75 ± 0.95	8.02 ± 0.65	1.26 ± 0.27	6.76
	Interface	54.13 ± 3.64	58.94 ± 0.70	19.41 ± 0.51	39.53
Bufo marinus	Homogenate	6.63 ± 0.65	7.29 ± 0.43	3.22 ± 0.65	4.07
	Interface		55.50 ± 1.36	33.22 ± 0.98	22.28
Turtle	Homogenate	3.23 ± 0.29	3.42 ± 0.97	0.32 ± 0.32	3.10
	Interface		16.97 ± 0.81	5.54 ± 0.27	11.43
Animal	Fraction	K^+-pNPPase activities (μmol/mg per h) (mean ± S.D.)			
		$-K^+$	20 mM K^+	10 mM K^+ + 1 mM ouabain	Ouabain-inhibited
Chicken	Homogenate	6.3 ± 0.02	6.6 ± 0.043	6.2 ± 0.03	0.4
	Fraction A	7.2 ± 0.014	10.3 ± 0.082	7.88 ± 0.033	2.4
Rana pipiens	Homogenate	10.9 ± 0.11	11.8 ± 0.08	10.4 ± 0.07	1.4
	Interface	31.9 ± 0.10	39.9 ± 0.072	32.9 ± 0.23	7.0

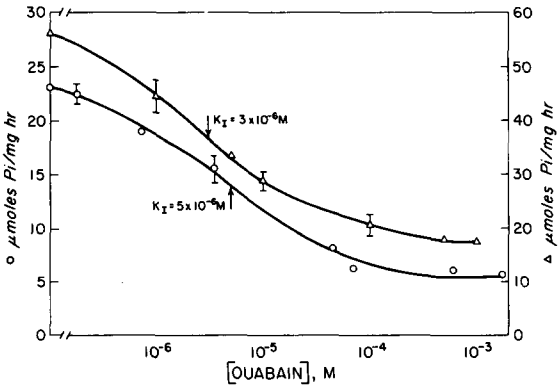

Fig. 1. The effect of ouabain on the $(Na^+ + K^+)$-ATPase activity of chicken (○) and frog (△) cardiac sarcolemma. The assay medium contained $MgCl_2$ (5 mM), NaCl (100 mM) KCl (20 mM) and histidine (50 mM). The enzyme was incubated with the inhibitor for 10 min at 37 °C before ATP (2 mM) was added. The reaction was stopped after 10 min with the addition of cold trichloroacetic acid. The concentration of ouabain giving half-maximal inhibition is $5 \cdot 10^{-6}$ for chicken (○) and $3 \cdot 10^{-6}$ for frog (△).

thought to monitor the same enzyme [40]. Other purification procedures for cardiac sarcolemma have yielded enrichments of 20-fold or greater [25,27,41,42] but in our hands they either did not provide sufficient material or aggregation occurred which prevented further separation. The concentration of ouabain which inhibits half of the maximum $(Na^+ + K^+)$-ATPase activity ($K_{0.5}$) was measured in chicken heart and *Rana pipiens* heart and is shown in Fig. 1 to be $5 \cdot 10^{-6}$ M and $3 \cdot 10^{-6}$ M ouabain, respectively.

B. [^{3}H]Saxitoxin binding

The results of [^{3}H]saxitoxin binding to *Rana pipiens* plasma membrane are shown in Fig. 2. The B_m for *R. pipiens* was 0.63 pmol/mg and the apparent K_d was $5 \cdot 10^{-9}$ M. This estimate of saxitoxin affinity is in good agreement with the concentration of tetrodotoxin needed to decrease the sodium current by 50% [12].

