Actions of Chiriquitoxin on Frog Skeletal Muscle Fibers and Implications for the Tetrodotoxin/Saxitoxin Receptor

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ABSTRACT Chiriquitoxin (CqTX) from the Costa Rican frog *Atelopus chiriquensis* differs from tetrodotoxin (TTX) only in that a glycine residue replaces a methylene hydrogen of the C-11 hydroxymethyl function. On the voltage-clamped frog skeletal muscle fiber, in addition to blocking the sodium channel and unrelated to such an action, CqTX also slows the activation of the fast potassium current in ~40% of the muscle fiber population. At pH 7.25, CqTX is as potent as TTX in blocking the sodium channel, with an ED_{50} of 3.8 nM. Its ED_{50}'s at pH 6.50 and 8.25 are 6.8 and 2.3 nM, contrasted with 3.8 and 4.3 nM for TTX. These differences are attributable to changes in the chemical states in the glycine residue. The equipotency of CqTX with TTX at pH 7.25 is explainable by an intramolecular salt bridge between the amino and carboxyl groups of the glycine function, all other surface groups in TTX and CqTX being the same. From available information on these groups and those in saxitoxin (STX), the TTX/STX binding site is deduced to be in a pocket 9.5 Å wide, 6 Å high, and 5 Å deep. The glycine residue of CqTX probably projects out of the entrance to this pocket. Such a view of the binding site could also account for the actions of STX analogues, including the C-11 sulfated gonyautoxins and the 21-sulfocarbamoyl analogues. In the gonyautoxins the sulfate groups are equivalently placed as the glycine in CqTX, whereas in the sulfocarbamoyl toxins the sulfate groups extend the carbamoyl side-chain, leading to steric hinderance to productive binding.

INTRODUCTION

Chiriquitoxin (CqTX) is an analogue of tetrodotoxin (TTX) found in the skin and eggs of *Atelopus chiriquensis*, a harlequin frog of the central highlands of Costa Rica (Kim, Brown, Mosher, and Fuhrman, 1975; Pavelka, Kim, and Mosher, 1977). In

those earlier studies, CqTX was known to retain the basic structure of TTX but to differ in the C-11 position by having a large substituent. In mouse lethality assays (Kim et al., 1975) and on the isolated frog skeletal muscle fiber it had the same specificity of action and potency as TTX (Kao, Yeoh, Goldfinger, Fuhrman, and Mosher, 1981). However, insufficient material was then available for a full clarification of the chemical structure or a definitive study of its actions on specific ionic channels. Repeated attempts to collect more material were unsuccessful until June 1988. Using current separation methods and a high pressure liquid chromatography TTX analyzer, CqTX was isolated and purified. Its structure has also been determined (Yotsu, Yasumoto, Kim, Naoki, and Kao, 1990).

We report here our studies of the actions of this new batch of CqTX on the functions of the sodium and potassium channels of the voltage-clamped, isolated frog skeletal muscle fibers. The significance of these studies extends beyond a mere comparison of CqTX with TTX. In recent years the structure–activity relations of some analogues of both TTX and saxitoxin (STX) have been clarified to a point where most active surface groups of these toxin molecules have been identified (for summary, see Kao, 1986; also Hu and Kao, 1991; Yang, Kao, and Yasumoto, 1992a; Yang, Kao, and Oshima, 1992b). Because the actions of CqTX are unique among TTX analogues, knowledge of its structure and actions permits us to draw some inferences on the probable physical dimensions of the TTX/STX binding site, and also to speculate on its possible location.

MATERIALS AND METHODS

CqTX was isolated from the skin of A. chiriquensis and its structure was determined by various spectroscopic methods, including mass spectrometry, ^{1}H- and ^{13}C-NMR spectrometry, and infrared spectrometry. Some details in stereochemical configurations were confirmed by derivatization experiments. The chemical studies can be found in Yotsu et al. (1990). The structure of CqTX is shown in Fig. 1. In comparison with that of TTX, the only difference is that a methylene hydrogen of the C-11 hydroxymethyl group of TTX is replaced by a glycine function in CqTX. Stated differently, CqTX has all the structural features of TTX, except that it has an additional –CH(NH$_2$)-COOH attached to the C-11 position.

The biological studies were performed on short segments of single isolated frog skeletal muscle fibers in a Vaseline gap voltage-clamp method (Hille and Campbell, 1976). Details of the procedures and methods of data acquisition and analysis can be found in other papers (Hu and Kao, 1991; Yang et al., 1992a). The actions of CqTX on both I_{Na} and I_K were studied.

