Interactions of Neosaxitoxin with the Sodium Channel of the Frog Skeletal Muscle Fiber

S. L. HU and C. Y. KAO

From the Department of Pharmacology, Downstate Medical Center, State University of New York, Brooklyn, New York 11203

ABSTRACT Neosaxitoxin (neoSTX) differs structurally from saxitoxin (STX) in that the hydrogen on N-1 is replaced by a hydroxyl group. On single frog skeletal muscle fibers in the vaseline-gap voltage clamp, the concentrations for reducing the maximum sodium current by 50% (ED_{50}) at pH's 6.50, 7.25, and 8.25 are, respectively, 4.9, 5.1, and 8.9 nM for STX and 1.6, 2.7, and 17.2 nM for neoSTX. The relative potencies of STX at the different pH's closely parallel the relative abundance of the protonated form of the 7,8,9 guanidinium function, but the relative potencies of neoSTX at the same pH's vary with the relative abundance of the deprotonated N-1 group. In constant-ratio mixtures of the two toxins, the observed ED_{50}'s are consistent with the notion that the two toxins compete for the same site. At pH's 6.50 and 7.25, the best agreement between observed and computed values is obtained when the efficacy term (ϵ) for either toxin is 1. At pH 8.25 the best agreement is obtained if the efficacy is 1 for STX but 0.75 for neoSTX. The marked pH dependence of the actions of neoSTX probably reflects the presence of a site in the receptor that interacts with the N-1 -OH, in addition to those interacting with the 7,8,9 guanidinium and the C-12 hydroxyl groups. Considering the three-dimensional structure of the STX and neoSTX molecules, the various site points are probably located in a fold or a crevice of the channel protein, where the extracellular orifice of the sodium channel is located.

INTRODUCTION

Saxitoxin (STX) is an important neurobiological tool because of its high affinity and high specificity binding to voltage-gated sodium channels of many excitable membranes. Recent studies suggest that its binding site is located near the external orifice of the sodium channel (Kao and Walker, 1982; Kao, 1983; see also Moczydlowski et al., 1984*a*; Strichartz, 1984), rather than some distance into the channel itself (Hille,

Address reprint requests to Dr. C. Y. Kao, Department of Pharmacology, State University of New York, Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203.

Dr. Hu's present address is Ciba-Geigy Corp., Summit, NJ 07901

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1975). However, an alternative view is that the binding site is not in the permeation path of the channel (Green et al., 1987). As the primary structure of the sodium channel is known (Noda et al., 1984), a better understanding of the binding of STX to its receptor could help to locate the site and provide a landmark on the large protein molecule to aid further structural clarification.

In the absence of a covalent marker for this site, a useful approach is to deduce complementary requirements in the binding site from structure–activity relations of the toxin molecules (see summary in Kao, 1986). Of the two guanidinium functions in STX, the 7,8,9 group has been identified as the active group involved in blocking the sodium channel (Kao et al., 1983). The role of the 1,2,3 guanidinium group is unclear, but it was not believed to be involved in channel blockade (Kao and Walker, 1982; Kao, 1983; Kao et al., 1983). Neosaxitoxin (neoSTX) differs structurally from STX only in having the hydrogen on N-1 replaced by a hydroxyl (Shimizu et al., 1978; Koehn et al., 1981), and therefore could be useful for probing the role of the 1,2,3 group.

Previous studies on neoSTX showed that at acidic and neutral pH's it was about equally as potent as STX, but that at alkaline pH's it was markedly and unexpectedly weaker than STX (Kao et al., 1983). Strichartz (1984) confirmed the low potency of neoSTX in alkaline conditions, but reported that under acidic and neutral conditions it was much more potent than STX. Largely on the basis of these observations, he proposed a covalent reaction between the C-12 -OH's of these toxins with some groups in the receptor as the basis of their binding. However, this proposal is at variance with key chemical information, because derivatives of STX (Koehn et al., 1981) and of neoSTX (Wichmann, 1981) where the C-12 groups have been reduced to alcohol configurations and cannot form covalent bonds are still active (Wichmann, 1981; Koehn et al., 1981; Kao et al., 1985). In the course of re-examining the actions of neoSTX, we found evidence that the N-1 group probably participates in binding to the channel protein. This information strongly implies certain structural features of the receptor site that had not been suspected before.

Preliminary accounts of this information have appeared (Hu and Kao, 1986; Kao, 1986).

MATERIALS AND METHODS

These experiments were done on short segments of single fibers of the semitendinosus muscle of the English frog *Rana temporaria,* using the vaseline-gap voltage clamp method (Hille and Campbell, 1976). In these experiments, an internal solution containing potassium glutamate and EGTA (see below) was applied in the end pools instead of the CsF used by Hille and Campbell. These EGTA-treated fiber segments did not contract upon depolarization. All experiments were carried out at room temperature, ~22°C.

In all experiments the fibers were held at −90 mV. Leakage and capacitive currents were compensated for by using an analog circuit. Fibers were discarded when the leakage current became unstable. Depolarizing steps of 10-mV increments were applied until ~20 mV beyond the reversal potential of the initial current. Current traces around the maximum I_{Na} and around E_{Na} were recorded; the maximum I_{Na} was used to compare the actions of toxins, and the current at E_{Na} (I_K) was used for assessing the health of the fiber as well as for checking the selectivity of action of the toxins on I_{Na}. No corrections were made to compensate for the series resistance.

In the presence of an uncompensated series resistance, the voltage control during the flow of

large membrane currents could be inaccurate, but the error would decrease as I_{Na} was reduced when increasingly higher concentrations of toxins were applied. A small systematic error could exist in the steepness of the dose–response curves. However, the ED_{50} was not affected. This point was tested in a small series of fibers as follows: A concentration of neoSTX close to the ED_{50} was tested on two groups of fibers, one in the usual Ringer's solution containing 110 mM Na^+ and the other in a modified Ringer's solution containing 40 mM Na^+ and 70 mM choline chloride. The average density of I_{Na} before toxin was 3.9 mA cm^{-2} in 110 mM Na^+ and 1.7 mA cm^{-2} in 40 mM Na^+. Since the ED_{50} for neoSTX at pH 7.25 was 2.7 nM (see Results), 3.0 nM was applied. The I_{Na} normalized to the pretoxin value was 43.3 ± 2.7% (means ± SEM, n = 8) for fibers in 110 mM Na^+ and 45.9 ± 4.3% (n = 11) for fibers in 40 mM Na^+. The difference between the means is statistically insignificant.

