SITE-SPECIFIC ANTAGONISTS TO TETRODOTOXIN AND SAXITOXIN

FINAL REPORT

C. Y. KAO

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C. Y. Kao

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Tetrodotoxin (TTX) and saxitoxin (STX) are important neurobiological tools because of their unique high-specificity and high-potency blockade of voltage-gated sodium channels. Although chemically different, their acticns are identical. The biophysical mechanism of their actions have been exhaustively studied, but the chemical nature of the actions remains unknonw. By studying the structure-activity relations of some natural and synthetic analogues of both TTX and STX on the voltage-clamped frog skeletal muscle fiber, we have deduced that the TTX/STX binding site is a pocket 9.5 Å (width) x 6 Å (height) and 5 Å (depth), located in a fold of the sodium-channel protein, close to the external orifice. There are 7 anchoring sites, a - g, which ion-pairs or hydrogen-bonds with surface groups in the toxin molecules. TTX and STX share one ion-pairing and 4 H-bonding sites in common. Such a binding site also explains the actions of the TTX analogue, chiriquitoxin, and STX analogues, gonyautoxins and sulfocarbamoyl saxitoxins. A specifically labelled ^{3}HTTX of 2900 Ci/mol has been synthesized for use in continuing efforts to locate the binding site.

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INTRODUCTION

Project goals

Tetrodotoxin (TTX) and saxitoxin (STX) are two of the most potent agents known which are capable of blocking the voltage-gated sodium channel of excitable cellular membranes, and thereby causing generalized paralysis. For this reason, there is a need to develop effective and specific antidotes for possible defensive purposes. The objective of this project is to generate more knowledge about the TTX/STX binding site, on which rational designs of specific antidotes might be based.

To accomplish this goal, efforts in this project have proceeded on two separate but interrelated tracks: (a) refinement of our knowledge of the physical dimensions, chemical structures, and location of the binding site by complementarity considerations of the structure-activity relations of various TTX and STX analogues; and (b) synthesis of simpler compounds which might interact with the binding site, as agonists or antagonists.

Background

Tetrodotoxin (TTX) and saxitoxin (STX) are important neurobiological tools because of their specific reaction with the voltage-gated sodium channel of many excitable cells. They are among the most lethal, rapidly-acting, low molecular weight, non-protein toxins known, each with a LD_{50} of about 10 ug/kg in most mammalian species (see Kao, 1966). In the cold-war era of the recent past, their extreme potency and potential availability concerned the U. S. Army authorities whose support was responsible, to a significant extent, for the fundamental research which led to the discovery of the basic mechanism of actions of these toxins, and their current status as important neurobiological tools.

Paralysis and death attributed to these toxins can be traced back in history for centuries. Poisoning caused by TTX occurs most frequently in Japan, because of a cultural predilection there for puffer fish (fugu) as a culinary delicacy. Toxic amounts of TTX are generally localized in the ovaries, eggs, and liver, and consumption of those parts can lead to fatal poisoning. TTX has also been found in a Californian newt, a Costa Rican frog, two Australian octopuses, and several fishes unrelated to the puffers. A possible explanantion for this puzzlingly diverse interspecies distribution may reside in a recent finding that TTX may have a microbial origin, because it can be detected in the culture-medium of some bacteria (summarized in Yasumoto, Nagai, Yasumura, Michishita, Endo, Yotsu, and Kotaki, 1986). To date, TTX is the only molecule known to be involved in poisonings.

Poisoning attributable to saxitoxin is known as **paralytic shellfish poisoning**. Historically confined to the Pacific and Atlantic coasts of the north American continent, this poisoning is now found with increasing frequency in all parts of the world. The causative agent is a unicellular dinoflagellate of the Alexandrium species, which blooms in oceanic or estuary waters, and is concentrated by filter-feeding molluscs. Such molluscs rely on calcium channels for their neuromuscular functions, and are not affected by the toxins. Consumption of such tainted molluscs by other species, including man, which depend on sodium channels for physiological function, leads to paralysis. A strongly suspected mechanism for the geographical dissemination is the loading

of ballast water containing cysts of offending organisms in endemic areas in oil tankers or other maritime vessels, and the subsequent discharge of such loads in previously uninfested areas. In contradistinction to TTX, about 20 different molecules of closely related structures have been identified in the family of paralytic shellfish poisons (Oshima, Sugino, and Yasumoto, 1989). For conveninece, I use the term saxitoxins for a generic reference to this family of molecules, and saxitoxin (STX) for the best known of them.

The two toxins are chemically very different, and each of them is structurally unique. TTX has a perhydroquinazoline nucleus, containing an unusually stable cyclic guanidinium function and two internal oxygen bridges which contribute to a rigid 5-ring structure. STX has a purine nucleus containing two guanidinium functions, and a 4-atom third ring. Both toxins have an abundance of -OH groups on the accessible surfaces, In spite of the vast differences in structure, the two molecules have virtually identical biological actions.

Although their paralyzing effects have been recognized for many years, they first came into sustained research interest in the early 1960's, in part because of the successful isolation of highly purified samples of each toxin, and in part because of their extraordinary potency in blocking electrical activities in isolated nerve and muscle fibers. In those years, the Hodgkin-Huxley theory of ionic basis of excitation was gelling, and the voltage-clamp technique for studying specific ionic conductances was becoming widely adopted. Thus, the extraordinary potency of these toxin was amenable for a novel type of analytical study. Virtually from the first voltage-clamp experiment, it became clear that these toxins had a unique effect unknown until then for any other substance. TTX was the agent first studied in detail, and STX proved to have the same actions in later work.

These toxins affected only the sodium current and nothing else. In Hodgkin and Huxley's original proposal, the sodium, potassium and leakage currents were operational parameters in an empiricial quantitative formulation of the excitation process. The notion of such ions passing through aqeuous channels in specific protein molecules was not even conceived of. Indeed, at the time of the first voltage-clamp studies of TTX, there was a raging controversy as to whether the sodium and potassium currents were really separate entities or whether they were different kinetic manifestations of some similar basic process. Thus, the impact of the observation that TTX reduced, and even completely obliterated, the sodium current, without nary an effect on the potassium current was immediate and enormous. The observation not only showed that the two currents resulted from two distinct processes, but also ushered in the molecular phase of neurobiology. Until then, excitation phenomena were studied by recording electrical events, because there was no known marker substance which recognized a molecular event in bioelectrically excitable tissues with such specificity and potency. Soon after, against great odds, a TTX-binding protein was isolated from several nerve and muscle preparations (summarized in Levinson, Duce, Urbane, and Recio-Pinto, 1986). Eventually, this protein was purified, and recognized as having many properties of the native sodium-channel. In 1984, the primary structure of the sodium channel was deduced from cDNA studies (summarized in Numa and Noda, 1986). At present, there are some suggested possible organizations of this 2000-amino acid protein (e.g. Guy and Conti, 1990), but no direct information on the 3-dimensional structure.