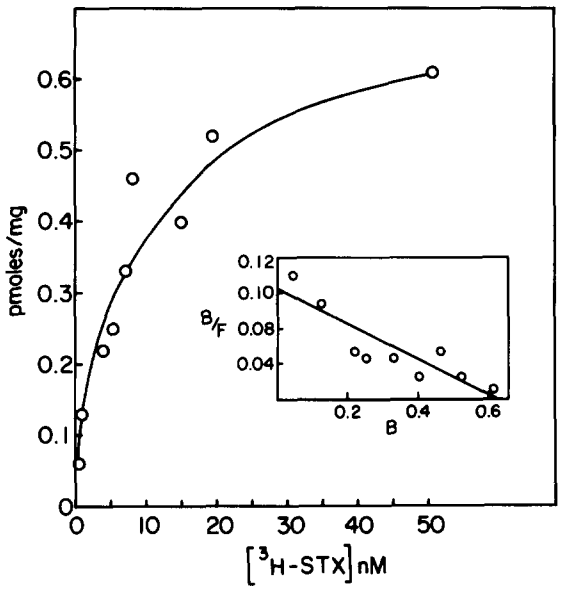

Fig. 2. Saturable binding of [^{3}H]saxitoxin to *R. pipiens* heart plasma membrane. The linear component was measured in the presence of $4 \cdot 10^{-5}$ M saxitoxin and subtracted from the total bound to give the saturable component. The saturable data were fit by eye. The Rosenthal plot was fitted with a linear regression; $B_m = 0.63$ pmol/mg and $K_d = 5.1$ nM. (The specific activity of [^{3}H]saxitoxin used was $7.1 \cdot 10^{16}$ dpm/mol.)

The results of [^{3}H]saxitoxin binding to Fraction A (membrane vesicles) from the sucrose gradient of chicken heart are shown in Fig. 3. The binding of [^{3}H]saxitoxin was linear up to $3 \cdot 10^{-7}$ M. When [^{3}H]saxitoxin binding was measured in the presence of $4 \cdot 10^{-5}$ M unlabelled saxitoxin, binding was again linear but with a smaller slope, indicating the presence of a binding site which saturated in the range greater than $2 \cdot 10^{-7}$ M but less than $4 \cdot 10^{-5}$ M. In order to improve both the signal-to-noise ratio of the measurements and to use less labelled saxitoxin we used a displacement assay [43] to estimate the affinity of chicken heart sarcolemma for saxitoxin. In this assay increasing amounts of saxitoxin were added to aliquots with constant protein and total [^{3}H]saxitoxin. If the total [^{3}H]saxitoxin concentration is much less than the dissociation constant, a useful variant of the Hill equation [44] holds.

$$\log\left(\frac{B/B_m}{1-(B/B_m)}\right) = \log K_I - \log[\text{Saxitoxin}] \qquad (2)$$

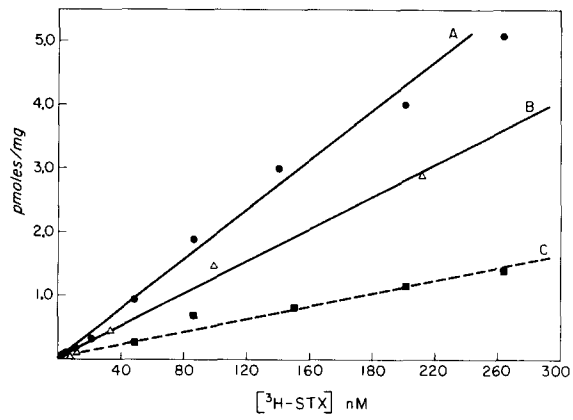

Fig. 3. A direct plot of [^{3}H]saxitoxin binding to chicken heart membranes. The binding of [^{3}H]saxitoxin alone is shown in A (●), binding in the presence of $4 \cdot 10^{-5}$ is shown in B (△), and the displaceable component of binding (A − B) is shown in C (■).