Any influence of shifts of the *I-V* relations along the voltage axis that occurred upon toxin action was circumvented by experimentally finding the maximum I_{Na} under all conditions (see also Wagner and Ulbricht, 1975). For any individual fiber, the E_{Na} remained constant to within 5 mV throughout the experiment.

The Ringer's solution bathing the fiber exterior consisted of (in mM/liter): 110 Na^+, 2.5 K^+, 1.8 Ca^{2+}, 116.3 Cl^-, and 5 HEPES. The pH was adjusted to 6.50, 7.25, or 8.25 with NaOH. The internal solution consisted of 115 K^+, 5 Na^+, 115 glutamate, 5 $H_2PO_4^-$ and 3 EGTA. The pH was adjusted to 7.4.

The general procedure was to determine the maximum I_{Na} in toxin-free solutions as a basis of comparison. Then various concentrations of toxins were introduced until a steady-state reduction in the maximum I_{Na} was reached in each. It should be noted that at the voltage at which maximum I_{Na} was elicited, I_K was small and developing so gradually that at the time I_{Na} peaked (~0.7 ms) no detectable I_K had developed (Fig. 1). Therefore, even with the potassium conductance intact in the fibers used, there is little error in our assay that can be attributed to an overlap of the sodium and potassium currents.

From such observations, dose–response relations were constructed based on the normalized residual maximum I_{Na} (I'_{Na}/I_{Na}). Each individual fiber could be used for three or four trials in increasing concentrations of toxins without a need for recovery in between. In most cases a washout and recovery were obtained at the end to make certain that the observed toxin effects were reversible. Generally, 15 min was allowed for the washout, by the end of which 80–90% of the pretoxin maximum I_{Na} has recovered. The recovery of I_{Na} followed an exponential time course with a time constant of ~1 min. At 10 min the recovery was virtually complete; lengthening the washout period to 40 min did not appreciably increase the degree of recovery. These observations suggest that the toxin effects observed in the present experiments were due primarily to the sodium channels in the sarcolemma, and that any contribution from sodium channels in the transverse tubules was minor. The degre of recovery varied, influenced in part by the concentration of toxin used. We found tetrodotoxin (TTX, Hebei preparation, Hu and Kao, 1985; available from Calbiochem Corp., San Diego, CA) to be the most fully reversible, with recoveries of 90–100%; recovery from STX was slightly less complete, ~90%, whereas recovery from neoSTX was least complete, ~75%.

The toxin concentrations chosen embraced the central portions of the dose–response curve on both sides of the inflection point. Usually, the same concentrations were used at different pH's so that the results could be compared statistically. In some cases, additional concentrations were used to cover extended portions of the curve. The toxin effects were also shown in Hill plots, in which log $(1 - P)/P$ ($P = I'_{Na}/I_{Na}$) was plotted against log [toxin]. The results were fitted by least-squares linear regression lines, with very good fit in every case. The ED_{50}'s were determined from these plots when $(1 - P)/P = 1$. Standard errors of estimates were also obtained from the regression analysis. Tests of significance of differences between means were performed on the basis of these standard errors (Arkin and Colton, 1953). At $(1 - P)/P = 1$, the

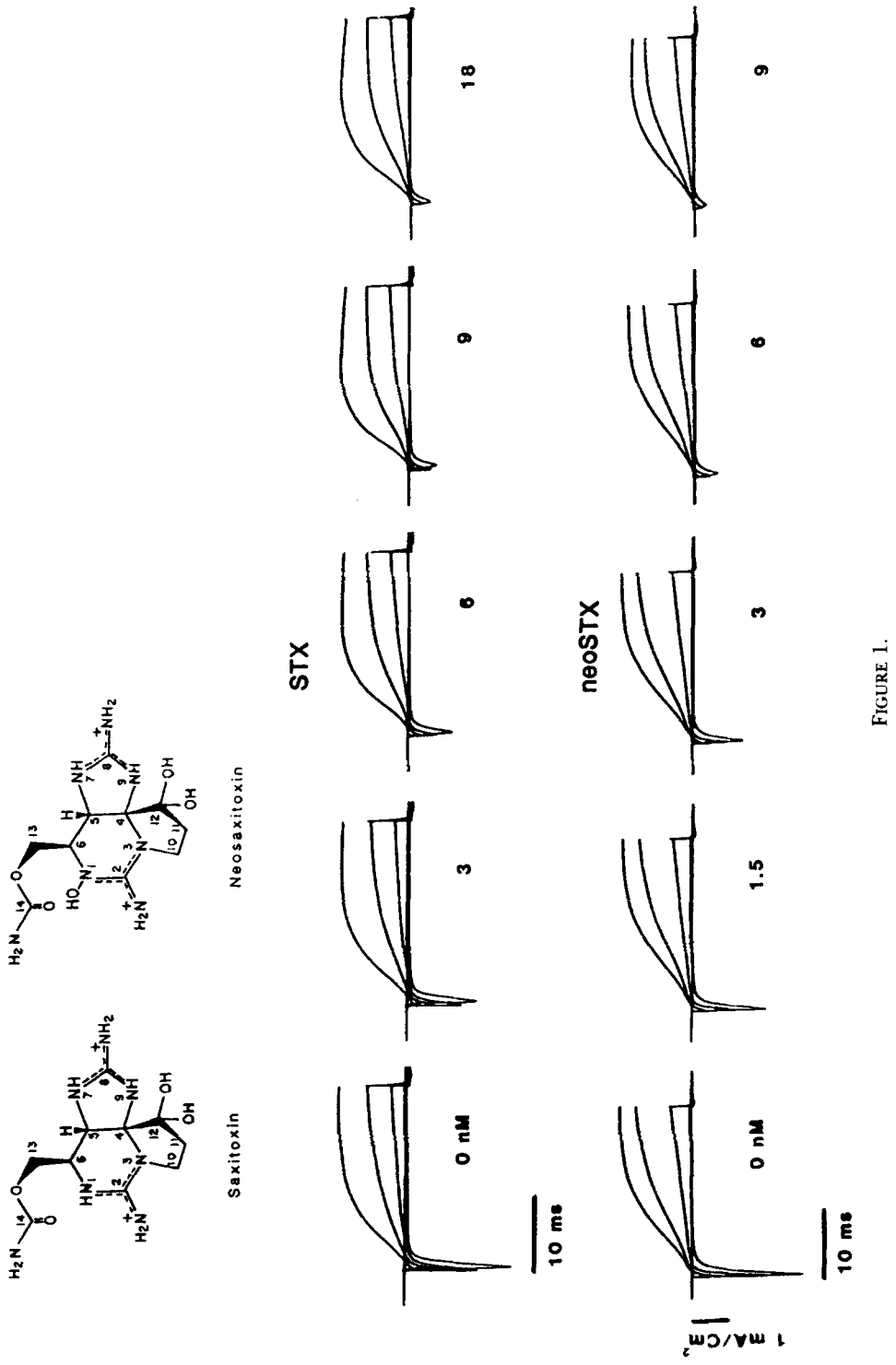
Neosaxitoxin
Saxitoxin
STX
neoSTX
0 nM
3
6
9
18
0 nM
1.5
3
6
9
10 ms
10 ms
1 mA/cm²

FIGURE 1.

standard errors of estimate covered a concentration range equivalent to ±1 SD of the ED_{50}. The standard error of the mean of the ED_{50}'s, as listed, was one-half of the covered concentration ranged divided by the square root of the number of samples.