The biophysical mechanism of the TTX or STX blockade of the sodium channel has been studied exhaustively. These toxin molecules do not affect the gating process, by which an appropriate voltage change initiates some conformational change in the sodium-channel molecule to permit passage of Na^+ through a central core in the protein molecule. They do not affect the kinetics of activation or inactivation of the sodium-channel processes, but simply reduce the maximum possible conductance of the channel. The pictoral representation of the block is that the toxin molecules in some way physically interferred with the passage of Na^+ ions through the channel.

The chemical nature of the TTX/STX action is unknown, largely because of difficulties with the chemistry of these molecules. TTX is virtually insoluble, except in slightly acidified water, and the structure of STX became known years after its action was understood. Most difficult of all, modifications of the structures of these toxins were difficult, in part because they were available in minute amounts and at very high costs, and in part because the biological action is lost with many modifications. All that can be deduced is that the guanidinium function in the toxin molecules is protonated (positively charged) at pH 7 - 7.4, and that the charge is somehow involved in the blockade. For nearly two decades after the first experiments on the TTX action on the sodium channel, our understanding of the chemical nature of the TTX/STX action rested on speculation: Kao and Nishiyama (1965) first suggested the imporance of the guanidinium group and proposed a physical obstruction model. Hille (1975) elaborated on this hypothesis, and located the anionic charge which ion-paired with the guanidinium functions in the selectivity filter of the sodium channel.

In the early 1970's, because of improved separation technology, several natural analogues of TTX and STX were discovered, and several successful modifications were made in the parent toxins. The first analogues studied included chiriquitoxin (CqTX), nortetrodotoxin (norTTX), neosaxitoxin (neoSTX), decarbamoylsaxitoxin (dcSTX), and saxitoxinols (STX reduced at the C-12 position) (Kao and Walker, 1982; summarized in Kao, 1986). Direct experimental evidence identifying some groups which were indispensable for biological actions were first obtained from STX analogus. By careful examination of molecular models, stereospecific similarities were found in TTX. These groups are:

TTX	STX
1,2,3 guanidinium	7,8,9 guanidinium
C-9 -OH	C-12 -OH
C-10 -OH	C-12 -OH

From this modest beginning, it became obvious that the TTX and STX molecules must bind to a site close to the external orifice of the sodium channel, and not at the selectivity filter which is deep inside the channel. Further work, much of it supported by this contract, has since identified nearly all the active groups on the surface of the two toxin molecules. As a result, the physical dimensions and possible shape of the binding site have been rationally deduced. This work and other parts of the project are detailed in the following sections.

STRUCTURE-ACTIVITY RELATIONS OF TETRODOTOXIN AND SAXITOXIN ANALOGUES

Material and methods used

Fig. 1 shows the structure of TTX and the various analogues which have been tested. Fig. 2 shows similar features for STX and various analogues. The resources needed for isolation and structural characterization of these analogues have always been beyond the reach of this laboratory. Therefore, all chemical work (except some recent work to be detailed in a later section) was done by colleagues in voluntary collaborations. In the case of TTX, the collaboration began with Prof. Harry S. Mosher of the Department of Chemistry, Stanford University in 1962 and lasted until his retirement in 1989. It continued, with some chronological overlap with Prof. Takeshi Yasumoto of the Department of Food Chemistry, Tohoku University, Faculty of Agriculture, Sendai, Japan, and is still continuing. In the case of STX, it began with Dr. Edward. J. Schantz of the US Army Chemical Corp in 1963, and through him with Prof. Heinrich K. Schnoes of the Department of Biochemistry, University of Wisconsin, Madison, WI until the middle 1980's. The colleagues there included some of his graudate students, notably Frank E. Koehn (now Dr.). Most recently, Dr. Y. Oshima of Prof. Yasumoto's department have become a valuable colleague on studies of some newer STX analogues. From the outset, it was understood that for our biological work to be meaningful, the toxins must be pure, and the structures known as far as was possible.

In the Mosher and Schnoes laboratories, characterization was based primarily on NMR spectroscopy. For analogues derivatized from TTX or STX and synthetic compounds, this approach was entirely feasible, but for natural analogues which might exist only in minute quantities, it is more difficult. However, the discovery of chiriquitoxin in a Costa Rican frog in the Mosher laboratory, and the isolation of several gonyautoxins in the Schnoes laboratory are notable exceptions to this general statement. In the Yasumoto laboratory, which began work in this area years after Mosher's pioneering work on the structure of TTX, a significant difference is the reliance on a chromatographic method for separation and initial identification, followed by NMR and mass spectrometry. The chromatographic method became possible because of important advances in separation technology. The present procedure is based on reverse-phase HPLC, using C-18 columns, and fluorescence determinations of a post-column derivatized product of TTX or STX. In the NMR characterization, about 1 mg of material is needed; in the HPLC method, when reference compounds have first been isolated, quantities as minute as 10 ng can be determined.

In our biolgical studies, we relied on various electrophysiological methods for determining the functions of the sodium channel. Initially, in the early 1970's, I had no discrete funding support to study TTX/STX, and had to use frog muscle fibers in my primary laboratory in Brooklyn. At that time, no voltage-clamp methods were available which could adequately control the fast sodium current in muscle fibers, and we relied on the maximum rate of rise (max dV/dt) of a constant-velocity action potential as an indirect means of gauging the effects of toxins on the sodium channel. Max dV/dt is the capacitive current, which is, at that instant, equal and opposite to the ionic current. It does not distinguish currents through different ionic channels. However, under the conditions of our experiments, and taking appropriate precautions in using internal controls, at the moment when max dV/dt is reached, most ionic current

is due to Na^+ influx, as very little potassium current had even begun.

In the late 1970's, after the first structure-activity studies were completed (Kao and Walker, 1982), and with the support of discrete funding, the actions of several analogues, then available at one time, were studied on the squid giant axon, using an axial-wire voltage-clamp method. By this method, specific ionic channels could be studied. Later, in order to extend work to beyond the squid season, the main focus was shifted back to the frog skeletal muscle fiber, but now using a vaselin-gap voltage-clamp method (Hille and Campbell, 1976).

The general principles of the methodology is straightforward. The sodium and potassium currents (I_{Na} and I_K) were determined on small regions (less than 200 um) of short segments of isolated single skeletal muscle fibers. For standardized comparisons, currents at two voltages were monitored: (a) the maximum I_{Na} (inward current) at whatever voltage (usually within a few millivolts) needed to elcit it was used for assessing the effect of toxins on the I_{Na}, and (b) the current at E_{Na} (at which no net I_{Na} occurred) is outwards, and represents I_K. This current is useful for gauging the health of the isolated fiber, and for determining the specificity of action of the toxin on sodium and potassium currents. In most cases, I_K remained unchanged even when I_{Na} is completely obliterated. However, with some analogues of TTX, the activation of the fast I_K is significantly slowed (see below).