Muscle fibers were held at −90 mV and depolarized by step increments until ~20 mV beyond the E_{Na}. To reduce errors attributable to series resistance, experiments were conducted in a bath solution containing only 44 mM Na$^+$. As a result, the E_{Na} was at +20 to +30 mV. The lower external Na$^+$ concentration does not alter the ED$_{50}$ of the toxin (Hu and Kao, 1991). Current traces showing maximum I_{Na} and at E_{Na} were selected for detailed comparisons. The current at E_{Na} was I_K, and was used in previous studies of other analogues of TTX and STX primarily to assess the health of the fiber. However, CqTX also slows the activation of the fast I_K. Thus, the current records taken at E_{Na} were also useful for determining the rate of activation of I_K under various conditions. Time constants of the activation of the fast I_K were determined on the same fiber in the control and toxin-affected states (see Results). t tests for significance of differences of these data were based on pair comparisons.

The maximum I_{Na}'s in toxin-affected conditions were compared with those in control (no toxin) states, and the normalized relation (I'_{Na}/I_{Na}) was used for dose–response curves. The

small amount of I_K present at this voltage does not affect the ED_{50} determinations (Yang et al., 1992a). Least-squares linear regression lines were fitted to data in Hill plots of log $(1-P)/P$ $(P = I'_{Na}/I_{Na})$ vs. log toxin concentration. Standard errors of estimate were also obtained. ED_{50}'s and their standard errors were taken at $(1-P)/P = 1$ (Hu and Kao, 1991). t tests for significance of differences in the sodium channel blocking effect were based on unpaired group comparisons.

RESULTS

General Description

Although it is generally held that TTX affects only the sodium channel, recent studies on two analogues of TTX modified in the C-6 position indicate that there may be

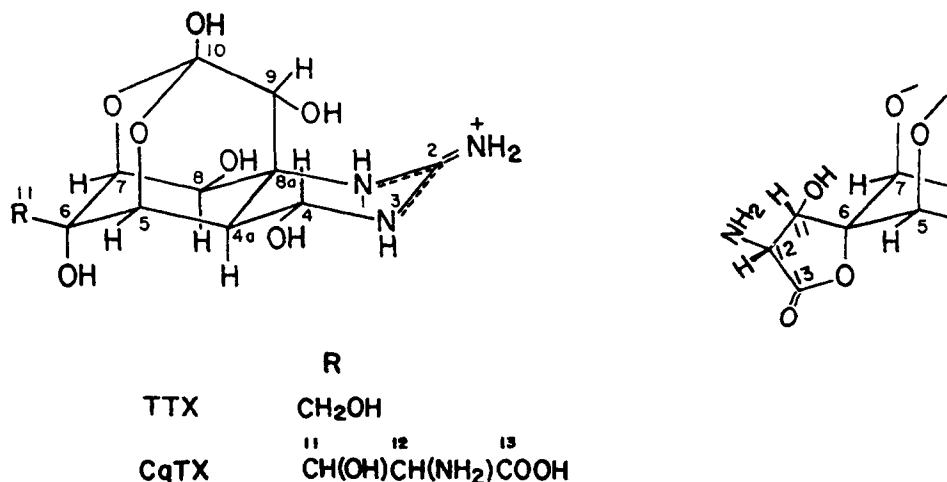|	R
TTX	CH₂OH
CqTX	CH(OH)CH(NH₂)COOH

FIGURE 1.   Chemical structure of CqTX compared with that of TTX. Note that CqTX contains all the features of TTX except that it has an additional glycine moiety on C-11. In TTX, and probably also in CqTX, the C-10 −OH group has a pKa of 8.8. In CqTX, the C-12 amino group has a pKa of ~9 and the C-13 carboxyl group has a pKa of ~2. These properties probably influence the sodium channel blocking action of CqTX at various pH's.

some exceptions (Yang et al., 1992a). In about half of the muscle fibers from *Rana temporaria*, TTX and 11-deoxyTTX, which retains the general configuration of the groups around C-6, significantly slow the activation of the fast I_K. 6-*epi*TTX, in which the -OH and the hydroxymethyl group of C-11 are in an epimeric configuration from that in TTX, does not cause significant slowing of the I_K. Fig. 1 shows that CqTX shares the general structural features of TTX at the C-6 position; Fig. 2 shows that in some fibers it also shares the effect of slowing the fast I_K. In such fibers, while I_{Na} is markedly reduced and eventually blocked, the fast I_K is also appreciably slowed. At 10 ms of a depolarizing step the I_K can be reduced by variable amounts, but at 20 ms it has usually reached the same amplitude as in the control state.