The experiments on interactions between STX and neoSTX were done at pH's of 6.50 and 8.25, because at these pH's differences in the actions of the two toxins were the largest within the physiological range of pH in which we were interested (see Results). At each pH the fibers were treated sequentially with a mixture of the two toxins in increasing concentrations. The mixture was made up such that the ratio of the concentrations of the two toxins ($\alpha = [T_2]/[T_1]$) closely matched the ratio of their individual ED_{50}'s ($r = ED_{50,2}/ED_{50,1}$). Dose–response curves were constructed from normalized maximum I_{Na} in a mixture as a function of the concentration of the more potent toxin. The theoretical potencies of such mixtures based on the assumption of two toxins acting on the same site or two toxins acting on different sites were calculated according to relations developed previously (Ariens et al., 1964; Kao and Walker, 1982). These computed potencies were compared with the observed potencies obtained from the regression lines in the Hill plots.

RESULTS

To facilitate the following presentation, we first restate briefly the salient chemical properties of STX and neoSTX. The N-1 of neoSTX (Fig. 1) deprotonates with a pK_a of 6.75 (Shimizu et al., 1978). The pK_a of the 7, 8, 9 guanidinium in STX is 8.25 and that in neoSTX is 8.65, whereas the pK_a of the 1, 2, 3 guanidinium in both compounds is > 11 (Rogers and Rapoport, 1980; Schantz et al., 1975; Shimizu et al., 1981). The C-12 −OH groups in STX are in a gem-diol configuration (HO-C-OH, hydrated ketone) which exists in equilibrium with the unhydrated ketone (C = O, carbonyl form). At pH 7 they are almost all in the gem-diol form; at pH 7.8, 76% is in this form, the fraction declining in more alkaline conditions (Shimizu et al., 1981). Deuterium or tritium can be exchanged from the solvent onto the C-11 methylene through a ketone-enol tautaumerism at C-12 (Rogers and Rapoport, 1980). The rate of exchange is apparently faster in neoSTX than in STX (Shimizu et al., 1981). There is, however, no quantitative information on any details of the ketone–ketone hydrate equilibrium in neoSTX similar to that for STX. The two guanidinium functions are in a purine nucleus, and are placed at ~130° to each other (see perspective view in Fig. 8; see also Kao and Walker, 1982; Kao, 1983).

STX

As a basis for comparison with neoSTX, the effects of STX are shown in Fig. 1. As the I_{Na} was reduced, the I_K remained virtually the same through four different doses of STX. From records like these, dose–response relations were constructed at pH's 6.50,

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Figure 1. (*opposite*) Current records from two individual fibers treated with different concentrations of STX and neoSTX at pH 7.25. Holding potential for each fiber was −90 mV. Temperature was ~22°C. Largest current in each panel was maximum I_{Na}. Note that at voltage and time of maximum I_{Na}, no I_K was yet detectable. Note also the stability of I_K and the specificity of toxins for I_{Na}. Inset shows structures of STX and neoSTX. Difference is on N-1, with −H in STX and −OH in neoSTX. pKa's for 7, 8, 9 guanidinium are 8.25 for STX and 8.65 for neoSTX; pKa for 1, 2, 3 guanidinium is > 11; and pKa for N-1 −OH in neoSTX is 6.75.

7.25, and 8.25 (Fig. 2 *A*). The observations were also displayed in a Hill plot of log $(1 - P)/P$ vs. log concentration (Fig. 2 *B*). These pH's were selected because of their relation to key chemical properties of the STX and neoSTX molecules, 6.50 being on the acid side of the pK_a of N-1 –OH in neoSTX, and 8.25 being the pK_a of the 7, 8, 9 guanidinium of STX. pH's 7.25 and 8.25 have also been used in a previous study (Kao et al., 1983), with which some of the present results might be compared. No other pH's were studied, because at pH's lower than 6.50 proton blockade of the sodium channel can be expected (e.g., Hille, 1984), whereas at pH's above 8.8 chemical degradation of STX and neoSTX would intervene.

The ED_{50}'s of STX at pH's 6.50, 7.25, and 8.25 are, respectively, 4.9 ± 0.2 (51), 5.1 ± 0.2 (31), and 8.9 ± 0.4 (58) nM (means ± SEM followed by number of samples in parentheses). Confirming previous observations, the dose–response curves can be readily fitted with a theoretical bimolecular scheme with an exponent close to 1.0.

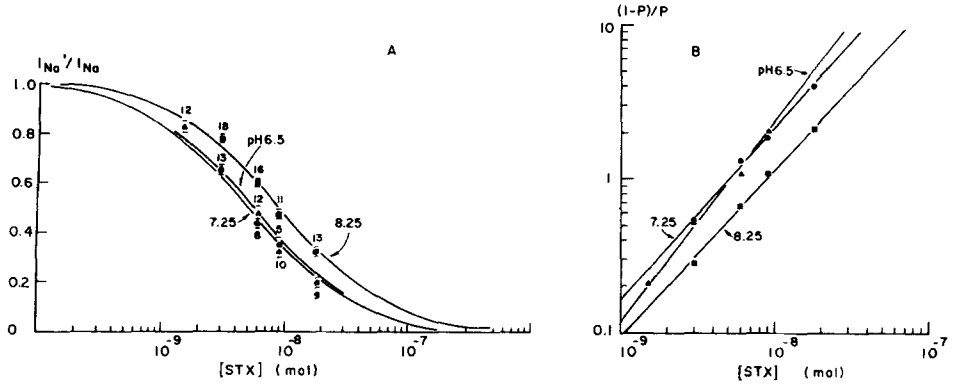

FIGURE 2. Dose–response relations of STX at different pH's. (*A*) Normalized residual maximum I_{Na} vs. [STX]. Points are means ± SEM, with attached number showing fibers used for each point. Solid lines are drawn according to a bimolecular reaction scheme where $I_{Na}'/I_{Na} = 1 - 1/(1 + ED_{50}/[\text{toxin}])$. (*B*) Hill plot of same data, log $(1 - P/P)$ vs. log [STX], where $P = I_{Na}'/I_{Na}$. Lines are drawn by least-squares linear regression. Slopes of lines are 1.25, 1.12, and 1.09, respectively, for pH 6.50, 7.25, and 8.25.