The reduce I_{Na} in the toxin-affected state (I'_{Na}) is compared with those in the pre-toxin and the post-toxin recovered states (I_{Na}) in the same fiber. The normalized ratio (I'_{Na}/I_{Na}) is then plotted against log concentration of toxin to show a dose-response relation. This relation is sigmoidal, and is well fitted by the function describing a bimolecular reaction scheme:

$$T + R \rightleftharpoons TR$$

where T = toxin, R = receptor, and TR = toxin-receptor complex. From the scheme, the response is:

$$I'_{Na}/I_{Na} = 1 - \{ 1 /(1 + ED_{50} / [Toxin]) \}$$

where [Toxin] = concentration of toxin.

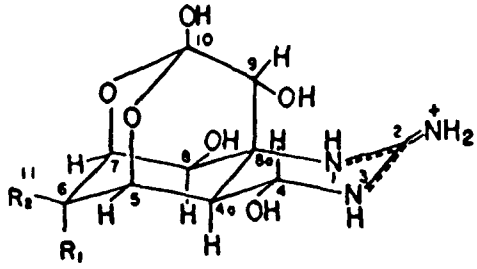
Additionally, the data are also handled in a Hill analysis where log $P/(1-P)$ ($P = I'_{Na}/I_{Na}$) is plotted against log concentration. In this method, the dose-response relation can be readily fitted with a linear least-squares regression line, the slope of which (always close to 1.0) confirms the bimolecular nature (no cooperativity) of the reaction. The concentration of the toxin analogue needed to reduce I_{Na} to one-half of the control value (ED_{50}) can be easily determined in Hill plots from $P/(1-P) = 1$. ED_{50}, so obtained, are single values, and cannot be used readily for comparisons. However, the Hill plot also allows ready determinations of the errors of estimate, from which standard errors of estimate can be appended to the ED_{50} for comparing the effects of different analogues (Hu and Kao, 1991).

Relative potencies of various analogues can be rank-listed against the parent toxin (TTX or STX), and some idea can be gained of the importance of a structural modification on the Na^+-channel blocking action of the compound. More exacting quantitation of the relative potencies is of uncertain value,

because the underlying nature of the chemical bond (non-covalent, ion-pairing and hydrogen-bonding) makes such efforts very difficult and nebulous. Indeed, the similar groups in TTX and STX identified in this project appear to be rather different from those identified by Danziger and Dean (1985), using a theorectical quantum chemical approach.

Tetrodotoxin analogues

When this project began, active groups on TTX were recognized mainly by comparison of stereochemical similarities with STX. These groups were the 1,2,3 guanidinium and the C-9 and C-10 -OH's. Two sets of experiments shortly after that provided direct experimental support for the roles of the -OH's. The significance of the C-9 -OH was confirmed in studies on 2 natural analogues, 4-epiTTX and 4,9 anhydroTTX (Kao and Yasumoto, 1985).



	R_1	R_2
TTX	OH	CH_2OH
6-*epi* TTX	CH_2OH	OH
11 deoxy TTX	OH	CH_3
CqTX	OH	$CH(OH)CH(NH_2)COOH$
11-oxoTTX	OH	$CH(OH)_2$

Fig. 1. Structure of tetrodotoxin and analogues studied.

The role of the C-10 -OH was affirmed by studying the effect of pH on the potency of TTX. The C-10 -CH group of TTX protonates with a pKa of 8.8. Within a physiological range of pH, the abundance of the protonated form can be varied to a limited extent. By comparing the Na^+-channel blockade of TTX on the voltage-clamped squid axon at different pH's, the potency was shown to depend on the abundance of the protonated species of -OH (Hu and Kao, 1986).

The 1,2,3 guanidinium group in TTX has a pKa of ca. 11, and is always protonated (with a cationic charge) within the physiological range of pH's. Its role in channel blockade is not testable with available means.

6-epitetrodotoxin (6-epiTTX) and 11-deoxytetrodotoxin (11-deoxyTTX).

The C-6 end of the TTX moelcule was thought to be unimportant for channel blockade, because norTTX, norTTX alcohol and chiriquitoxin were only slightly

less active or equally as active as TTX (see Kao, 1986). However, work done on this project with two new natural analogues, and a fresh sample of chiriquitoxin have overturned that conclusion. 6-epiTTX and 11-deoxyTTX are both natural analogues found in an Okinawan newt, Cynops ensicauda (Yasumoto, Yotsu, Murata, and Naoki, 1988). The former had the -OH in an epimeric position from that in TTX, whereas the latter had a methyl function instead of hydroxymethyl in the C-11 position. 6-epiTTX is 4% as active as TTX, and 11-deoxyTTX is 1% as active. Considerations of energetics indicate that the reduced potencies are attibutable to the loss of a hydrogen- bond in each case (Yang, Kao and Yasumoto, 1992).

Chiriquitoxin (CqTX)

Among TTX analogues, chiriqiutoxin (CqTX) has a special place. As the first biologically active TTX analogues found, it sparked a re-examination of the structure-activity relations of TTX, a study which had been abandoned earlier because of our inability to modify TTX and still retain significant degrees of activity. CqTX is found in the the skin and eggs of a harlequin frog of Costa Rica (Kim, Brown, Mosher and Fuhrman, 1975; Pavelka, Kim and Mosher, 1977). It was known to be structurally similar to TTX except for a large substituent on the C-6 end of the molecule. It was equally as active as TTX in mouse-lethality assays (Kim et al., 1975), and on the max. dV/dt of the action potential in frog skeletal muscle fibers (Kao and Yeoh, 1977). However, in those first studies, insufficient amounts were available to solve either the chemical structure or the specific ionic conductances affected. Repeated attempts at collecting more material were unsuccessful, until June 1988.

Under primary support from an NIH grant, I went to San Jose, Costa Rica on a few days notice of the availablility of the frogs (Atelopus chiriquensis), and collected 971 skins. This material was fixed in isopropanol, refrigerated, and sent to Japan for extraction, isolation, and structural determination. Mari Yotsu undertook the work as a Ph.D. thesis research project under the supervision of Prof. Takeshi Yasumoto. By spring 1989, a new batch of CqTX had been isolated and purified, and the structure determined. This work on the chemistry of CqTX is published (Yotsu, Yasumoto, Kim, Naoki and Kao, 1990).

The earlier chemical studies, especially with regards to the basic similarites between TTX and CqTX, was confirmed by the new work. In addition, the new study showed that the substituent was on C-11, and that it was in the form of a glycine replacing one of the methylene hydrogens of the hydroxymethyl function in that position.