The log functions allow more balanced weighing of data using high and low saxitoxin concentrations than the usual linear plots. Data from such an experiment are shown in Fig. 4. From these plots the K_d was estimated to be $1.75 \cdot 10^{-6}$ M saxitoxin. Since [^{3}H]saxitoxin is much less than

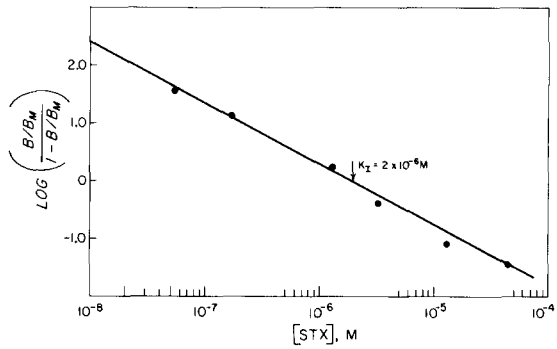

Fig. 4. A variant Hill plot of [^{3}H]saxitoxin binding to chicken heart membranes. Total binding of the [^{3}H]saxitoxin was measured at $3 \cdot 10^{-7}$ M and displaced with saxitoxin at increasing concentrations. The non-displaceable component was measured with $1.3 \cdot 10^{-4}$ M saxitoxin and subtracted from the total binding. The best fit for the linearized data is:

$$\log[(B/B_M)/(1-(B/B_M))] = -5.69 - 1.05\log[\text{saxitoxin}]$$

where B is the amount of bound [^{3}H]saxitoxin and B_M is the maximum bound at the fixed [^{3}H]saxitoxin concentration. The K_I for the plot is $2 \cdot 10^{-6}$ M and the K_d is $1.75 \cdot 10^{-6}$ M.

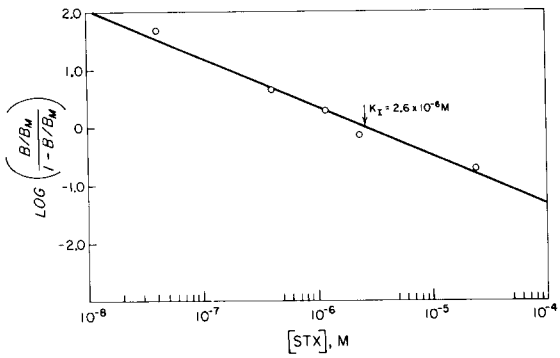

Fig. 5. A variant Hill plot of [^{3}H]saxitoxin binding to turtle heart membrane. The binding was measured as in Fig. 4. The best fit for the linearized data was:

$$\log[(B/B_M)/(1-(B/B_M))] = -5.58 - 0.88\log[\text{saxitoxin}]$$

The K_d for saxitoxin binding is $2.3 \cdot 10^{-6}$ M.

the K_d, B_m was calculated from the relationship

$$B_m = \frac{K_I \cdot B}{[[^3\text{H}]\text{saxitoxin}]} \quad (3)$$

to be 8.6 pmol/mg protein. A similar value, 8.9 pmol/mg, was estimated from the data in Fig. 4 assuming a K_d of $1.75 \cdot 10^{-6}$ M and fitting this to Eqn. 1.

The turtle heart was chosen as an evolutionary intermediate between the frog heart, which demonstrated a relatively high affinity for saxitoxin, and the chicken heart which had a low affinity. No evidence for a high-affinity binding site was seen in this preparation. The variant Hill plot from a displacement assay is shown in Fig. 5: the K_d was estimated at $2.3 \cdot 10^{-6}$ M saxitoxin and $B_m = 11$ pmol/mg. The empirical slopes of -1.05 for chicken and -0.88 for turtle heart for the variant Hill plot support the commonly accepted 1 : 1 binding stoichiometry for the interaction between saxitoxin and the sodium channel.

TABLE III

THE DISTRIBUTION OF [^{3}H]SAXITOXIN BINDING SITES IN *RANA PIPIENS* MYOCARDIAL FRACTIONS

The method of membrane fractionation used in this experiment has been reported earlier [26] and is briefly described in the methods. Specific binding was measured at $1.5 \cdot 10^{-8}$ M [^{3}H]saxitoxin. Binding is expressed as total pmol from a preparation of 60 hearts.

