Saxitoxin Binding Sites in Frog Myocardial Cytosol

Abstract. *Cytosolic fractions of frog heart homogenates contain large amounts of a soluble, large molecular weight protein that binds the specific neurotoxin saxitoxin with the same high affinity as does the plasma membrane. Another neurotoxin, tetrodotoxin, which ordinarily is competitive with saxitoxin, does not displace saxitoxin from the cytosolic sites or from plasma membrane–enriched vesicular fractions even when its concentration exceeds that of saxitoxin by a factor of 1000. Thus, cytosolic sites are similar to membrane sites in this respect. The vesicular fraction accounts quantitatively for the amount of saxitoxin bound by whole ventricles, so that no appreciable losses seem to occur. Therefore, the cytosolic site probably is a membrane site precursor, although other possibilities cannot be ruled out. In any case, the occurrence of a soluble molecule closely related to the sodium channel provides opportunities for further study of the structure of the sodium channel.*

In nanomolar concentrations, the neurotoxins saxitoxin (STX) and tetrodotoxin (TTX) selectively block but do not alter the kinetics of the voltage-dependent sodium current in most excitable membranes (*1*). They have no other known effects. The two toxins competitively bind 1 : 1 to the sodium channel site (*2*) with high affinity (dissociation constant $K_D \sim 5 \times 10^{-9} M$). They block the sodium current in frog atria equally well (*3*). Because of their high specificity and affinity, STX and TTX have often been used as Na^+ channel markers. Furthermore, TTX or STX binding sites from the membranes of nerve tissue (*4*), brain tissue (*5*), eel electroplax (*6*), and rat skeletal muscle (*7*) have been solubilized by detergents.

It has been reported (*8*) that a supernatant of beef brain and heart muscle homogenates associates with prepared liposomes and confers on them a transient, veratrine- and TTX-sensitive increase in the rate of ^{22}Na uptake. However, the centrifugation used (100,000*g* for 30 minutes) was not sufficient to eliminate small plasma membrane vesicles from the supernatant fraction.

We report here high-affinity STX binding in the cytosolic fraction of homogenates of ventricles and atria from the northern frog, *Rana pipiens pipiens*. Isolated, washed ventricles or atria were homogenized with 10 to 20 strokes of a Teflon pestle. The plasma membrane fraction was collected over a step sucrose gradient of density 1.13 g/cm^3 by centrifugation at 100,000*g* for 2 hours (*9*).

Binding of [^{3}H]STX (*10*) in all subcellular fractions was measured by equilibrium dialysis in the presence and in the absence of excess unlabeled STX or TTX and in the supernatant fraction (P_2A) by the gel filtration method (*11*). The saturable binding of whole tissue was also measured. Figure 1 shows saturable STX binding to a component of the soluble fraction ($K_D \sim 5$ n*M*). There were about 1.2 pmole of binding sites per milligram of soluble protein or about 12.5 fmole of binding sites per milligram (wet weight) of ventricular tissue.

About five times more [^{3}H]STX binding per unit of tissue was observed in the soluble fraction of the homogenates than in intact ventricles or the plasma membrane fractions (Table 1). The density of STX binding sites in whole intact tissue is equivalent to the STX binding activity measured in the plasma membrane fraction when both are normalized to membrane area. We found that 5 to 20 times more binding appeared in the homogenate than in the intact tissue. Of the 52 percent of the homogenate sites recovered in the more purified cytosolic and plasma membrane fractions, 43 percent appeared in the cytosolic fraction and only 9 percent appeared in the plasma membrane vesicular fraction.

When cytosolic fractions were centrifuged at 250,000*g* for 12.5 and 16 hours, 40 and 60 percent of the STX binding sites appeared in the pellets. These correspond to S^0 values of 2.3 and 2.6 svedbergs. From the formula, molecular weight = $S^0RT\rho/D^0(\rho - 1)$, where R is the gas constant, T is temperature, ρ is specific density, and D^0 is the diffusion coefficient, one can calculate molecular weights of about 170,000 and 200,000 by using $D^0 = 5 \times 10^{-7}$ and $\rho = 1.07$. These values were chosen from the extremes of possible values to maximize the calculated molecular weights. Even so, the calculated molecular weights are too small to constitute the smallest possible vesicles. If particle densities and diffusion constants are chosen from the usual values of lipid-free proteins, molecular weights in the neighborhood of 30,000 may be calculated. Such small molecular weights do not agree with the gel filtration data discussed below.