Biologically, CqTX was known to be equally as potent as TTX at around the body pH of 7 - 7.4. In a re-examination of the actions of CqTX (Yang and Kao, 1992), the influecne of pH on the channel-blockade in frog muscle fibers was compared with that seen with TTX. The ED_{50}'s of CqTX for blocking Na^+-channels at pH's 6.50, 7.25, and 8.25 are respectively: 6.8, 3.8, and 2.3 nM. By comparison, those for TTX are: 3.8, 3.1, and 4.3 nM (Hu and Kao, 1991). Some variations are expected when assays are done on different batches of frogs in different seasons of the year. For a better comparison of the ED_{50}'s at pH 7.25, a series was done on TTX at the time that CqTX was assayed. The ED_{50} for TTX was 4.1 nM, which is statistically no different from that of CqTX. Two features are readily evident from these observations: at neutral pH, CqTX and TTX are equally potent, and the influence of pH on the potency of CqTX is different from that on TTX.

These observations can be explained by considering the chemical properties of the glycine moeity in CqTX. The terminal (C-13) carboxyl group has a pKa of ca. 2, and the C-12 amino group, a pKa of ca. 9. At pH 7.25, most of the C-13 group is deprotonated, while the C-12 group is protonated. Any intramolecular salt-bridge between these vicinal functions would largely remove them from any charge-interactions with binding sites in the Na^+-channel protein. Since all the other aspects of the CqTX are the same as TTX, the equipotency of the two toxins is understandable.

Any consideration of the potency of CqTX at acid pH's must be tempered with the understanding that some surface negative charges are present around the external orifice of the Na^+ channel. Acid pH's reduce the density of such charges, and thereby lower the local concentrations of positively charged toxins, such as TTX, STX, and/or neoSTX. However, the effect is small, and readily recognizable because the observed potency is lower than at neutral pH when it is expected to be higher. For CqTX, the potency at pH 6.50 is too low to be completely accounted for by the changes in surface negative charges. In acid pH's, the C-13 carboxyl group and the C-6 -OH in CqTX forms an intramolecular lactone (Yotsu et al., 1990). At pH 6.50, there is probably a shift of the equilibrium between CqTX and CqTX lactone such that more of the lactone species are present than at pH 7.25. Such a shift could explain the lower potency at pH 6.50.

At alkaline pH's, a lowered potency of TTX is explained by the lesser amount of the protonated C-10 -OH group, a change which probably also occurs in CqTX. The markedly higher potency actually observed is explained by the probable emergence of more protonated C-12 amino group which then participated in bonding to receptor.

11-oxotetrodotoxin (11-oxoTTX)

During studies of the chemistry of TTX, the primary alcohol on C-11 was seen as a ready target for modification. Attempts at oxidizing this group led to norTTX, but a predicted aldehyde was never found. 11-oxoTTX is a hydrated aldehyde of TTX, discovered in a south Pacific puffer fish, Arothron nigropunctatus (Khora and Yasumoto, 1989). The discovery proves that a C-11 aldehyde can exist, and that it occurs in a stable form as a hydrate, 11-oxoTTX. Its potentials as a synthon for this project is enormous. The natural compound was obtained in too small a quantity for us to evalaute its biological actions. Dr. B. Q. Wu, Senior Research Scientist on this project, succeeded in producing 11-oxoTTX synthetically from TTX (details below). The synthetic and natural 11-oxoTTX are identical in all chemical and physical characteristics.

The actions of 11-oxoTTX (synthetic) was studied on the voltage-clamped frog skeletal muscle fiber. It is specific in blocking the Na^+ channel. Unique among all TTX analogues studied, it was 3-5 times more potent than TTX, with an ED_{50} of 0.7 nM (Wu, Yang, Kao, Yotsu and Yasumoto, 1991).

Collaborative studies with Dr. S. R. Levinson of University of Colorado Medical Center, Denver on binding to purified Na^+-channels from the electroplaques indicate that 11-oxoTTX binds about twice as firmly as TTX. Studies on dog brain Na^+ channels in lipid bilayers (Dumas, Andersen, Wu and Kao, 1992)) indicate that the higher potency can be attributed primarily to a slower dissociation rate constant. My explanation of these properties is that the additional -OH group on C-11, as compared with TTX, provides one more site for hydrogen-bonding to receptor.

The observations on these four C-6 modified analogues of TTX provide a new view to the possible shape and dimensions of the binding site in the Na^+-channel protein. This view will be detailed in a later section. 11-oxoTTX is an especially important analogue because of its usefulness for further synthetic work. As an example, we have successfully synthesized a specifically labelled ^{3}HTTX by incorporating the tritium label covalently into one of the methylene hydrogens on C-11. This radiolabelled TTX is anywhere from 6-60 times more active than the sparingly available diffusely labelled TTX, and unlike the tritium label in ^{3}HSTX, the label is stable and does not exchange with solvent protons. We expect to be using this specifically labelled ^{3}HTTX for further studies on the location of the TTX/STX binding site. In fact, exploratory experiments have been started with Dr. Levinson and Dr. Peter Kao of Department of Medicine, Stanford University Medical Center.

Saxitoxin analogues

In the first study of structure-activity relations of STX analogues (Kao and Walker, 1982), the importance of the C-12 -OH's were recognized by the use of saxitoxinol, which had one or another of those -OH reduced. The 7,8,9 guanidinium, and not the 1,2,3 guanidinium was identified as the active guanidinium, because neoSTX which had a modification on N-1 was found to be equally potent with STX. This conclusion was reached essentially by exclusion.

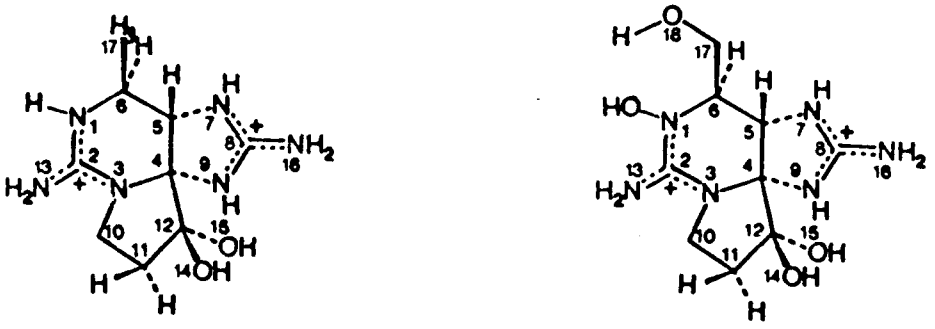


Fig. 2. Structures of saxitoxin analogues studied. Left, deoxydecarbamoylSTX. Position 17 has a methyl group. Right, DecarbamoylneoSTX.