This feature is also seen in the Hill plots, where the slopes are 1.2, 1.1, and 1.1 for pH's 6.50, 7.25, and 8.25, respectively.

The relative potencies of STX at pH's 7.25 and 8.25 is 1.75, in close agreement with a previous finding on the squid axon (1.79; Kao et al., 1983). This ratio is very close to the relative abundance of the protonated form of the 7, 8, 9 guanidinium (1.80), reaffirming a conclusion that the 7, 8, 9 group is involved in blocking the sodium channel. At pH 6.50, 98% of the 7, 8, 9 group is protonated, compared with 90% at pH 7.25. If the interaction of STX with the receptor depended only on the abundance of the charged 7, 8, 9 guanidindium, one would have expected an ED_{50} of 4.7 nM at pH 6.50. The observed ED_{50} of 4.9 nM shows that at pH 6.50 STX is slightly less potent than at pH 7.25. Although the difference between the observed ED_{50}'s at pH's 6.50 and 7.25 is not statistically significant ($P > 0.3$), a similar observation has been made by a number of investigators before (e.g., Wagner and

Ulbricht, 1975; Barchi and Weigel, 1979; Strichartz, 1984). Such a discrepancy also occurs for both neoSTX and TTX. A plausible explanation is that at pH 6.50 the density of the negative surface charges is lower, resulting in a reduced attraction of the positively charged STX to the binding site (e.g., Wagner and Ulbricht, 1975).

neoSTX

Like STX, neoSTX blocked only the I_{Na} (Fig. 1). The ED_{50}'s of neoSTX determined from Fig. 3 are 1.6 ± 0.1 (36), 2.7 ± 0.1 (42), and 17.2 ± 0.6 (68) nM, respectively, for pH's 6.50, 7.25, and 8.25. Thus, confirming Strichartz's observation (1984) at near neutral or acidic pH's, we found that this sample of neoSTX was more potent than STX at pH 7.25 (see Discussion). That the relative potency of neoSTX to STX at pH's 6.50 and 7.25 reported here is less than one-half of that reported by Strichartz for frog nerve bundles may be attributed in part to the different tissues used, as the

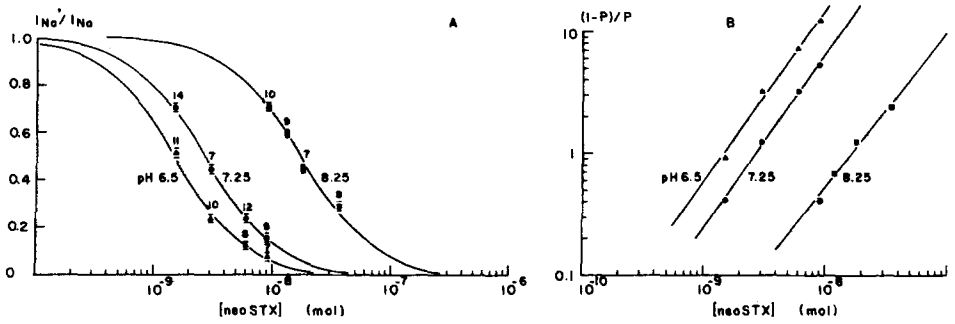

FIGURE 3. Dose–response relations of STX at different pH's. Conventions are the same as those in Fig. 2. (*A*) Normalized residual maximal I_{Na} vs. [neoSTX]. For better fit, curves were steepened with a power of ~1.4 in bimolecular scheme, but ED_{50} is unaffected (see Kao and Walker, 1982). (*B*) Hill plot. Slopes of linear regression lines are 1.41, 1.43, and 1.27, respectively, for pH's 6.50, 7.25, and 8.25.

sodium channels in muscle and nerve are different isoforms (Barchi, 1986; Numa and Noda, 1986).

At pH 8.25, neoSTX was appreciably less potent than STX on the frog muscle fiber, as it was on the squid axon (Kao et al., 1983) and on the frog nerve bundle (Strichartz, 1984). If the abundance of the protonated 7, 8, 9 guanidinium function were the only determinant of potency, neoSTX, in which the pK_a of this group is 8.65, should have been ~20% more potent than STX. On the other hand, because its net charge changes from +1.6 to +0.6 as pH changes from 6.5 to 8.25, the local concentration of neoSTX at the binding site could possibly decrease by a factor of 2.7, thereby affecting its potency (see below). However, the observed change in potency by a factor of 10.8 is far greater. Therefore, the lowered potency is most probably caused chiefly by the state of the N-1 −OH in neoSTX.

TTX

Changes in pH can affect the density of negative surface charges and any negative charge due to specific groups in the toxin-binding site, which will in turn influence

the local concentration of the cationic toxin molecules. The possible effects of pH on surface charges can be calculated in several ways, using different assumptions. Here we adopt an experimental approach to this problem by examining the influence of pH on the binding of TTX, in addition to those of STX and neoSTX. It should be recalled that the only guanidinium function in TTX has a pK_a of over 11, and is therefore almost all protonated within the physiological range of pH's. However, the C-10 −OH of TTX deprotonates with a pK_a of 8.8, and could cause TTX to occur predominantly in either a cationic or zwitterionic form. Thus, to help resolve the question of the influence of surface charge on toxin effects, three toxin molecules with identical capabilities of blocking the Na^+ channel are available, with net charges from +1 (TTX) to +2 (STX), and varying from +0.6 to +1.6 (neoSTX).