The cytosolic STX binding site is precipitated by 70 percent $(NH_4)_2SO_4$. Concentrating the soluble fraction tenfold by using an Amicon ultrafiltration system with an XM-300a filter results in a five- to tenfold reduction in absorbance at 260 μm (nucleic acids) but only a 20 percent loss in STX binding. As shown in Fig. 2, A and C, the peak of the STX binding component eluted from our Sephacryl column at 180 ml, which corresponds to a Stokes radius of approximately 52 Å (Fig. 2D). When the cytosolic fraction was concentrated fourfold and chromatographed (Fig. 2B), an additional and presumably much larger binding component eluted in the void volume but was not present when the samples were more dilute (Fig. 2, A and C). About 30 percent of the [^{3}H]STX binding sites were recovered in the eluate. However, STX

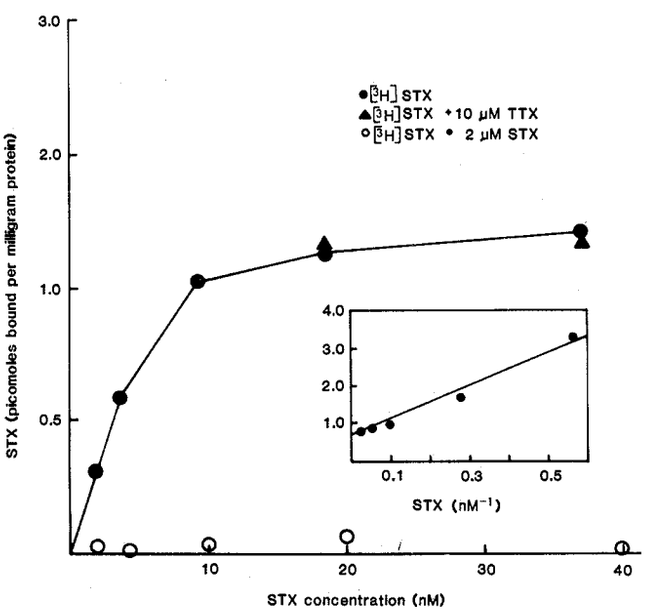

Fig. 1. Cytosolic binding of STX, as measured in the supernatant fraction of frog heart ventricle homogenate centrifuged at 100,000*g* for 2 hours. The binding capacity is about 1.4 pmole per milligram of supernatant protein, with a K_D of about 5 n*M*.

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binding was not lost when the samples were stored at 4°C for the same length of time as the column run. When similar samples were subjected to isoelectric focusing in a sucrose column for 72 hours, only 5 percent of the binding was lost.

The lability, during column chromatography, of TTX receptors solubilized by detergent from electric eel electroplax membranes has also been reported (*6*). Our loss of binding during gel filtration, the apparent low density of the STX binding entity revealed by centrifugation, and the apparent aggregation of the entity with concentration are compatible with the hypothesis that the soluble binding component is associated with phospholipid.

Whether the cytosolic STX binding sites originate in the plasma membrane or in the cytoplasm is of primary interest and has important ramifications. STX binding protein could have been released into the soluble phase during homogenization as a result of many factors, including protease activity, phospholipase activity, changes in the ionic environment, and mechanical disruption.