In later work on both the squid giant axon (Kao, James-Kracke and Kao, 1983) and the frog skeletal muscle fiber (Hu and Kao, 1991), the active guanidinium group was identified affirmatively by studying the effect of pH on the potency of STX. The 1,2,3 guanidinium had a pKa of 11, and almost all of it

exists in the protonated (cationic) form within the physiological range of pH's. Moreover, the relative abundance of the species at pH's 7.25 and 8.25 is essentially 1.0, making it impossible to alter its concentration as a means of testing its role in channel blockade. However, the 7,8,9 guanidinium has a pKa at 8.25. The relative abundance of the protonated (cationic) species of the group at pH 8.25 is 0.5, and that at pH 7.25, 0.9. The relative abundance at the 2 pH's is 1.80. Careful examination of the relative potencies of STX at the two pH's show that STX is more potent at pH 7.25 than pH 8.25, and that the relative potencies at the two pH's is 1.79 in the squid giant axon, and 1.77 in the frog skeletal muscle fiber. Such good agreements between the relative abundance of the protonated species and the relative potencies strongly identifies the 7,8,9 guanidinium as the active guanidinium involved in channel blockade. By contrast, the discrepancy between these prameters for the 1,2,3 guanidinium excludes that group.

Neosaxitoxin (NeoSTX)

This is a natural analogue of STX, different only in having the hydrogen on N-1 replaced by a -OH. Around neutral pH, it has generally been found to be about equally potent as STX, either in mouse-lethality assays (see discussion and references in Hu and Kao, 1991) or on isolated nerve or muscle preparations (e.g. Kao and Walker, 1982). However, in a re-examination of the actions of neoSTX, using a highly purified sample, neoSTX was found to be 1.4 times as potent as STX at pH 7.25. At pH 6.50, it is still slightly more potent than STX, but at pH 8.25, it is marked weaker than STX. To understand these observations, it should be recalled that the N-1 -OH deprotonates with a pKa of 6.75. The potency of neoSTX at different pH's turns out to follow the state of the N-1 -OH more than that of the 7,8,9 guanidinium. Thus, the new evidence shows that the N-1 group does have some influence. I now believe that the N-1 -OH is situated close to an anionic site in the receptor, with which it hydrogen-bonds when in the protonated state (acid and neutral conditions), and by which it is charge-repelled when in a deprotonated state (alkaline conditions)(Hu and Kao, 1991).

One problem in the field of research on neoSTX is that the potencies reported in the literature are rather variable. We addressed this problem in a later study on a decarbamoylated analogue of neoSTX (Yang, Kao and Oshima, 1992; see below). However, I will mentioned this point here in conjunction with neoSTX. Unlike STX, neoSTX is apparently more difficult to purify, because the potency of pure samples in the literature differ by a factor of 3. The specific toxicity of the purest samples should be about 6000 Mouse Units/mg. In mouse-lethality assays, neoSTX and STX are generally found to be about equally lethal, even though on isolated muscle fibers at near-neutral pH neoSTX is about 40% more potent. The reason for the discrepany is that the precision of the mouse-lethality assay is 20%, and the difference between STX and neoSTX is probably not sufficient to be reliable in such assays.

Deoxydecarbamoylsaxitoxin (doSTX).

To understand the significance of this compound, it is necessary to recall that the carbamoyl function in STX can be removed either by acid hydrolysis (Ghazarrosian, Schantz, Schnoes and Strong, 1976), or enzymatically (Sullivan, Iwaoka, and Liston,1983). The resulting decarbamoylSTX (dcSTX) was among the first STX analogues to be studied by us (Kao and Walker, 1982), and found to retain 20% of the biological activity on frog muscle. Later, in a study on the

squid giant axon, this degree of activity was confirmed (Koehn, Schnoes and Kao, 1983). In addition, the full activity was restored when the dcSTX was recarbamoylated. It was thought then that the moderate degree of biolgical activity might be due to the -OH group remaining on C-13 in the dcSTX molecule.

The opportunity to test this notion came when doSTX became available. This compound was isolated from the mussle, Mytilus edulis, which had become contaminated with Gymnodinium catenatum in Tasmania, Australia (Oshima, Hasegawa, Yasumoto, Hallgraff and Blackburn, 1987). It differs from dcSTX in having a methyl group on C-13. As expected, because a methyl group does not participate in hydrogen-bonding, doSTX is considerably weaker than dcSTX. On the voltage-clamped frog muscle fiber, it has an ED_{50} of 618 nM, or 0.008 that of STX (Yang et al., 1992b). Energetically, the Gibbs free energy of binding, ΔG, decreased by 12 kJ/mol, consistent with the loss of a hydrogen-bond.

Decarbamoylneosaxitoxin (dcneoSTX).

This compound was isolated from Spondylus butleri which had been contaminated with Pyridinium bahamensis in Palau (Harada, Oshima, Kamiya and Yasumoto, 1982). The reason for investigating its biological activity (Yang et al., 1992b) is to see whether there are any interactions between the carbamoyl side-chain and the N-1 hydroxyl of neoSTX, an issue made somewhat more urgent because of the finding that the N-1 -OH apparently participated in binding to receptor (Hu and Kao, 1991, see above).

In comparing the actions of the pair STX and dcSTX with neoSTX and dcneoSTX, there is one striking difference. Whereas dcSTX is 0.2 as active as STX at pH 7.25, dcneoSTX is 0.004 as active as neoSTX. We explain the difference in the following way. The C-13 -OH on the decarbamoylated analogues are located stereochemically close to the N-1 group. In STX, this steric relation is apparently innocuous. In neoSTX, where the N-1 -OH has a pKa of 6.75, at pH 7.25, the group is predominantly in the deprotonated form, An intramolecular hydrogen-bond between the C-13 -OH and the deprotonated N-1 $-O^-$ would effectively remove the C-13 -OH from any interaction with receptor groups.

Stereospecific similar groups and implications for the TTX/STX binding site

Observations on 6-epiTTX and 11-deoxyTTX show that there are receptor site capable of interacting with the C-6 and C-11 -OH groups. Since the active groups of TTX occur on all surfaces of the molecule, the binding site has to be in a fold or a crevice of the sodium channel protein. Fig. 3 shows a representation of such a hypothetical binding pocket with sites **a - g** designated as anchor points complementary to active groups in the toxin molecule.

Site **a** is an anionic site (probably a deprotonated carboxylate) which ion-pairs with the cationic guanidinium. Sites **b** and **c** are hydrogen-acceptors, capable of forming hydrogen bonds with the C-9 and C-10 -OH's. Site **d** is probably a deprotonated carboxylate, capable of ion-pairing or charge-repelling the N-1 -OH of neoSTX. Site **e**, **f**, and **g** are hydrogen acceptors interacting with the -OH's on C-4, C-6, and C-11 of TTX.