The potency of TTX at pH's 6.50, 7.25, and 8.25 on the frog skeletal muscle are shown in Fig. 4, where the ED_{50}'s are 3.8 ± 0.1 (43), 3.1 ± 0.2 (17), and 4.3 ± 0.2

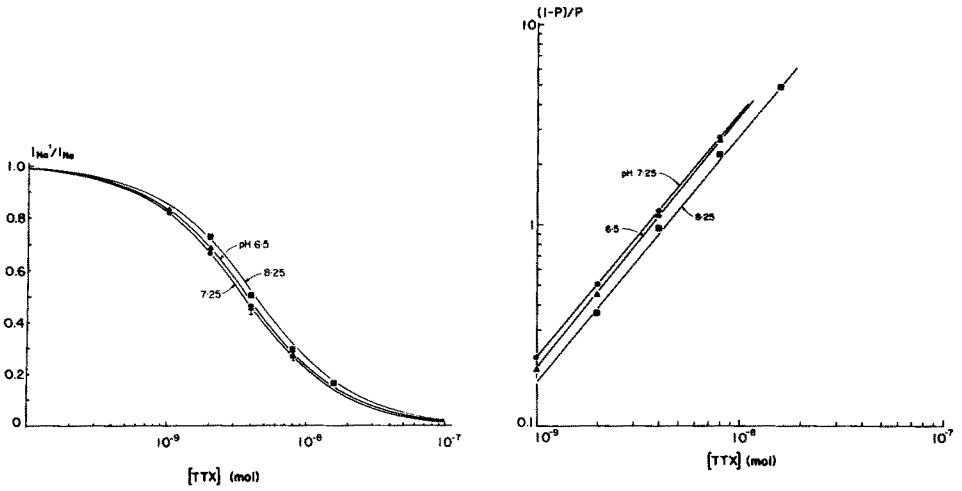

FIGURE 4. Dose–response relations of TTX at different pH's. Conventions are the same as those in Fig. 2. (*A*) Normalized residual maximal I_{Na} vs. [TTX]. Solid lines are drawn according to a biomolecular reaction scheme. (*B*) Hill plot of the same data. Linear regression fit gives slopes of 1.28, 1.21, and 1.26 for pH's 6.50, 7.25, and 8.25, respectively.

(26) nM, respectively. The relative potency at 7.25 and 8.25 is 1.23, and the relative abundance of the C-10 −OH at these pH's is 1.24. Similar to the observation on STX and neoSTX, at pH 6.50 TTX is slightly less potent than expected ($P < 0.001$).

Effect of pH on Toxin Potencies

In Fig. 5, the observed potencies of TTX, STX, and neoSTX at pH's 6.50, 7.25, and 8.25 are compared with those that might be expected on the basis of certain assumptions of the chemical states of the toxin molecules. For each toxin, the observed ED_{50} at pH 7.25 was taken as the reference, and the expected potencies for other pH's were calculated from it on the basis of the Henderson–Hasselbalch relation. For TTX, the expected potency is based on the degree of protonation of the C-10 −OH function. For STX and neoSTX, two different assumptions are used. In

one case, the expectation is based on the relative abundance of the protonated form of the 7, 8, 9 guanidinium. It is evident that for STX the observed and the expected potencies are in close agreement, whereas for neoSTX they are not. For the latter, expected potencies were also calculated on the basis of the abundance of the deprotonated N-1 −OH group. Clearly, for neoSTX the observed pH dependence of potencies is in much closer agreement with the state of the N-1 −OH group than it is with that of the 7, 8, 9 guanidinium.

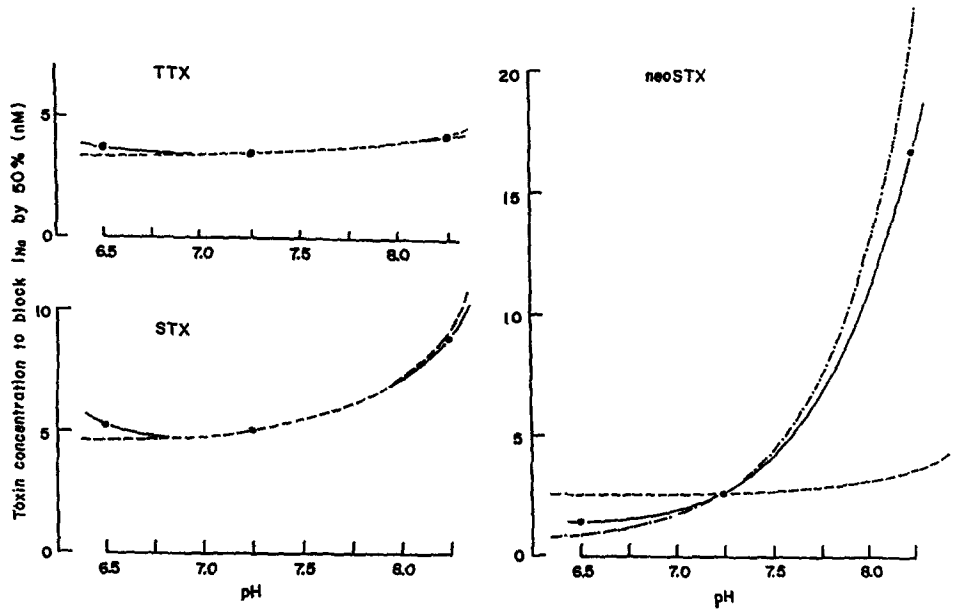

FIGURE 5. pH dependence of potency of TTX, STX, and neoSTX. Solid lines and filled circles are experimental results. Dashed lines are toxin concentrations expected to cause 50% reduction of maximum I_{Na} computed on the basis of relative abundance of the protonated form of the C-10 hydroxyl group of TTX and of the 7, 8, 9 guanidinium group in STX and neoSTX. The concentration at pH 7.25 was used as reference, and those for other pH's were calculated on the basis of applicable pK_a and the Henderson–Hasselbalch relation. For neoSTX, the broken line represents expectation based on the abundance of deprotonated N-1 −OH. For each toxin, the slight discrepancy between observed and expected potencies at pH 6.50 is probably due to a slight decrease in the density of surface negative charges. For neoSTX, the discrepancy at pH 8.25 may be caused by competition between active and inactive species of the neoSTX molecule (see Discussion).

For each toxin molecule, it is worth noting how well the observed and expected potencies coincide, even though the net charge on the molecule is rather different. These observations indicate that within the pH range of 6.50–8.25 there is little evidence that the surface charges were affected sufficiently to influence toxin binding in any substantial way. They also show that the important determinant of binding is the abundance of the active species of the molecule and not the net electric charge of the whole molecule (Chabala, L., and O. S. Andersen, personal communication).

Therefore, our results can be interpreted in terms of how different states of the toxin molecules interact with basically the same receptor site.

Interactions between STX and neoSTX

The apparent involvement of the N-1 group in affecting the potency of neoSTX but not STX prompted us to examine whether STX and neoSTX competed for the same binding site. The results led to a previously unexpected aspect of the binding of neoSTX to the receptor. In these experiments, constant ratio mixtures of STX and neoSTX were tested in the same way as single toxins had been. The procedures were modeled on those described in detail in Kao and Walker (1982). The expected ED_{50}'s were computed for the cases where both toxins acted on the same binding site, and where the two toxins acted on different sites. The outcome was then compared with the experimental results.