Proteases and phospholipases can cause the release of membrane proteins that retain enzymatic activity. To address the possible involvement of proteases, phospholipases, or the ionic composition of the homogenizing buffer in the release of substantial amounts of membrane protein during homogenization, we used a variety of homogenizing media. The control medium contained 100 m*M* KCl, 10 m*M* tris-maleate, and 2 m*M* $CaCl_2$ and was adjusted to *p*H 7.4 at 4°C. In some solutions 100m*M* NaCl or 200 m*M* sucrose replaced the KCl. Each solution was also made up without the $CaCl_2$. In addition, 1 and 3 m*M* EDTA, $10^{-4}M$ phenylmethylsulfonyl fluoride, $1.3 \times 10^{-5}M$ pepstatin, $3.6 \times 10^{-5}M$ leupeptin, 1 m*M* dithiothreitol, and 10 m*M* iodoacetate, which have been used to inhibit different kinds of proteolysis and phospholipase activity in various systems, were added to the Ca^{2+}-free control medium. These media did not inhibit the appearance of the soluble STX binding site.

Overall, the likelihood of a membrane origin for the cytosolic STX binding site seems small because other plasma membrane markers from the homogenate are purified by a factor of 14 to 19 in our plasma membrane fraction and do not appear in the cytosolic fraction (*9*). These markers include ouabain-sensitive Na^+,K^+-adenosinetriphosphatase, 5′-nucleotidase, and alkaline phosphatase. The latter two enzymes reportedly are released by phospholipase activity or mechanical disruption in other tissues. The binding capacity of the plasma membrane fraction is consistent with the binding capacity measured in the intact tissue.

That binding of STX to the cytosolic and vesicular sites is not competitively inhibited by TTX, while such competition exists in the intact tissue, must be explained whether the cytosolic and membrane sites are the same or not. The underlying issue is whether or not our results indicate that the cytosolic site is a different polypeptide than the membrane-bound sodium channel site. Three points bear on this issue. First, the density of STX binding sites on the vesicular membrane is the same as that for the whole tissue. Second, our estimates of the size of the cytosolic site (200,000 to 400,000 daltons) do not conflict with size estimates of solubilized membrane sites in other tissues. Third, TTX effects on heart action potentials may be structure- or voltage-dependent.

Table 1. Tritiated STX binding capacities of frog myocardial fractions. The values (means ± standard deviations) were calculated from 1.1 pmole of STX per milligram of protein as measured, taking 4.5×10^{-6} mg of protein per square millimeter of membrane and 5.3×10^2 square millimeters of membrane per milligram of tissue (wet weight).

Fraction	[^{3}H]STX bound (fmole) per milligram (wet weight) of tissue	TTX competition
Intact ventricle	3.6 ± 0.3	Yes
Homogenate after low-speed centrifugation	29.0 ± 9.6	No
Supernatant after 2-hour centrifugation at 100,000*g*	12.5 ± 2.9	No
Plasma membrane vesicles	2.6 ± 0.6	No