As noted above, we have previously identified 3 stereospecific similar groups in the TTX and STX molecules. When the 1,2,3 guanidinium group of TTX is aligned with the 7,8,9 guanidinium of STX in such an orientation that the C-9

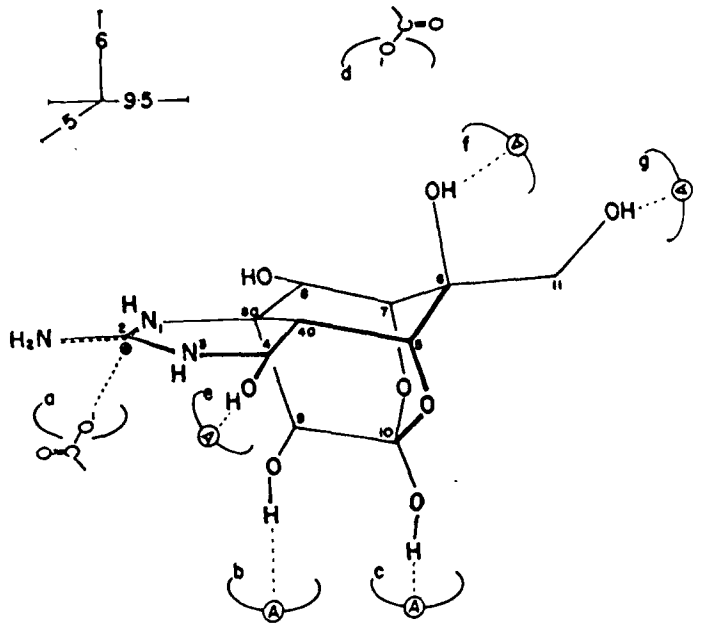


Fig. 3. Perspective view of a molecule of TTX in its postulated binding site. Anchoring points are designated a - g. See text for details. (A) denote hydrogen-acceptors. Scale in left upper corner are in angström units.

and C-10 -OH's of TTX are aligned with the C-12 -OH's of STX, then the C-6 end of the TTX molecule lines up with the carbamoyl side-chain of STX. When the 7,8,9 guanidinium of STX is aligned complementary to site a, then sites b and c readily line up complementary to the C-12 -OH's. When these 3 anchor points are aligned, the C-13 -OH of deSTX is closely positioned to site f, and the end amino group in the carbamoyl side-chain is lined up complementary to site g (Fig. 4).

From these suprising fits for two rather dissimilar molecules, we deduce that the TTX/STX binding site probably is a pocket 9.5 Å (width) x 6 Å (height) x 5 Å (depth). Steric factors as well as charge-interactions influence the

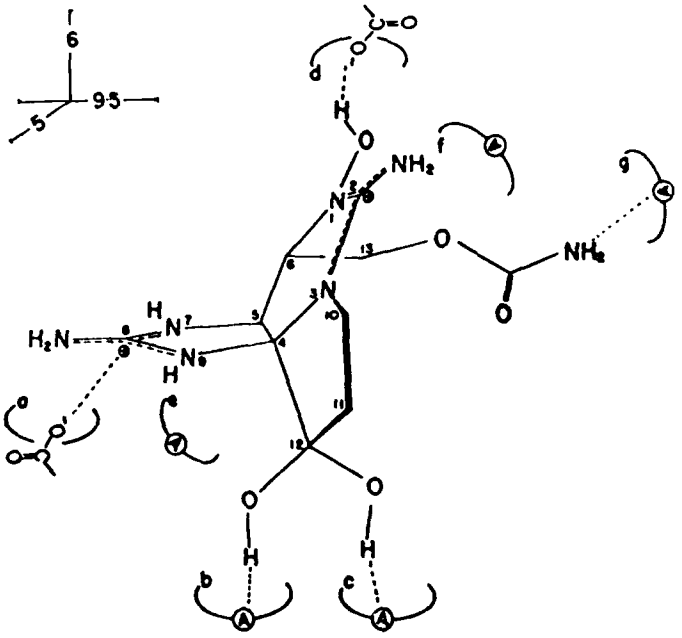


Fig. 4. Perspective view of a molecule of STX in its postulated binding site, which is same as that for TTX.

correct docking orientation of a toxin molecule onto its receptor site. CqTX

presents a specially challenging problem. The glycine function on C-11 is bulky; yet CqTX has the same potency as TTX. The explanation is probably that the glycine moeity projects out of the entrance to the binding pocket, and is somewhat removed from reactive anchoring points (Fig. 5).

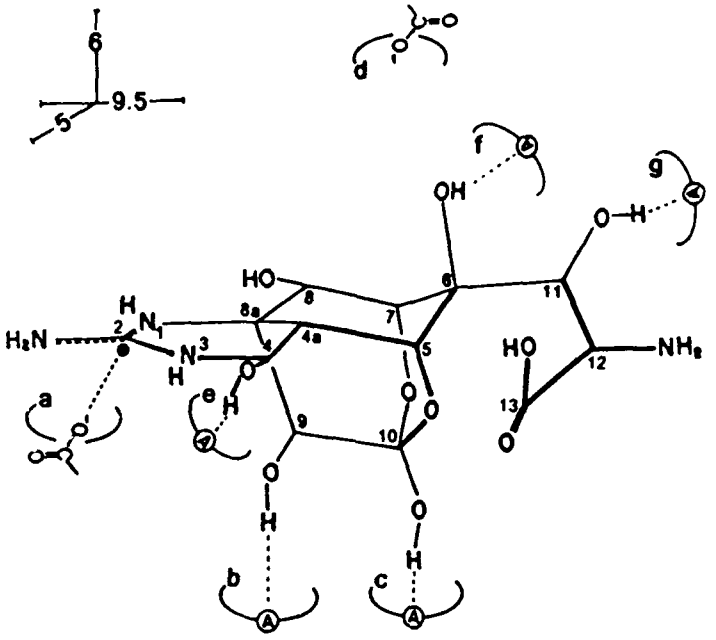


Fig. 5. Perspective view of a molecule of CqTX in the TTX/STX binding site. Glycine function on C-11 projects out between anchor-points c and g. CqTX has all other groups as in TTX: hence, its equipotency with TTX.

This view of the binding site can also explain the potencies of some STX analogues which have not been satisfactorily accounted for before. The gonyautoxins have large $-OSO_3$ groups on C-11, which add not only steric bulk but also strongly negative charges to a region very close to the critical C-12 -OH's. Yet their potencies are only slightly reduced. In the sulfocarbamoyl toxins, $-OSO_3$ groups are added to the end of the carbamoyl side-chain, seemingly far from any reactive points. Yet their potencies are markedly lower, and only become enhanced when the sulfate group is hydrolyzed off.