Supernatant 1	200	Supernatant 2	140	Supernatant 3	0
		Interface 2	50	Pellet 3	45
		Pellet 2	5		
		Total	195	Total	45

C. Distribution of binding sites in frog homogenates

The distribution of saxitoxin binding sites from the initial homogenate of *R. pipiens* heart is given in Table III. A large fraction of the initial homogenate binding is seen in the supernatant from 2 h centrifugation at $132\,000 \times g$. The ratio of binding sites in Supernatant 2 to Interface 2 (Table III) ranged from 5 : 2 to 10 : 1 in different preparations. The apparent K_d was $5 \cdot 10^{-9}$ M and B_m was 1.2 pmol/mg for this cytocolic fraction. Further centrifugation of this supernatent for 12 and 16 h at $250\,000 \times g$, failed to pellet more than 60% of the binding sites arguing against these sites having a membrane origin [22].

For this study cytosolic fractions were prepared from *R. pipiens*, *R. catesbeiana*, and *B. marinus* in a sucrose medium using a Polytron for the initial disruption. Fig. 6 shows the saturable [^{3}H]saxitoxin binding component, measured with gel filtration, from *R. pipiens* cytosolic fraction.

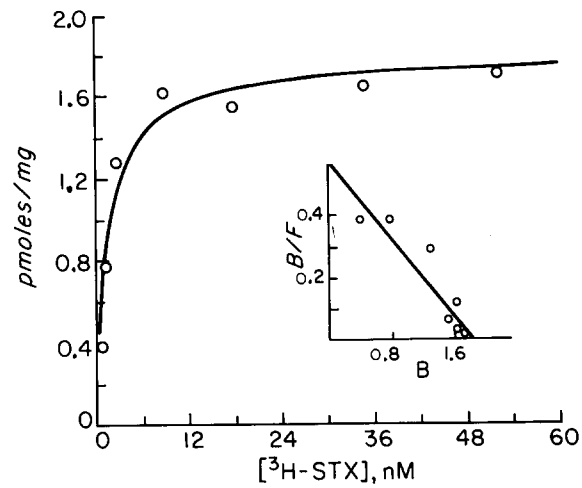

Fig. 6. Saturable binding of [^{3}H]saxitoxin to *R. pipiens* myocardial cytosol. The linear component was subtracted from the total binding and the saturable component was fitted as described in the text; the best fit gives a site density of 1.8 pmol/mg and K_d of $1.7 \cdot 10^{-9}$ M. The insert shows a Rosenthal plot of the data: A linear regression was used to estimate the site density, 1.9 pmol/mg and $K_d = 2.1 \cdot 10^{-9}$ M.

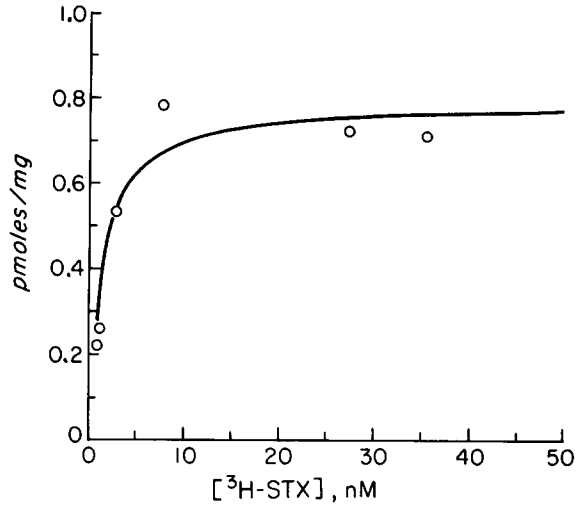

Fig. 7. Saturable binding of [^{3}H]saxitoxin to *B. marinus* heart cytosolic fraction. The linear binding component was measured as described in Fig. 6 and subtracted from the total binding to give the saturable component. The best fit to this data gave a site density of 0.79 pmol/mg and $K_d = 1.4 \cdot 10^{-9}$ M.