Effect of CqTX on Potassium Current

Because I_K is rather variable from fiber to fiber and even in the same fiber at different times, we used an arbitrary criterion to determine whether a toxin slowed the fast I_K. The time constants (τ) of the first 2 ms of the I_K of the pretoxin and recovered states were separately determined and then averaged. The averaged control τ was compared with that of the toxin-affected state. If the latter exceeded the former by 10%, we selected that fiber as having had its I_K slowed. Table I gives a summary of these comparisons. Of 36 fibers used for this study, 14 showed some slowing of the I_K. At 1
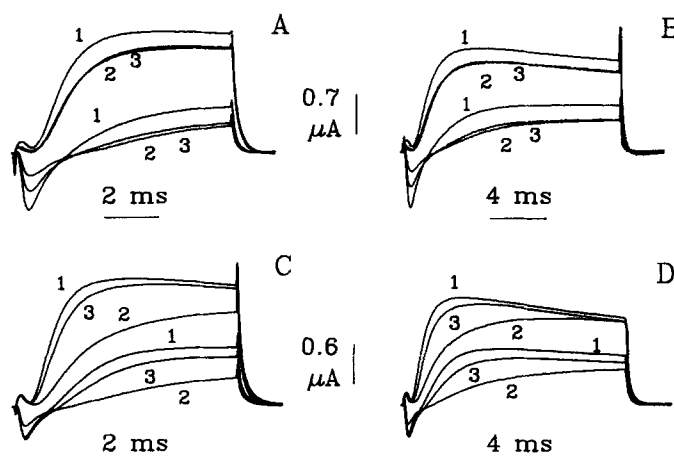

FIGURE 2.   Effect of CqTX on I_{Na} and I_K of frog skeletal muscle fiber. Two different fibers (*A* and *B* from one; *C* and *D* from the other) in bath medium containing 44 mM Na^+. Holding potential −90 mV. Currents produced by two voltage steps are shown, one showing maximum inward I_{Na} and the other, at E_{Na}, showing I_K. In all panels, traces marked *1* denote the control state. In *A* and *B*, traces marked *2* denote responses to 4 nM CqTX, and traces marked *3* denote responses to 8 nM CqTX. In *C* and *D*, traces marked *2* denote responses to 2 nM CqTX, and traces marked *3* denote the recovered state. *A* and *C* are at fast sweep; total duration is 10 ms. *B* and *D* are at slower sweep; total duration is 20 ms. In addition to reducing I_{Na}, CqTX can also slow fast I_K without appreciably reducing total I_K. This feature is best shown in *C* and *D*, where the seemingly reduced I_K at 10 ms (*C*) is no different from the control state at 20 ms (*D*). The effect of CqTX is nearly fully reversible (*C* and *D*). In *A* and *B*, increasing concentrations of CqTX increased reduction of I_{Na}, but had no similar effect on the slowing of I_K. See text for additional reasons why the slowing of I_K is independent of block of I_{Na}.

nM CqTX generally did not cause any significant slowing of the I_K, even though it could reduce I_{Na} by ~20% (see Fig. 5). In 2–40-nM concentrations CqTX often caused some slowing, but the degree of slowing was not dependent on the concentration (Fig. 2, *A* and *B*). In those fibers in which slowing occurred, I_K usually recovered when CqTX was washed out. On subsequent application of another dose of CqTX, usually of a higher concentration, a lesser degree of slowing of I_K often occurred.

No influence of TTX. At pH 7.25, the ED_{50}'s of CqTX and TTX are the same, and like TTX, CqTX does not affect the onset or decay of the I_{Na} (see below). These

TABLE I

Effect of CqTX on the Rate of Activation of the Fast Potassium Current

Concentration	Fibers	Time constant (τ) of fast I_K		P
		Control	Toxin	
nM		*ms*		
1	12	0.61 ± 0.02	0.62 ± 0.02	> 0.05
2	10	0.61 ± 0.02	0.69 ± 0.04	< 0.05
4	22	0.60 ± 0.01	0.67 ± 0.02	< 0.05
8	8	0.63 ± 0.02	0.73 ± 0.02	< 0.05
16	8	0.45 ± 0.02	0.56 ± 0.04	< 0.05

All values are means ± SEM, with Fibers referring to the number of fibers used. Altogether 36 fibers were used, some of them for multiple doses of CqTX. Most fibers used for 16 nM came from frogs in May; fibers for all others came from frogs in July–September. P denotes P value in paired t test.

observations suggest that the affinity of the two toxins for the binding site are similar. The following experiments on the interaction between CqTX and TTX were conducted with concentrations of TTX 10–100-fold higher than that of CqTX to skew the mass-action equilibrium in favor of TTX. Even under these conditions, the effect of CqTX on I_K was not influenced by the presence of TTX, whether TTX was applied before or after CqTX.