Molecular modelling shows that in gonyautoxins, the $-OSO_3$ moeities are similarly placed as the glycine function in CqTX. Therefore, they may also project out of the pocket, and have little influence on the potency (Fig. 6A). In the sulfocarbamoyl toxins, however, the $-OSO_3$ groups extend the carbamoyl chains so much that they interfer with a proper docking of the toxin molecules into the binding-site (Fig. 6B). Not surprisingly, when those extensions are hydrolyzed off, the resultant toxins are freed from the steric hinderance, and are considerably more potent.

Site-directed mutagenesis experiments on the rat brain sodium channel II show that in the short segment 2 between S5 and S6 in all four repeats, there are negatively charged sites which are important for the binding of TTX or STX (Terlau, Heineman, Stühmer, Pusch, Conti, Imoto and Numa, 1991). When the anionic glutamate or aspartate is changed to the uncharged glutamine or

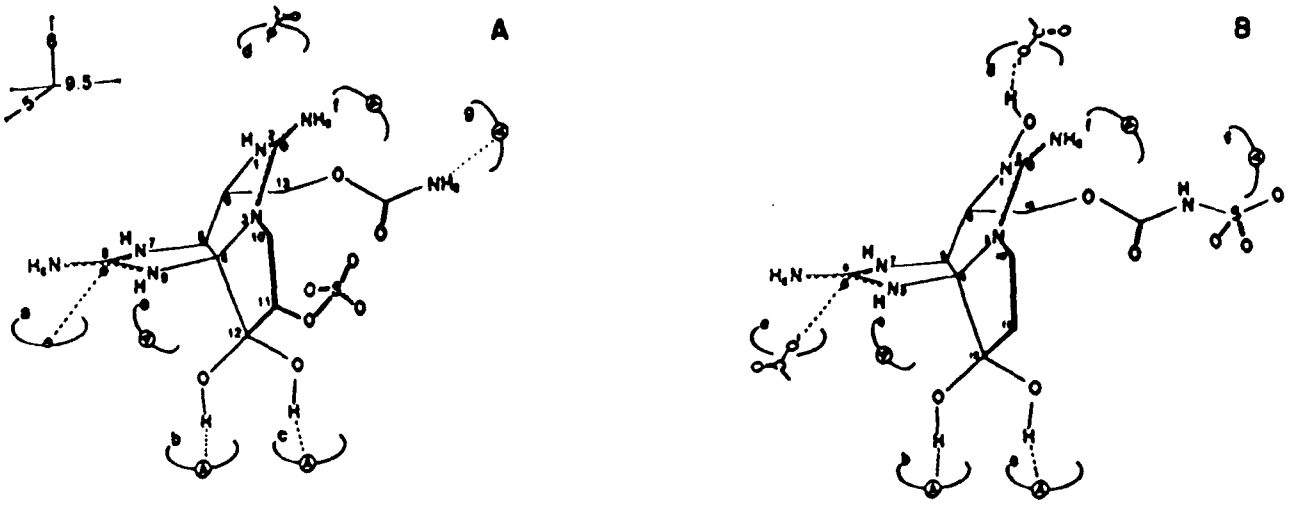


Fig. 6. Perspective view of a molecule of a gonyautoxin (left) and a sulfocarbamoyl-STX (right) in TTX/STX binding site. For gonyautoxins, sulfate group projects out between anchoring-points c and g, accounting for minor effects on potency. For sulfocarbamoyl toxins, sulfate group extend length of carbamoyl function to cause steric hinderance to toxin docking.

asparagine, TTX or STX sensitivity is lowered by 3 orders of magnitude (Noda, Suzuki, Numa and Stühmer, 1987; Terlau et al., 1991). I have attempted to model this region from residue 383 to 390, and cannot see any obvious structural features which correspond to a pocket for the TTX/STX binding site. The only notable point is that if glutamate 387 corresponded to our site a, then the carbonyl oxygen in asparagine 388 would correspond to our sites b and c. If the different repeats were arranged in a concentric model (Guy and Conti, 1990), then the TTX/STX binding site could straddle two repeats, and overhanging amino acid residues can form a pocket with the postualted anchoring points. Clearly, for a fuller identification of the binding site, marker substances based on TTX or STX would have to be made. With the headstart gained during the contract, we are continuing with such efforts (see below).

CHEMICAL MODIFICATIONS OF TETRODOTOXIN

These efforts represent the second track of this project (see Introduction). We did not attempt to work with STX in these ventures, because we could not get enough material to work with any degree of confidence. We focussed on TTX, because it is available in very pure conditions from commerical sources. Prof. H. S. Mosher also attempted to synthesize some new cyclic guanidinium compounds for this project. Dr. B. Q. Wu has embarked on, and is continuing with, synthesis of a different class of compounds which possess possible reactive groups complementary to some of the anchoring points we have postulated to exist in the TTX/STX binding site. Also, Dr. G. S. Wu is synthesizing photoactivatable derivatives of TTX, which we hope to use for locating the binding site.

Material and methods used

All the methods are standard, and the material are obtained from commercial sources. The only unique feature meriting special mention is the HPLC TTX analyzer which we constructed, based on published descriptions (Yasumoto and Michishita, 1985). The unit is based on a reverse-phase HPLC system, using postcolumn derivatization and fluorometric detection of product. It is used for both analytical and preparative purposes. The HPLC hardware is a Waters system. The column is a C-18 type (Shodex ODS5, 0.46 x 25 cm). For analytical purposes, the mobile phase consists of 0.06 N heptaflurobutyric acid in 0.001 N HOAc, adjusted to pH 5 by NH_4OH, and flowing at 0.4 ml/min. The column output is mixed with an aqueous solution of 4N NaOH at 135° C, and then cooled by a jacket of running tap water at ca. 10° C. The output is then sent to a fluorometer (Gilson, model 121), excited at 310 - 410 nm, and the emission detected at 480 - 520 nm.

For preparative purposes, the mobile phase is 20 mM sodium-1-heptasulfonate and 5 mM H_3PO_4-NH_4OH (pH = 7.2), flowing at 0.8 ml/min.

With a new column, the sensitivity of this system is ca. 10 ng of TTX. In routine use, when the column has been used, the working sensitivity range is 50 - 100 ng TTX.

11-oxoTTX

After the existence of this compound was established by the discovery of it as a natural analogue (metabolite ?) of TTX (*Khora and Yasumoto*, 1989), we began our efforts to produce it synthetically from TTX. We oxidized TTX by several methods, including hydrogen peroxide, ferrous sulfate (Fenton's reagent), and dimethylsulfoxide (Pfitzner-Moffat reaction). Although 11-oxo TTX was produced with Fenton's reagent, the subsequent purification and desalting turned out to be very difficult. The Pfitzner-Moffat reaction (using DMSO and dicyclohexyl carbodiimide, DCCI) proved to be more productive.