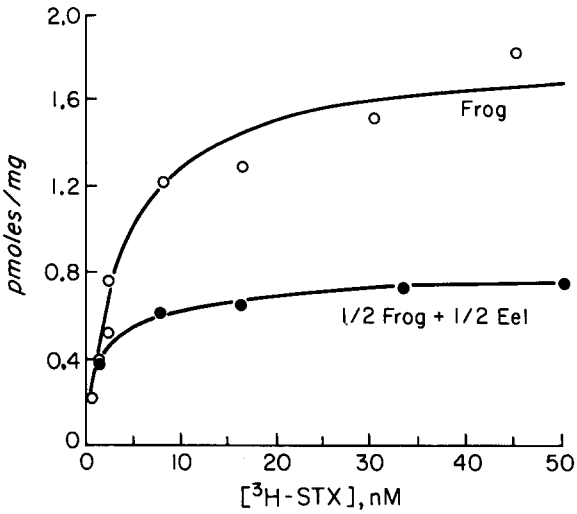

Fig. 8. Saxitoxin binding of *R. catesbeiana* myocardial cytosol and the cytosol from a combined homogenate of *R. catesbeiana* and *Electrophorus electricus* electric organ. Homogenate fractions were prepared as described in the text. Equal amounts of protein from the two homogenates were incubated for 30 min at 25 °C. [^{3}H]Saxitoxin binding was measured in cytosolic fractions following centrifugation of the homogenates at 132000 × *g* for one hour. (○) represents binding to the frog myocardial cytosol and (●) is binding to a combined eel and frog fraction. The site density for the frog alone was 1.8 pmol/mg and the density for the combined fraction was 0.79 pmol/mg. Eel cytosol alone showed no saxitoxin binding.

TABLE IV

A SURVEY OF TETRODOTOXIN COMPETITION AGAINST [^{3}H]SAXITOXIN BINDING IN VARIOUS TISSUE PREPARATIONS

The binding was measured with gel filtration or equilibrium dialysis as described in the text. The concentration of tetrodotoxin (TTX) used was 10^{-5} M; binding curves were done using 10^{-5} M saxitoxin (STX) to determine the linear binding component.

Tissue	Fraction	Apparent K_d for [^{3}H]STX binding	Competition by TTX
Rana pipiens heart	Sarcolemma	$5.1 \cdot 10^{-9}$ M	–
	Cytosol	$2.1 \cdot 10^{-9}$ M	–
Bufo marinus	Cytosol	$1.4 \cdot 10^{-9}$ M	–
Adult chicken heart	Sarcolemma	$1.7 \cdot 10^{-6}$ M	+
	Cytosol	no binding	
Turtle heart	Sarcolemma	$2.3 \cdot 10^{-6}$ M	+
	Cytosol	no binding	
Electrophorus electric organ	Cytosol	no binding	
Rabbit heart	Cytosol	no binding	
Rat brain	Cytosol	no binding	

The apparent K_d in the low ionic strength sucrose medium was $1.7 \cdot 10^{-9}$ M; the site density was 1.8 pmol/mg. These results confirm that the presence of the soluble site in *R. pipiens* is not dependent on either the mode of homogenization or the ionic strength of the homogenizing medium. A similar binding site was found in the cytosolic fractions from both *R. catesbeiana* and *B. marinus*, see Figs. 7 and 8. The K_d values for binding were $1.4 \cdot 10^{-9}$ M for *B. marinus* and *R. catesbeiana*, respectively, and the site densities were 0.8 pmol/mg and 1.8 pmol/mg. Cytosolic fractions was prepared from adult chicken heart, turtle heart, *Electrophorus electricus* electric organ, rabbit heart, and rat brain. None of these preparations showed binding in the Supernatant 2, cytosolic fraction, see Table IV.