Fig. 3 is an example of a high concentration of TTX that did not prevent CqTX from slowing the fast I_K. The I_{Na} was fully blocked by 40 nM of TTX, and the fast I_K
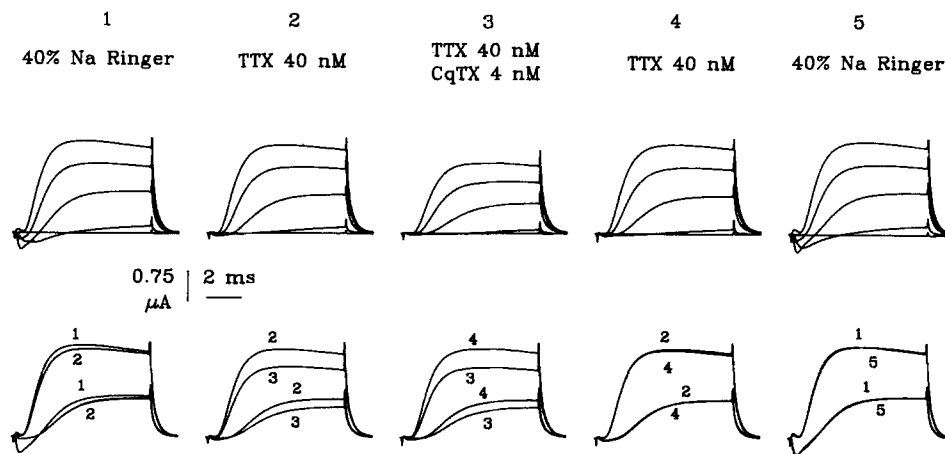

FIGURE 3.   Prior action of TTX has no effect on the slowing of I_K by CqTX. (*Top row*) Current traces in various conditions sequentially as marked on top. Holding potential −90 mV; currents result from step voltages of −65, −40, −15, −10, and +35 mV. (*Bottom row*) Superimposed current tracings show different effects, with the number of the trace referring to the conditions marked above the top row. 40 nM TTX (panel 2) fully blocked I_{Na} but only slightly and insignificantly affected fast I_K. An additional CqTX (panel 3) markedly slowed fast I_K. This effect was reversible (panel 4). When TTX was removed (panel 5), both I_{Na} and I_K reversed fully.

was slightly slowed (panels *1* and *2*). When 4 nM of CqTX was applied, no additional effects on I_{Na} could be detected. The fast I_K was appreciably slowed, and the I_K at 10 ms was substantially lower than before (panels *2* and *3*). When CqTX was removed, the rate of activation of I_K recovered to that in TTX (panel *4*; compare with panel *2*). When TTX was also removed, both I_{Na} and I_K fully recovered (panel *5*; compare with panel *1*). This absence of influence of a prior application of TTX was demonstrated just as clearly in another fiber. Because the fibers were in very good condition, and the effects were clear, we did not repeat this sequence on more fibers.

High concentrations of TTX did not reverse the effect of CqTX on the I_K. In 10 fibers, I_{Na} was reduced by an ED_{50} dose (4 nM) of CqTX. In every fiber the fast I_K was appreciably slowed and the I_K at 10 ms was reduced. Then a high concentration of TTX (100 nM in four fibers and 400 nM in six fibers) was applied. While I_{Na} now became fully blocked, no further change in the I_K was seen. In every case the toxins were allowed to act for at least 8 min to reach steady state. Since the effect of TTX on I_K was usually less than that of CqTX, if the high concentration of TTX had reversed the effects of CqTX on I_K, one might have expected some recovery. Instead, the I_K at 10 ms in CqTX was 1.07 ± 0.11 μA (mean ± SEM of 10 fibers), compared with 0.99 ± 0.11 μA in a combination of CqTX and TTX.