Figs. 6 and 7 show the dose–response relations of these experiments at pH's 6.50 and 8.25, as compared with those of the single toxins. Table I shows the ratios of the

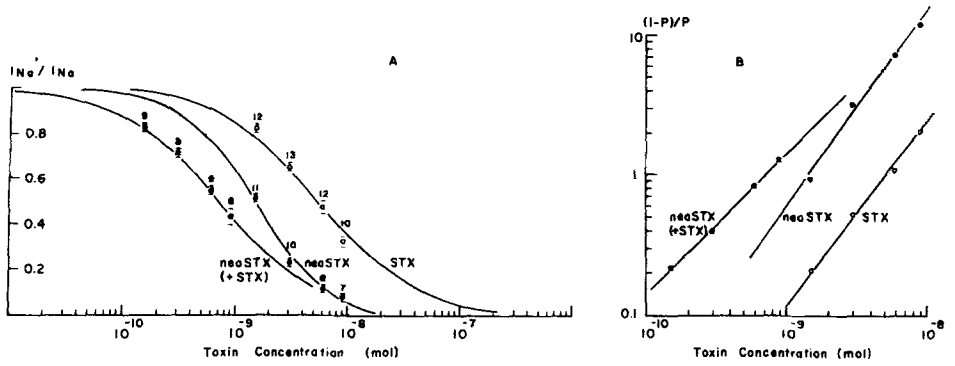

FIGURE 6. Dose–response relations of STX, neoSTX, and constant-ratio mixtures of the two toxins at pH 6.50. At this pH, N-1 −OH of neoSTX is mostly protonated with no charge. The data for single toxins are the same as those shown in Figs. 2 and 3. Assuming $ED_{50} = K$, $r = K_2/K_1 = 3.58$. Mixtures of toxins in a ratio of ~3.5 are: [STX]/[neoSTX] = 0.53 nM/0.15 nM; 1.05/0.3; 2.1/0.6; 3.15/0.9. The observed ED_{50} for the mixture (as neoSTX equivalent) is in good agreement with the ED_{50} computed for the two toxins competing for one site.

concentrations of the toxins (α), and of their ED_{50}'s (r) in the mixture, with the more potent toxin designated as T_1 and the less potent toxin as T_2. The observed potency shown denotes the concentration of T_1 in the mixture that is required to half the maximum I_{Na}. Also shown are the expected ED_{50}'s for the case of single-site action and that for different-site action. For each pH the observed ED_{50} is closer to that expected of a single-site action by these two toxins than it is to that of a different-site action.

It should be pointed out that the expected ED_{50}'s calculated according to Eqs. 2 and 3 in Kao and Walker (1982) are based on the assumption that the efficacy (ϵ) of both STX and neoSTX is 1 (i.e., $ED_{50} = K_d$; see Discussion). For the results at pH 6.50, the close agreement (to within 2%) between the expected and observed ED_{50}'s justified this assumption. For the results at pH 8.25, the observed ED_{50} exceeds that expected by 32%, a discrepancy which suggests that the assumptions used were not

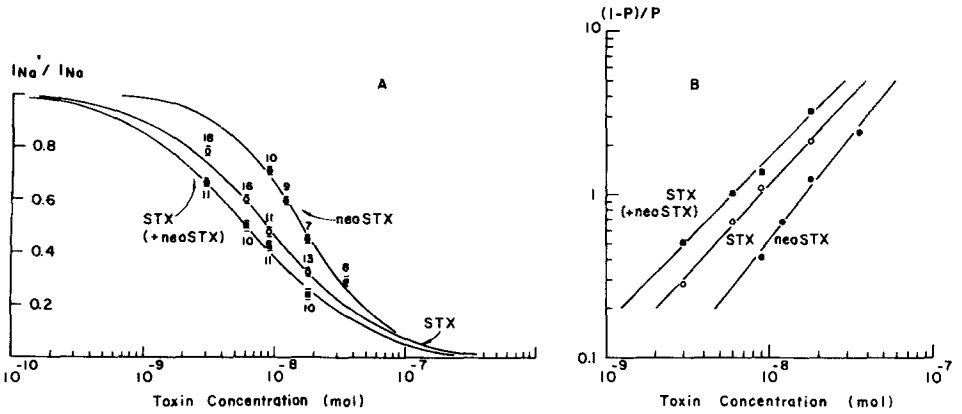

FIGURE 7. Dose–response relations of STX, neoSTX, and constant ratio mixtures of the two toxins at pH 8.25. At this pH, N-1 −OH of neoSTX is mostly deprotonated, with a negatively charged oxygen. Conventions are similar to those in Fig. 6. r = 1.89. Mixtures of a ratio of ~1.8 are: [neoSTX]/[STX] = 5.4 nM/3 nM; 10.8/6; 16.2/9; 32.4/18. Observed ED_{50} (as STX equivalent) fits the expected ED_{50} for single-site action better than for two-site action, even with a discrepancy of 32%. However, if the efficacy of neoSTX were 0.75 and that for STX were 1, then the observed ED_{50} fits the expected ED_{50} for the single-site case. See text for details.

entirely valid. However, Eq. 3 in Kao and Walker (1982) for the case of two toxins acting on different sites can be replaced with the following relation, which is, in fact, a more general form:

$$f = \frac{([T_1]/K_1)(\epsilon_1 + \epsilon_2\alpha/r)}{1 + ([T_1]/K_1)(1 + \alpha/r)}$$

TABLE I

Actions of Constant-Ratio Mixtures of STX and neoSTX

A. pH 6.50: $r = ED_{50,STX}/ED_{50,neoSTX} = 3.58$; α = [STX]/[neoSTX] = 3.5

ED_{50} (nM of neoSTX)

Observed	Expected	
	One site	Two sites
0.73	0.74*	0.61*

B. pH 8.25: $r = ED_{50,neoSTX}/ED_{50,STX} = 1.89$; α = [neoSTX]/[STX] = 1.8

ED_{50} (nM of STX)

Observed	Expected	
	One site	Two sites
6.04	4.56*	3.78*
	6.05‡	4.81‡

*Assuming efficacy is 1.0 for both STX and neoSTX.