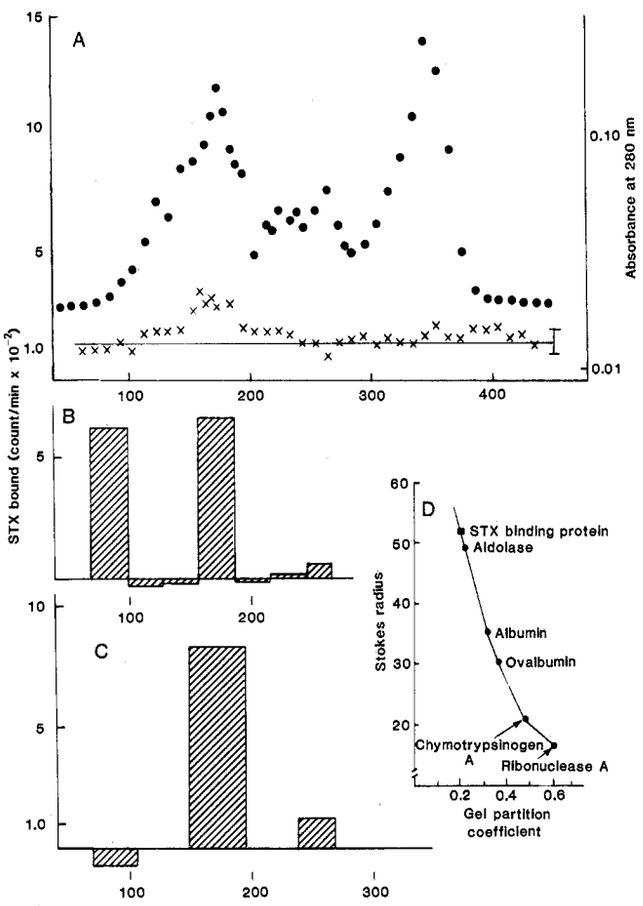

Fig. 2. Gel filtration chromatography of cytosolic proteins. In a series of experiments, the supernatants of heart homogenates were centrifuged at 100,000*g* for 2 hours and filtered through a 3 by 65 cm column of Sephacryl S-200 gel. The concentration of [^{3}H]STX used throughout was 20 n*M*. (A) The crosses show the [^{3}H]STX binding; the horizontal line shows the mean and the vertical bar the standard deviation for values obtained in the presence of 2 μM unlabeled STX in every fifth fraction; and the circles show the protein concentration in the fractions. (B) Histogram showing [^{3}H]STX binding to column fractions in an experiment in which the applied sample was concentrated fourfold over that used in the column run in (A). (C) Histogram showing the results of an experiment performed like that in (B), but at the same protein concentration as in (A). The early "aggregation" peak disappears. The ordinate in (B) and (C) is the decrease in [^{3}H]STX observed after 2 μM unlabeled STX was added to the assayed fractions. (D) Mobility of the STX-binding protein in our column compared to that of some standard proteins.

One might hypothesize that the cytosolic and vesicular sites are the same as those on the intact cell's membrane but behave as they do because the affinity of the sodium channel site for TTX is dependent on voltage or some other feature of the intact cell and because neither the vesicular nor the cytosolic sites have the same conformation as that of the intact cell's membrane site. Since the amount of membrane-bound STX does not change with isolation of the membrane in a variety of solutions, STX binding probably is not characterized by a similar dependence.

Alternatively, one might hypothesize that the cytosolic and intact membrane sites are fundamentally different. Then, if the sodium channel site affinity is lost during membrane isolation procedures, STX binding to vesicles could be due to adsorption of the cytosolic sites onto the vesicles. In both locations the difference in affinities for TTX and STX would be a property of the cytosolic sites. According to this hypothesis, the similarity of the number of sites in the intact cell's plasma membrane and in the derived vesicles is simply fortuitous. The hypothesis that the sites are related seems more likely to us.

In conclusion, we have found a tissue with either an exceptionally labile sodium channel or a cytoplasmic binding site with high affinity for STX. If the latter is true, as seems more likely, then we have found either an exception to STX's specificity for sodium channels or a soluble structure related to the sodium channel site.

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References and Notes

1. T. Narashashi, J. Moore, S. Scott, *J. Gen. Physiol.* **47**, 965 (1964).
2. B. Hille, *ibid.* **151**, 199 (1968).
3. J. Connor, L. Barr, E. Jakobsson, *Biophys. J.* **15**, 1047 (1975).
4. R. Henderson and J. H. Wang, *Biochemistry* **11**, 4565 (1972).
5. B. K. Kreuger, R. W. Ratzlaff, G. R. Strichartz, M. P. Blaustein, *J. Membr. Biol.* **50**, 287 (1979).
6. W. S. Agnew, S. R. Levinson, J. S. Brabson, M. A. Raferty, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2606 (1978).
7. R. L. Barchi, S. A. Cohen, L. E. Murphy, *ibid.* **77**, 1306 (1980).
8. N. K. Lishko, M. K. Malysheva, A. V. Stefanov, *Dokl. Biophys.* **248**, 179 (1979).
9. L. Barr, J. A. Connor, M. M. Dewey, J. Aprille, P. V. Johnston, *Biochim. Biophys. Acta* **345**, 336 (1974).
10. J. M. Ritchie, R. B. Rogart, G. R. Strichartz, *J. Physiol. (London)* **261**, 477 (1976).
11. S. R. Levinson, C. J. Curalalo, J. Reed, M. A. Raftery, *Anal. Biochem.* **99**, 72 (1979). Our correction ratio was as follows: [^{3}H]STX bound per milligram P_2A (equilibrium dialysis) to [^{3}H]STX bound per milligram P_2A (gel filtration) = 1.31 ± 0.05. We thank G. R. Strichartz for his gift of [^{3}H]STX.