No influence of $[Na^+]_o$ or of direction of I_{Na}. In addition to the apparently independent actions of TTX and CqTX on the I_K, other evidence shows that the effect of CqTX on the I_K is unrelated to functions of the sodium channel. In a Na^+-free bathing solution, CqTX caused as much slowing of the I_K as it did in 44 mM Na^+. Moreover, slowing of I_K was seen whether the I_{Na} was inward or outward.

The slowing of the fast I_K by CqTX is qualitatively similar to that seen with TTX and 11-deoxyTTX in about half of the muscle fiber population (Yang et al., 1992a). From all the above observations, we conclude that the slowing of the fast I_K is not related to the sodium channel blocking action of these toxin molecules, but that it is somehow related to the configurations of the C-6 groups common to all three. See Discussion for further comments.

Effect of CqTX on Sodium Current

Similar mechanism to that of TTX. Because CqTX slows the I_K in an appreciable fraction of the fiber population, a question arises as to whether it might have slowed the inactivation of I_{Na} such that some overlap of currents might be responsible for slowing the I_K. To examine this problem, experiments were performed on fibers in which the I_K had been blocked by applying Cs^+ to the end pools and allowing it to diffuse into the nodal area. Fig. 4 shows a typical example of such an experiment. In the control state, only the inward I_{Na} was seen. Upon action of 4 nM of CqTX the I_{Na} was reduced but there was no prolongation of the decay phase. When the toxin-affected I_{Na} is scaled to match the peak of the control I_{Na}, the currents are superimposed, with no detectable differences in either the onset or decay kinetics. The same results were seen in nine other fibers. Thus, the mechanism of action of CqTX on I_{Na} is apparently similar to that of TTX.

Dose–response relation. Fig. 5 shows the dose–response relation of CqTX on the I_{Na} at pH 7.25. As in the case of TTX, the data fit well a bimolecular reaction scheme where one toxin molecule interacted with one receptor site. This point is reinforced
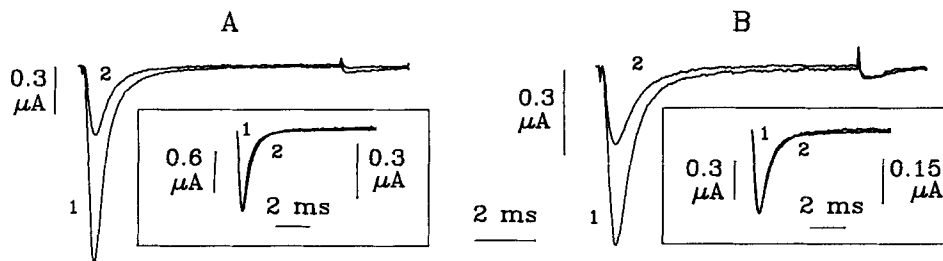
FIGURE 4.   CqTX does not affect the rate of inactivation of I_{Na}. Two different fibers, both with I_K blocked by Cs^+. Holding potential -90 mV; step voltages to $+10$ mV. Traces marked *1* are maximum I_{Na} in control state; traces marked *2* are maximum I_{Na} in 4 nM CqTX (ED_{50}). (*Insets*) Traces *2* up-scaled to have peaks coincide with those of traces *1*, showing the absence of effect of CqTX on the kinetics of onset or decay of I_{Na}.

by the slope of 1.1 in the Hill plot where $\log (1-P)/P$ ($P = I'_{Na}/I_{Na}$) is plotted against log toxin concentration. The ED_{50} of CqTX of 3.8 nM is not statistically significantly different from that of TTX, which is 4.1 nM (see Yang et al., 1992*a*).

Effect of pH on the dose–response relation. Because various toxin molecules contain ionizable groups, alterations in pH have been useful in correlating chemical changes with potency differences. Also, because the actions of CqTX are rather similar to those of TTX, it is instructive to examine the influence of pH on the actions of CqTX in comparison with those of TTX. In TTX, the C-10 $-OH$ has a pKa of 8.8. It is more potent at pH 7.25 than at pH 8.25, with a relative potency that agrees well with the relative abundance of the protonated species of the C-10 hydroxyl group (Hu and Kao, 1991). Although there is no direct information on the property of the C-10
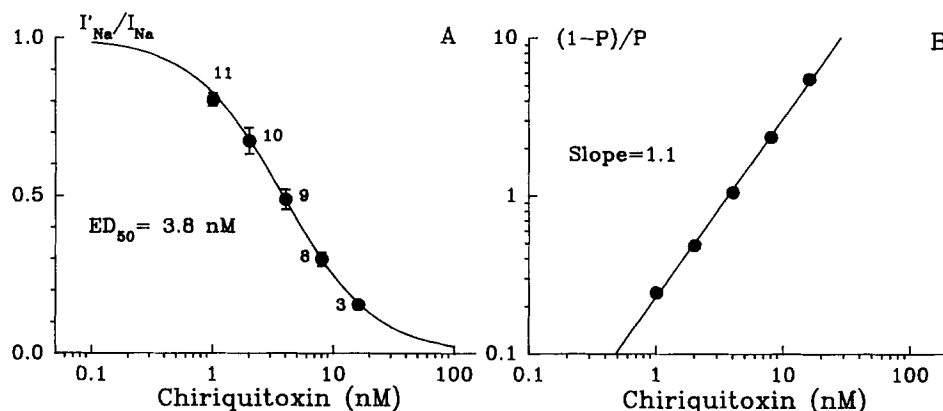