D. Origin of soluble amphibian site

To explore the possibility that an enzyme was present in the amphibian heart which was not blocked by any of the inhibitors used and which specifically 'clipped' the saxitoxin binding site from the membrane during homogenization we prepared homogenates from both *Electrophorus* elec-

tric organ and *R. catesbeiana* heart. Equal amounts of protein from eel electric organ and frog heart were incubated together at 25 °C for 30 min. After the incubation, the individual homogenates and the combined homogenate were centrifuged for an hour at 132 000 × *g*. The resulting supernatans were assayed for saxitoxin binding. Fig. 8 shows the results of binding assays on the combined cytosolic fraction as well as the *R. catesbeiana* alone. No binding was seen in the eel cytosolic fraction. The site density for the frog alone was 1.8 pmol/mg protein and the density for the combined fraction was 0.79 pmol/mg. The electric organ was homogenized in 200 mM choline chloride and 10 mM Tris at pH 7.4 and the frog heart was homogenized in the sucrose medium as described. This variation in the media may be responsible for the shift in the apparent K_d from $4.4 \cdot 10^{-9}$ in frog alone to $1.5 \cdot 10^{-9}$ M in the combined fraction. If any sites were released from the eel membrane the combined cytosol would show a site density of more than half the sites in the frog cytosol. In fact, slightly less than half the number of sites were found in the combined fraction arguing against the release of sites from the eel membrane. While this experiment does not rule out the possibility of an enzyme which is specific for amphibian cardiac sodium channel, the previous demonstration [22] that the saxitoxin site density in the intact frog ventricle is not different from the density of sarcolemma sites is evidence against the idea that the soluble site originates in the sarcolemma. Ouabain inhibited $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities were measured in the cytosolic fractions from amphibian heart; the levels of activity were undetectable suggesting the absence of significant amounts of plasma membrane in the cytosolic fraction.

E. Tetrodotoxin competition

The use of tetrodotoxin to displace [^{3}H]saxitoxin binding in order to estimate the amount of nonspecific binding is a common practice because in most nerve and muscle sodium channels, high concentrations of tetrodotoxin displace saxitoxin [6,7]. Table IV shows the results of displacement of [^{3}H]saxitoxin binding by 10^{-5} M tetrodotoxin in the amphibian sarcolemma fractions. The results show tetrodotoxin competition is present for the low-affinity site in chicken and turtle fractions but none of the amphibian fractions demonstrated competition by tetrodotoxin (Table IV). The fact that the in situ frog cardiac sodium channel has a high affinity for tetrodotoxin may mean that this site undergoes some alteration upon homogenization.

Discussion

A primary motivation for characterizing saxitoxin binding to cardiac membranes is to develop an enriched plasmalemma fraction from which sodium channel can be isolated and reconstituted. A sodium channel purification scheme based on saxitoxin binding as a marker for the channel requires a preparation with high-affinity saxitoxin binding such as frog heart. Electrophysiological measurements of tetrodotoxin and saxitoxin affinities to cardiac membranes indicated that mammalian myocardial sodium channels have very low affinities for the toxins and hence are not good candidates for a source of high affinity cardiac sodium channel, see Table I.

The enrichment of ouabain inhibited $(Na^+ + K^+)$-ATPase activity was used to assess the relative purity of our sarcolemma preparations. The enzyme activities are comparable to those obtained in other cardiac preparations [40,41]. Similarly the K_I values for ouabain inhibition, Fig. 1, in chick and frog cardiac sarcolemma agree moderately well with earlier values measured in calf heart [41].