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Splitting of the Circadian Rhythm of Activity Is Abolished by Unilateral Lesions of the Suprachiasmatic Nuclei

Abstract. *The circadian rhythm of activity in vertebrates often splits into two components after continuous exposure to constant light. This observation suggests that at least two circadian pacemakers underlie the activity rhythm. After unilateral ablation of the hypothalamic suprachiasmatic nuclei in hamsters, the splitting phenomenon was eliminated and a single rhythm of activity was established. The period of the new circadian activity rhythm differed from the periods of the split rhythm and that preceding the split. These results suggest an interaction between the bilaterally paired suprachiasmatic nuclei in the generation of the circadian rhythm of activity.*

Although circadian phenomena in the biological world have been described for centuries, the endogenous nature of circadian oscillators has been established only in the last 20 years. Elaboration of the biological basis of the circadian clock in multicellular organisms has been complicated by reports that both physiological and behavioral circadian rhythms are based on the interaction of more than one circadian pacemaker (*1, 2*). One of the strongest indications that there are multiple oscillators is provided by the observation that in many vertebrates the circadian rhythm of locomotor activity can dissociate, or "split," into two distinct bouts of activity (*1*). Any attempt to explain the biological basis for the generation of circadian rhythms in vertebrates must take into account the splitting phenomenon, although at present little is known about the physiological basis of splitting.

Splitting of the circadian locomotor rhythm occurs in various vertebrate species, including lizards, birds, and mammals (*1, 3*). In a study of the split rhythm in the golden hamster (*Mesocricetus auratus*), Pittendrigh and Daan (*1*) observed that (i) when splitting first occurs, the two activity components often free-run (that is, the activity bouts are not entrained) and have different circadian periods until they stabilize approximately 180° out of phase, and (ii) the attainment of the stabilized antiphase relationship between the bouts of activity is accompanied by a decrease in the circadian period (τ) of the split activity rhythm. On the basis of this splitting phenomenon and other observations, several investigators have suggested a system of at least two coupled oscillators as the underlying mechanism for the mammalian circadian pacemaker system (*1, 2, 4*).

Attention has focused on the hypothalamic suprachiasmatic nuclei (SCN) as the neural location of the circadian oscillators in mammals. The SCN receive a direct retinal innervation that relays information about the light-dark environment for the photic entrainment of endogenous circadian rhythms (*5*). Moreover, complete bilateral destruction of the SCN leads to the loss of a number of different circadian rhythms (*6, 7*). Although the SCN are a vital component of the circadian system, little is known about the complex neural interactions underlying the organization of the mammalian circadian system or how multiple circadian pacemakers might be coupled to each other to generate a single circadian pattern.

We have provided anatomical evidence that the two SCN are reciprocally innervated (*8*). This anatomical reciprocity indicates a physiological coupling, even though unilateral SCN lesions have shown little or no effect on a number of diverse circadian rhythms (*6, 7*); however, these studies had focused on entrainment and the generation of an intact circadian rhythm. We examined the effect of unilateral SCN ablation on split circadian activity patterns of golden hamsters maintained in constant light. The results suggest that both suprachiasmatic nuclei are required to produce the split activity pattern.

Animals were housed individually in cages equipped with activity wheels, and locomotor activity was recorded (*9*). Animals were maintained in constant light (*10*), and their activity records were examined periodically to determine when a split pattern of activity developed. Small electrolytic lesions aimed at a single SCN were made in 19 hamsters that had demonstrated many weeks of a split rhythm of locomotor activity (*11*). After the lesions were made, locomotor activity was recorded for several months in the same photic environment.

Because knowledge of the locus of the lesion was critical to the interpretation of the behavioral findings in this study, a rigorous histological procedure was used to determine the exact extent of the lesions. An injection of 4.0 μl of a 30 percent horseradish peroxidase (HRP) solution was given in the vitreous of the eye contralateral to the side of the lesion 24 to 48 hours before the animal was

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