The synthetically produced 11-oxoTTX is identical to the natural compound chromatographically and by NMR spectrum. The one characterization which still needs to be done is a determination of the mass by FAB mass spectroscopy. Because of various difficulties in making stable 11-oxoTTX, a total of 3-5 mg were collected eventually, instead of the target of 20 mg. Of the amount made, slightly more than 1 mg of highly purified material remains, to serve both as reference and as starting material for other reactions.

11-oxoTTX can be reduced by use of borohydride. We have regenerated TTX by this method, and the product is identical to the natural TTX in both NMR spectrum and in biological actions. The NMR spectral data of these compounds are shown in Table 1.

Although the selective oxidation of the primary alcohol on C-11 has been attempted before (Chicherportiche, Balerna, Lombet, Romey and Lazdunski, 1980), no report of a successful production of 11-oxoTTX has ever been published. In the Chicherportiche paper, the product is not an aldehyde but a ketone, and has one C atom less than is in 11-oxoTTX (their Fig. 1). No attempt was made to isolate the product, nor to characterize it properly.

Table 1 ^{1}H NMR spectral data of natural TTX, 11-oxoTTX and regenerated TTX

C	Natural TTX	11-oxoTTX	Regenerated TTX
4	5.47 (d, 8.8)	5.46 (d, 9.5)	5.60 (d, 9.6)
4a	2.33 (d, 9.2)	2.27 (d, 9.4)	2.46 (d, 9.6)
5	4.28 (br s)	4.33 (br s)	4.41 (br s)
7	4.06 (br s)	4.15 (br s)	4.19 (br s)
8	4.23 (br s)	4.23 (br s)	4.36 (br s)
9	3.93 (s)	3.93 (s)	4.06 (s)
11	4.01 (d 11)	5.70	4.14 (d 8)

Right column shows carbon atom number to which proton is attached. Data are in ppm in D_2O and CD_3COOD. In parentheses, type of signal and coupling constant; d = doublet, br = broad singlet, s = singlet. Data of regenerated TTX are shifted downfield by 0.13 ppm from those of natural TTX.

Specifically labelled ^{3}HTTX

In spite of an obvious need for a radioactive TTX as a useful ligand for the sodium channel, there is really no such substance available. The only agent used in a few laboratories is a diffusely labelled TTX, in which ^{3}H is introduced by the Wilzbach method. The yield of the reaction is around 1%, and extensive work-up is necessary to clean it from other decomposition material. The specific activity is around 0.5 Ci/mol.

Another published method involves introducing a ^{3}H label into the 4 or 9 position by cleavage of the ether bond of 4,9 anhydroTTX (Grünhagen, Rack, Stampfli, Fasold and Reiter, 1981). An uncharacterized product of high radioactivity was claimed. However, that claim is suspect, because there is no intelligent chemical rationale for the purported reaction scheme.

Still another type of ligand is available, and they are based on coupling radioactive amino acids via ethylene diamine to norTTX (Chicherportiche et al., 1980). The disadvantage of these compounds is that the amino acid and the coupling agent are larger than TTX. In view of the physical dimensions of the TTX/STX binding site we have deduced (see above), it is moot whether such ligands can be really occupying the TTX/STX receptor.

By reducing 11-oxoTTX with sodium borotritide (a ^{3}H labelled sodium borohydride), we produced a specifically labelled TTX of 2931 Ci/mol. The non-radioactive product was characterized by NMR and its biological actions were tested on voltage-clamped muscle fibers. This compound has been tested on the purified sodium channel of electroplaques, and found to have all the binding properties of TTX, but with at least 60 times higher signal levels. The methods of making such a ^{3}HTTX is now the subject of a pending patent application submitted through the Research Foundation of the State University of New York. The actual production of the radioactive form was performed only once, because of various logistical problems imposed on us by the lack of support from the department and institutional administrations. The overall yield of the reaction is 6%, much lower than we had expected. However, we are optimistic that the yield can be much improved and the specific activity increased.

Synthesis of cyclic guanidinium compounds with partial resemblence to TTX

This work was undertaken in the early part of the contract in

collaboration with Prof. H. S. Mosher. At that time, our thinking was focussed on the guanidinium end of the toxin molecule and the C-9 and C-10 -OH groups as being the primary reactive points of the TTX moelcule (and equivalent groups in the STX molecule). Under a subcontract, Prof. Mosher undetook to continue some earlier work we had done together towards synthesizing new compounds with structures resembling the guanidinium end of the TTX molecule. Basically, the rationale is based on the recognition that the group of C atoms bearing the two -OH's (and -OH's on other groups in TTX) and the -NH end can be viewed as amino sugars. If such amino sugars could be converted to guanidino sugars, then the scheme could be successful. Several synthetic approaches were adopted, and a new reagent, aminoiminomethanesulfonic acid was introduced to transform an amine into a monosubstitued guanidine under mild conditions (Kim, Lin and Mosher, 1988). Although several of the desired reactions took place with the desired product, as indicated by appropriate NMR spectra, none of them could be isolated in pure form for later biological testing. (Other details in Annual Report of May 1, 1989). This aspect of the work was suspended.

CONCLUSIONS AND FUTURE DIRECTIONS

Some goals of this project have been attained, and some others are in sight. In the studies on the structure-activity relations of TTX and STX analogues, we have touched virtually all the surface active groups of the toxin molecules, and have established a surprising number of stereospecific similarites between two otherwise different molecules. As a result, we have deduced the probable shape and dimensions of the TTX/STX binding site. What remains to be resolved is the location of the binding site on the sodium channel protein. Although some speculations have been made, the desired evidence must await actual identification of the amino acid residues.

At present, no marker substance is avaiable for such identification purposes. We have taken some important steps towards developing some marker substance. The most important step is our success in making 11-oxoTTX, the first truly reactive intermediary of TTX. From 11-oxoTTX, we have made the first specifically labelled radioactive TTX of high specific activity. The availability of this ligand is essential for future attempts to locate the amino acid residues in the TTX/STX binding site. Moreover, we are also using 11-oxoTTX for the synthesis of photoactivatable derivatives of TTX. These experiments are still in a very early stage, and will need to be carried further.

The aim of producing marker substances of the TTX/STX binding site overlaps with the goal of making simpler compounds which might mimic or antagonize the actions of TTX and/or STX. Attempts at making new guanidino sugars, to interact with our anchoring points **a**, **b** and **c**, have not been successful, because the products could not be isolated. Efforts to make compounds which can interact with anchoring points **a**, **f** and **g**, appear to be more successful, but the products lack the high specificity of action on only the sodium channels. This work is continuing, and likely to be successful to some degree.

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Patent

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Personnel

C. Y. Kao	Principal Investigator
Biqi Wu	Senior Research Scientist
Guosheng Wu	Senior Research Scientist
Lie Yang	Research Scientist
Jinliang Sui	Graduate Student Assistant
Suzanne Pemrick	Consultant