Our results from [^{3}H]saxitoxin binding measurements to the amphibian cardiac sodium channel, Fig. 2, support electrophysiological measurements indicating a high affinity for tetrodotoxin (see Table II). Estimates of tetrodotoxin affinity to the sodium channel of embryonic chick heart were determined from the inhibition of the sodium currents [45]. Iijima and Pappano [45] estimated the K_d at less than $3 \cdot 10^{-8}$ M for embryos up to 18 days. Marcus and Fozzard [46] compared the effect of tetrodotoxin on embryonic chick hearts to its effect on adult hearts. They reported a 50% reduction of the maximal upstroke velocity with 10^{-8} M tetrodotoxin on days 4, 8, 18 and adult ventricles. Although the relationship between the maximum rate of the action potential rise and

sodium conductance is not linear, these authors estimated a K_d for tetrodotoxin of $5 \cdot 10^{-9}$ M for all ages of chicken ventricles.

Our results on the inhibition of [^{3}H]saxitoxin binding by unlabelled saxitoxin to adult chicken cardiac membranes suggest the presence of a large population of low-affinity binding sites, Fig. 3. In fact, we saw no evidence for a high affinity site in adult hearts. Similar binding measurements were made by Rogart et al. [21] on 10–20-days chick hearts which estimate a K_d for saxitoxin binding of $(6–8) \cdot 10^{-9}$ M, 20–30-times higher than the saxitoxin affinity for chick brain, $K_d = 0.3 \cdot 10^{-9}$ M. The saxitoxin binding site density appears quite low in amphibian cardiac sarcolemma when compared with the 10-fold greater density of low affinity sites in the chicken and turtle. We found 5–10-times more saxitoxin binding in the amphibian cytosolic fraction than the membrane fraction suggesting a membrane origin for these soluble sites. However, when the density of membrane sites in the intact frog heart was compared to the density in the purified membranes normalized to membrane area the agreement was quite good [22]. The density of the low affinity sites in chicken and turtle is difficult to estimate because of the very low signal-to-noise ratio at concentrations approaching saturation. The approach used by Rogart et al. [21] to reduce the linear component of binding in heart may permit better estimates of the low affinity binding site densities with radiolabelled ligands but the true density of sodium channels in heart may ultimately be best estimated using patch clamp techniques.

Several points require further consideration in these experiments. First the age may determine the affinity for the toxin, younger chicks having a higher affinity, see Table I; secondly both our study and Rogart's [21] report the presence of a very large non-specific component of binding in these preparations. Rogart's approach was to reduce the non-specific components by using an assay with a high salt wash and to substitute cesium for sodium in the binding assays. Our approach was to enrich the membrane fraction and to use a displacement assay which estimates the apparent K_d for unlabelled saxitoxin, avoiding possible artifacts with labelled toxin especially at high concentrations. Despite the unresolved discrepancy in K_d values between Rogart et al. [21] and this report on chick heart our conclusions about the cardiac sodium channel in chicken are in agreement. Namely, there are at least two types of sodium channel; a high-affinity saxitoxin type seen in nerve, muscle and amphibian heart and a lower affinity channel seen in chicken and mammalian heart. Table I summarizes current information on the tetrodotoxin and saxitoxin affinities for various cardiac preparations using biochemical and electrophysiological techniques. Large discrepancies in both directions exist regarding the interaction of the toxins with cardiac sodium channels. Electrophysiological measurements using V_{max}, the maximum rate of rise of the action potential, may be more difficult to interpret than clamp currents nevertheless, the differences between the physiological and biochemical estimates of K_d values seem too large to be caused by that complication alone.

Catterall and Coppersmith [16] measured [^{3}H]saxitoxin binding to heart homogenates from six vertebrates including rat, bullfrog, chicken, salmon, guinea pig and monkey. In all these cases the K_d for tetrodotoxin was between 3.5 and 10.4 nM which reflect large discrepancies with the electrophysiological data, Table I. These authors concluded that the high affinity saxitoxin sites are not associated with the myocardial sodium channels studied in physiological experiments. Instead, they suggest that the source of high-affinity saxitoxin binding sites in vertebrate hearts is the autonomic nerve endings. Lombet et al. [47] reported K_d values of $2 \cdot 10^{-9}$ M for [^{3}H]en-tetrodotoxin bindings to guinea pig, rabbit, embryonic chick heart and rat heart homogenate. These results are also at variance with concentrations necessary to inhibit the sodium currents.