‡Assuming efficacy is 0.75 for neoSTX and 1.0 for STX.

where ϵ_1 and ϵ_2 are efficacies of toxins 1 and 2, respectively. For the results in pH 8.25, if the efficacy of neoSTX were lowered to 0.75, and that of STX were kept at 1, then the expected ED_{50} closely approached that observed (Table I). Further comments on this point follow in the Discussion.

DISCUSSION

Comparison with Earlier Work

Because of the lack of specific chemical analytical methods for STX and neoSTX, these toxins are still quantitated by bioassay, most frequently the mouse lethality assay (e.g., Wichmann et al., 1981). Absolute quantities are difficult to determine, but relative potencies with reference to that of STX are usually reliable and informative. The specific toxicity of STX (5,500 mouse units/mg of crystalline toxin) is generally agreed on by different investigators in the field, but that for neoSTX remains unsettled, with values from different laboratories differing by factors of ~2 (Hashimoto and Noguchi, 1989). Previously, we found neoSTX to be equipotent with STX at pH 7.3 on the frog muscle fiber (Kao and Walker, 1982), but that sample was obtained from a different source, and an independent assay suggested that it might have had a lower potency (Hall, 1982), which could account for the differences with the present results.

In contrast to observations on isolated tissues, in mouse lethality bioassays almost all investigators who have purified neoSTX have found it to be approximately equipotent with STX (Genenah and Shimizu, 1981; Wichmann et al., 1981; Hall, 1982; Hashimoto and Noguchi, 1989). The discrepancy may be explained by the following considerations. At pH 7.4, the probable intraperitoneal pH of the assay mouse, neoSTX is only 1.4 times more potent than STX (Fig. 5). As the precision of the bioassay is 20%, the difference in relative potencies of the two toxins might be too tenuous to be determined with confidence. It is also possible that neoSTX is converted to STX in the body (Shimizu and Yoshioka, 1981; Wichmann et al., 1981) but not in isolated preparations of nerves and muscles.

Possible Influence of Negative Charges in the Toxin-binding Site

Recent studies using batrachotoxin-activated sodium channels incorporated into lipid bilayers have shown the presence of a higher density of negative charges in the vicinity of the TTX/STX binding site (Green et al., 1987; Guo et al., 1987). The density of these negative charges is influenced by pH, and can, in turn, influence the local concentration of the cationic STX and neoSTX as well as TTX. The present results are based on equilibrium reactions that cannot easily differentiate between the effects of surface charges and those of specific charged groups in the toxin-binding site. However, our results show little evidence that these surface charges have been titrated to affect toxin binding between pH's 6.50 and 8.25.

Concerning the specific negative charge in the binding site, most indications suggest that it has a pK_a of 5 or lower. In the present experiments, this group should be virtually fully ionized at pH 6.50, and would exert only minor incremental effects when pH was changed to 8.25. Although there is as yet rather meager information on details, we believe that this anionic group could be involved in the ion-pairing with

the active guanidinium function (i.e., site *a* on Fig. 8) which we have identified as the 7, 8, 9 group in STX by the close relation between the pH dependencies of its chemical state and the potency. By analogy, we extended this identification to the 7, 8, 9 guanidinium in neoSTX because we beleive that by virtue of their close structural similarities, including the C-12 −OH's common to both STX and neoSTX, the docking orientation of these molecules (Dean, 1989) for forming toxin–receptor complexes must be very similar. Thus, the N-1 −OH of neoSTX is probably not proximately involved in interacting with this specific negative charge in the binding site. From these considerations we conclude that within the pH range of 6.50–8.25 the main determinants of potency are the chemical state of the toxin molecule and the abundance of the active species, but not changes in the receptor or the surrounding membrane.

Influence of the Chemical State of neoSTX on Its Reactivity

In the Results we referred to an efficacy term (ϵ) to explain some aspects of the action of neoSTX. In drug–receptor interactions, the dose–response curves depend on a combination of an affinity factor and an efficacy factor (Ariens et al., 1964; Dean, 1989). We use this term in a general sense to embody the meanings of both the "intrinsic activity" of Ariens (1954) and the "efficacy" of Stephenson (1956). Binding to the receptor does not automatically lead to the desired effect. Thus, a full agonist with an efficacy of 1 and a competetive antagonist with an efficacy of 0 could have the same affinity, but the agonist would produce the biological effect and the antagonist would not. In this and previous work, without probing into the detailed mechanisms between toxin binding and channel blockade, we have taken the simplest assumption that the efficacy factor is 1, meaning that during random collisions between the toxin molecules with the binding sites, each collision resulted in a productive binding (Dean, 1989) which led to a blockade of the sodium channel.

This assumption appears to be applicable to most instances shown in the Results, except that of neoSTX at pH 8.25. As shown in Table I, the only way for the observed results to fit a case of one-site action by both STX and neoSTX is if the efficacy of neoSTX were not 1 but 0.75. A plausible physical model for this situation is that only 75% of the collisions of neoSTX with the receptor site actually lead to productive binding and effective blockade of the sodium channel. Alternatively, all collisions might lead to binding, but only 75% of the binding is productive. The two possibilities might be distinguished by kinetic studies, in isolated systems, of diffusional or post-collisional delays and the rates of association and dissociation, but not by the equilibrium studies we have done in the present experiments. In either case, neoSTX at pH 8.25 could behave somewhat like a partial agonist under some conditions, but differ from it in being capable of blocking all I_{Na} at high concentrations. Experimentally, we have seen that at 10–20 times the ED_{50}, neoSTX at pH 8.25 can block all I_{Na} (Yang, L., and C. Y. Kao, unpublished observations). Perhaps for neoSTX the form with a protonated N-1 −OH is the channel-blocking species and the form with a deprotonated N-1 $-O^-$ is an ineffective species. Whether the ineffectiveness is due to low affinity or to incorrect docking orientation remains to be investigated. Nevertheless, the possibility of the coexistence of an active and an inactive species, with the resultant competition for the binding site, could underlie

the shortfall between the observed and expected potencies of neoSTX at pH 8.25 (Fig. 5).

Implications for the Structure of the Binding Site

In the surface–receptor hypothesis, STX is viewed as binding to some site very close to the external orifice of the sodium channel by the side of the molecule with the C-12 −OH groups (Kao and Walker, 1982; Kao, 1983). Because the 1, 2, 3 guandinium group is located on the opposite side of the molecule, it was considered not to be directly involved in blocking the channel.