FIGURE 5.   Dose–response relation of CqTX in blocking I_{Na} at pH 7.25. (*A*) Relative residual I_{Na} vs. log concentration of CqTX. The solid curve represents a bimolecular reaction scheme. The number attached to each point denotes the number of individual fibers from which data are obtained. Symbols denote mean values and vertical bars denote 1 SEM if larger than the symbol. The ED_{50} of 3.8 nM is not statistically different from that of 4.1 nM TTX (see text). (*B*) Hill plot, where $P = I'_{Na}/I_{Na}$. ED_{50} is determined when $(1-P)/P = 1$. A slope of 1.1 is consistent with a bimolecular reaction.

group in CqTX, because of similarities in chemical structures to TTX one might assume the pKa to be about the same as that of TTX. On this basis, one might expect CqTX to be similarly more potent at pH 7.25 than at 8.25.

Fig. 6 shows that, in actuality, CqTX is appreciably more potent at pH 8.25 (ED_{50}, 2.3 nM) than it is at pH 7.25 (ED_{50}, 3.8 nM; $P = 0.01$). Also, at pH 8.25 CqTX is significantly more potent than TTX (ED_{50}, 4.3 nM; $P < 0.001$, group comparison with data in Hu and Kao, 1991). These differences are probably not due to differences in protonation of the C-10 group, but are attributable to dissociable groups in the glycine function of CqTX.

At pH 6.50, increments in the protonated species of the C-10 group over that present at pH 7.25 are small, and the potency of TTX is slightly lower (ED_{50}, 3.8 nM), possibly because of a decrease in the density of surface negative charges (see discussion in Hu and Kao, 1991). For CqTX, however, the potency at pH 6.50 (ED_{50},
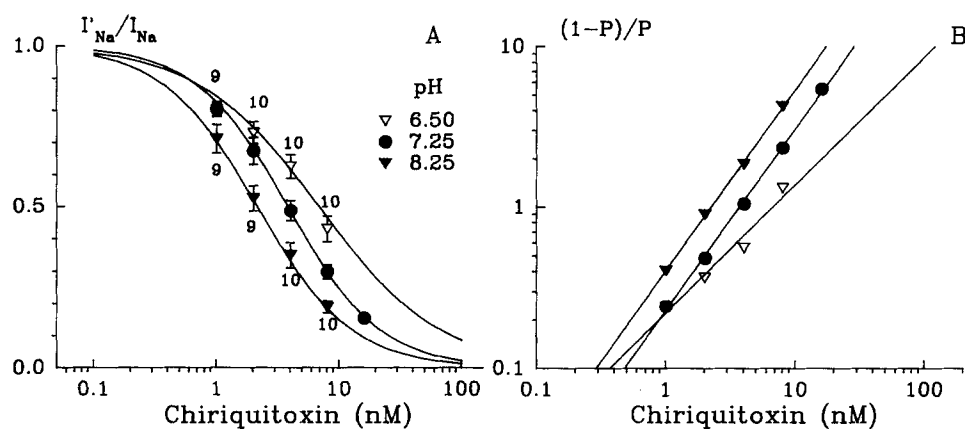

FIGURE 6.   Influence of pH on the dose–response relation of CqTX in blocking I_{Na}. Conventions are similar to those in Fig. 5, from which data at pH 7.25 are taken. Slopes in the Hill plot are 0.9, 1.1, and 1.1, and ED_{50}'s are 6.8, 3.8, and 2.3 nM for pH 6.50, 7.25, and 8.25, respectively. See text for explanations of different potencies.