Some of the present confusion about the cardiac sodium channel's sensitivity to tetrodotoxin and saxitoxin may arise from not distinguishing between these toxins. Most electrophysiologists have used tetrodotoxin to block sodium currents whereas saxitoxin is the more common biochemical probe. Binding competition between the two toxins, tetrodotoxin and saxitoxin, in heart tissue has not been well characterized. Tetrodotoxin does not displace [^{3}H]saxitoxin binding in frog myocardial homogenates or sarcolemma fractions, see Table

IV, yet tetrodotoxin does displace [^{3}H]saxitoxin in intact frog heart membranes. This behavior may suggest changes in the binding site during homogenization. Kao and Walker [48] recently investigated the actions of several saxitoxin analogues on frog muscle fibers. One of their conclusions was that saxitoxin and tetrodotoxin may bind to opposite sides of the membrane receptor with an overlapping central region. Thus, in tissues where no competition exists between the two toxins, subtle alterations in the overlapping region may have occurred. Many sodium channels are not tetrodotoxin sensitive. Twarog et al. [49] demonstrated that bivalve mollusc nerves have a low tetrodotoxin sensitivity which varies over several orders of magnitude depending on the species. Pappone [50] described two populations of sodium channels in denervated rat skeletal muscle; one had a K_d for tetrodotoxin of $5 \cdot 10^{-9}$ M and the other bound with a low affinity, $K_d = 10^{-6}$ M. Strichartz et al. [51] monitored the appearance of a tetrodotoxin-sensitive sodium channel in developing skeletal muscle cells in culture and the presence of a batrachatoxin catalyzed sodium flux and found that the batrachatoxin activated flux appears before the appearance of an saxitoxin binding site. These data suggest that although the saxitoxin binding may be useful as a marker in some tissues, its use as a sodium channel probe may be limited in other tissues, particularly heart. A more useful molecular probe may be cross-reacting monoclonal and polyclonal antibodies against well characterized high saxitoxin affinity sodium channels [52].

The observation of a high saxitoxin affinity binding site in amphibian cytosolic fractions introduced a third type of sodium channel. (The word type is used for lack of a more precise term; in fact the cytosolic site may be a precursor or degenerate form of the membrane channel and not a distinct type of channel). This cytosolic site was present in all amphibian cardiac preparations we looked at and was not present in homogenates from other excitable tissues. One approach to understanding whether this site is a form of sodium channel would be reconstitution into phospholipid vesicles with the demonstration of saxitoxin blockable ^{22}Na fluxes [10,53].

In conclusion, a number of saxitoxin binding sites have been reported in cardiac tissues. They include the high-affinity saxitoxin binding site in amphibian plasma membranes, the much lower-affinity site in mammalian and chicken plasma membranes, and the high-affinity cytosalic site in amphibian cardiac homogenates. Currently very little agreement is seen regarding the in vivo properties and the binding characteristics of these sites. Possibly the isolation procedures selected for a particular population and controlled experiments are needed to address this issue. Regardless of these variations the important issue is the relationship of biological function to chemical behavior. Two techniques are now available which should be useful in defining the functional and biochemical properties of cardiac sodium channels. The patch clamp technique circumvents many of the problems related to the series resistance of cardiac cells; this technique may permit direct estimates of the concentrations of saxitoxin needed to block sodium currents. The other important biochemical technique is the use of monoclonal or polyclonal antibodies as high affinity molecular tools for the purification of antigens. Polyclonal antibodies against rat muscle sodium channel demonstrate cross reactivity with rat heart [52]. Isolation of the cardiac channel may therefore be achieved without the use of saxitoxin. Once isolation of the channel is achieved, these structure-function relationships may be better addressed.

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