NeoSTX is most active and more potent than STX when the N-1 group is protonated with no charge. It is least active and less potent than STX when the N-1 group is deprotonated with a negatively charged oxygen. A possible explanation for

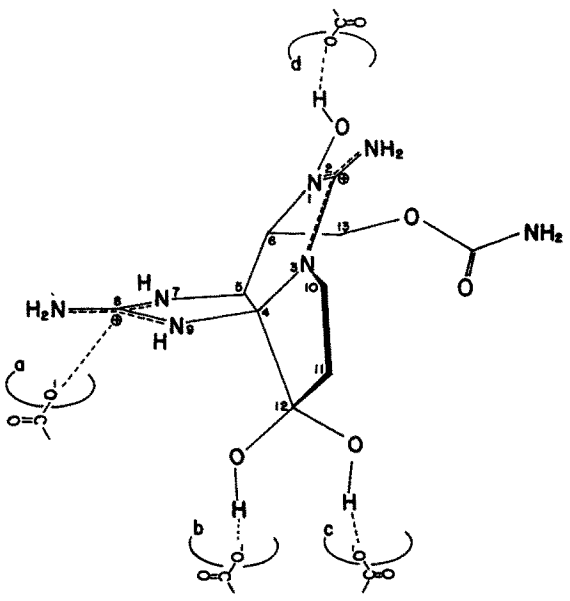

FIGURE 8. Perspective view of neoSTX and probable binding site points in the sodium channel. Sites *a–c* have been identified previously (Kao and Walker, 1982; Kao, 1983); site *a*, probably an ionized carboxylate function, ion-pairs with the 7, 8, 9 guanidinium, while sites *b* and *c*, as carboxylates or other hydrogen-acceptor functions, form hydrogen bonds with the C-12 −OH's. Site *d* is newly identified by the present results. Probably an ionized carboxylate function, it interacts with N-1 −OH of neoSTX, hydrogen-bonding while the group is protonated, and charge-repelling when the group is deprotonated.

these observations is that the N-1 −OH lies close to an anionic function in the sodium channel protein, with which it can form a hydrogen bond when in the protonated state, and by which it is repelled when in the deprotonated state. In Fig. 8, which illustrates this view, site *a* probably ion-pairs with the positively charged active guanidinium group. Sites *b* and *c* form hydrogen bonds with the C-12 −OH's. The present results suggest that site *d* can form a hydrogen bond with or repel the N-1 −OH of neoSTX. Sites *a* and *d* are probably deprotonated carboxylate functions in the sodium channel protein, whereas sites *b* and *c* could be similar groups or other hydrogen acceptor functions. As these site points (Dean, 1989) practically envelope the toxin molecule, the total toxin-binding site is not likely to be planar over the dimensions of the toxin molecule, but is probably in a pocket or crevice of the sodium channel protein.

Such a possibility escaped detection before, when only STX was studied. Nevertheless, if this structural detail of the binding site were to be valid for neoSTX, it has to be valid for STX as well. In STX, the 1, 2, 3 guanidinium with a pK_a of 11.2 is completely protonated within the physiological range of pH's. Whether the positive charge on this group has any influence on the binding of the whole toxin molecule is unknown, but it could exert a constant influence that would not be detectable by limited changes of pH. However, the possibility that neoSTX interacts differently, as does STX, could explain the higher potency of neoSTX at some pH's, and its lower potency at other pH's. This proposed mechanism is consistent with an observation that the dissociation rate constant for neoSTX is lower than that for STX on sodium channels incorporated in lipid bilayers at pH 7.4 (Moczydlowski et al., 1984*b*). Indeed, one might expect the dissociation rate constant for neoSTX to be pH dependent. Perhaps an "allosteric" mechanism of action of STX (Green et al., 1987) can also be explained, as blockage of access to a crevice could occur some distance away from the actual permeation path of the sodium channel.

Critique of Strichartz's Covalent-Bonding Hypothesis

Because he found neoSTX to be four to eight times more potent than STX, and because the C-11 protons in neoSTX exchanged more rapidly as compared with those in STX, Strichartz (1984) concluded that the two phenomena can be linked by the presence of a larger fraction of a carbonyl function at the C-12 position in neoSTX than in STX. He proposed a two-step mechanism by which STX first attached to the receptor by ion-pairing through the 7, 8, 9 guanidinium, and a subsequent dehydration of the C-12 gem-diol to the carbonyl form, which then formed a Schiff-base covalent bond with a secondary amine or an oxygen in the receptor.

This covalent mechanism differs substantially from another suggested mechanism by which the C-12 −OH groups attach to the receptor site through hydrogen bonds (Koehn et al., 1981; Kao and Walker, 1982; Kao, 1983; Kao et al., 1985). Because the primary structure of the sodium channel is known (Noda et al., 1984), these different suggestions could place the STX-binding site, and probably also the mouth of the sodium channel, in quite different parts of the channel protein. Therefore, it is worthwhile to consider Strichartz's proposed mechanism in some detail.

Aside from the fact that he relied entirely on changes in the compound action potentials of multifiber nerve bundles, Strichartz did not consider some important chemical properties of the toxin molecules. An important test of his covalent hypothesis is to know whether neoSTX, when reduced at the C-12 position, would still be active. This test would parallel the situation known for STX. When either one of the C-12 −OH's in STX is reduced, alcohol functions result that, energically, cannot form covalent bonds. Yet the reduced compounds, saxitoxinols, possess intrinsic activity, even through it is much less than that of STX (Koehn et al., 1981; Kao and Walker, 1982; Strichartz, 1984; Kao et al., 1985). We suggested that both C-12 −OH functions probably formed hydrogen bonds with acceptors in the receptor site (sites *b* and *c* in Fig. 8).

NeoSTX has been reduced to neosaxitoxinol by Wichmann (1981), who found it to be weakly active. The relative potency of neosaxitoxinol to neoSTX is comparable

with that of saxitoxinol to STX. Since the C-12 groups in neosaxitoxinol are in an alcohol configuration that cannot form covalent bonds, the intrinsic activity must be attributed to some noncovalent mechanism. In neoSTX the C-12 groups probably participate in hydrogen bonding in a similar manner as in STX.

Trinitrobenzenesulfonic acid, an amino-modifying reagent, which Strichartz (1984) and his co-workers (1984) found to interfere with the binding of [^{3}H]STX, also did not affect the reduction of I_{Na} by STX or TTX in voltage-clamped preparations of either frog nerves or muscles (Pappone, 1986).

From these considerations, we conclude that the participation of the N-1 −OH of neoSTX in binding to the receptor is sufficient to account for the slightly higher potency of neoSTX over that of STX in acidic and neutral pH's. Our proposed mechanisms has the added advantage of explaining the weakness of neoSTX in alkaline pH's, an observation that cannot be explained by Strichartz's covalent mechanism.

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