6.8 nM) is significantly less than that at pH 7.25 (ED_{50}, 3.8 nM; $P = 0.05$). Again, the reason for the difference must be sought in changes in the states of the glycine function.

DISCUSSION

Until the discovery of CqTX in 1975, no analogue or derivative of TTX was known to have anything but a small fraction of the potency of TTX. Thus, the chemical mechanism by which TTX blocked the sodium channel remained largely in the realm of speculation. CqTX was the first analogue of TTX to have substantial potency, and it rekindled studies of the structure–activity relations of the TTX family of molecules. Coincidentally, natural analogues of STX were also being discovered. The availability of analogues and derivatives of both TTX and STX fostered a re-examination of the

chemical mechanism of the sodium channel blockade. As shall become clear presently, because of its structure and its actions CqTX is of special importance in helping to consolidate a conception of the probable physical dimensions of the TTX/STX binding site.

Relation to Previous Work and Effect on I_K

The first study of the cellular actions of CqTX was made at a time when no voltage-clamp method capable of controlling the rapid sodium current was available for work on the frog skeletal muscle fiber (Kao and Yeoh, 1977). The maximum dV/dt of the action potential was used for an indirect assessment of sodium channel function, and constant current methods were used to study potassium channel function. In those experiments on muscle fibers of *Rana pipiens*, slowed repolarization of the action potential and a reduced level of delayed rectification were seen in some fibers at the same time that spike generation was impaired (Kao and Yeoh, 1977; Kao et al., 1981). Based on changes in the maximum dV/dt of the action potential, CqTX was judged to be as potent as TTX in blocking the sodium channel, but CqTX was also thought to have some effects on the potassium channel. The present results confirm that CqTX does have some effect on the potassium channel, but show that the effect is not a simple blockage as first believed. It is a more complex interference with the activation kinetics of the fast I_K and occurs in only about half of the population of muscle fibers. Because of variability, we do not have enough information at this time to fully explain that effect. All evidence in hand indicates that the effect on I_K is unrelated to the sodium channel blocking action. The effect on I_K is also seen with TTX and 11-deoxyTTX, but not with 6-*epi*TTX or neoSTX (Yang et al., 1992a). The common structural feature shared by CqTX, TTX, and 11-deoxyTTX is the configuration of the groups on C-6. Perhaps some potassium channel molecules, possessing some homology with the sodium channel (eg., Tempel, Papazian, Schwarz, Jan, and Jan, 1987) have a binding site for those groups.

Effect on I_{Na} and Influence of pH

In the sodium channel blocking effect, CqTX is unusual among TTX analogues in possessing a high degree of potency. With the exception of 11-oxoTTX (Wu, Yang, Kao, Yotsu, and Yasumoto, 1991), all other TTX analogues studied have varying degrees of reduced potency as compared with that of TTX, but CqTX is equally as potent as TTX. However, the pH influence on the potency of CqTX is rather different from that of TTX. Based on known chemical properties of CqTX (Fig. 1) we offer the following explanations for these differences:

In the glycine moiety of CqTX, the C-12 amino group has a pKa of ~9, and the terminal (C-13) carboxyl group has a pKa of ~2 (Yotsu et al., 1990). At pH 7.25 the C-12 amino group is mostly protonated, whereas the C-13 carboxyl group is deprotonated. An intramolecular salt bridge between those vicinal groups could largely remove them from any charge interactions with receptor groups in the sodium channel. As all the other groups in CqTX are the same as those in TTX, including the C-11 -OH group that forms a hydrogen bond with a receptor site (Yang et al., 1992a), the equipotency of CqTX and TTX is not difficult to understand,

provided a satisfactory explanation can be found for the absence of steric influences of the glycine moiety (see below).

Why is the pH influence on potency so different between TTX and CqTX? For TTX and STX between pH 6.50 and 8.25, differences in the chemical states of the toxin molecules are the most important factors in influencing potency (Hu and Kao, 1991). In TTX, changes in pH between 6.50 and 8.25 primarily affect the protonation of the C-10 -OH group, a condition that probably pertains to CqTX as well. In CqTX, however, the glycine function has two additional ionizable groups sensitive to pH changes. Under acidic conditions, the C-13 carboxyl group in CqTX tends to form an intramolecular lactone with the C-6 -OH (Yotsu et al., 1990; see also Fig. 1). Although pH 6.50 is less drastic than the conditions of those chemical studies, there may be a change in the equilibrium between CqTX and CqTX-lactone, shifting more toward the lactone form than it does at pH 7.25. In the lactone form, the C-6 -OH is lost and the C-11 −OH is fixed, probably in a different position from that in TTX. As both C-6 and C-11 −OHs participate in hydrogen bonds with site points in the receptor (Yang et al., 1992a), a shift in the equilibrium toward the lactone could account for the reduced potency of CqTX at pH 6.50.

At pH 8.25 TTX is less potent than at pH 7.25, but CqTX is appreciably more potent. Whereas the ED_{50}'s of TTX and CqTX at pH 7.25 are not significantly different, those at pH 8.25 are. These differences can be attributed to a decline in the protonated species of the C-12 amino group, a weakening of the intramolecular salt bridge with the terminal carboxyl, and an increasing role of the C-12 amino group in hydrogen bonding with receptor groups. However, it should be recognized that when the ED_{50} for reducing I_{Na} is taken as the K_d of the toxin receptor interaction, the difference in the ED_{50}'s at pH 7.25 and 8.25 reflects a difference in the Gibbs free energy of binding ($\Delta G = -RT \ln K_d$) of only ~ 1.5 kJ/mol.

Implications for the TTX/STX Receptor

In addition to electronic factors, steric factors also influence the docking orientation of a toxin molecule onto its receptor. In earlier work, three stereospecifically similar groups that participate in biological activity have been identified in the TTX and STX molecules (summarized in Kao, 1986): the 1,2,3 guanidinium of TTX and the 7,8,9 guanidinium of STX, the C-9 and C-10 hydroxyls of TTX, and the C-12 hydroxyls of STX. The N-1 hydroxyl of neoSTX was found to have an influence in binding to the receptor under some conditions (Hu and Kao, 1991). More recently, two analogues each of TTX (6-epiTTX and 11-deoxyTTX) and STX (deoxydecarbamoylSTX and decarbamoylneoSTX) have provided significant new information on the active groups in the TTX and STX molecules (Yang et al., 1992a, b). Briefly, the C-6 end of the TTX molecule was found to be active; also, when the active guanidinium groups and the pair of −OHs (see above) of the two toxin molecules were aligned, the carbonyl oxygen of STX corresponded to the C-6 −OH of TTX, while the amino group in the carbamoyl function of STX corresponded to the C-11 −OH of TTX. Since these active groups present themselves on all surfaces of the toxin molecules, we deduced that the TTX/STX binding site must be located in a crevice or a fold of the sodium channel protein.

Combining the present results with the earlier deduction, the TTX/STX binding

site can be viewed as being a pocket ~9.5 Å wide, 6 Å high, and 5 Å deep (Fig. 7; also Kao and Yang, 1992). In it are seven site points (designated sites *a–g*), which interact with various surface groups of either the TTX or the STX (and neoSTX) molecule. The CqTX molecule could have an additional reactive site point complementary to the C-12 $-NH_2$. However, the most impressive feature of CqTX is that in spite of the additional steric volume provided by the glycine function, it is very similar to TTX in potency. One possible explanation is that when bound productively to the receptor, the CqTX molecule is so oriented that the glycine function faces, and possibly protrudes out of, the entrance to the binding site, hence the minor contributions of the additional reactive groups on CqTX.

An attractive aspect of such a view of the binding site is that it can also explain two features regarding STX analogues that have not been satisfactorily accounted for before. Since TTX and STX bind to the same receptor, this ability to accommodate a group of chemically different compounds adds to the general plausibility of the proposed view. Among STX analogues there are two sulfur-containing subfamilies, the C-11 (numbering system for STX) gonyautoxins, which contain large sulfate substituents on the C-11 position, and the 21-sulfocarbomoyl toxins, which contain a sulfate end on the carbomoyl side-chain (for summary, see Oshima, Sugino, and Yasumoto, 1989). For gonyautoxins, the $-OSO_3$ substituents on C-11 add both large steric volumes and strongly negative charges rather close to the critical C-12 gem-diols, yet their potencies are only minimally reduced (Kao, Kao, James-Kracke, Koehn, Wichmann, and Schnoes, 1985). On the other hand, the bulk and charges of the $-OSO_3$ groups in the sulfocarbomyl toxins at the end of a side-chain profoundly lower the potency. Both of these features can be explained by the proposed view of the TTX/STX receptor as situated in a pocket.

Molecular modeling shows that when the active guanidinium groups of TTX and STX are aligned, as are the C-9 and C-10 $-OHs$ of TTX with the C-12 $-OHs$ of STX, the C-11 sulfate groups of the gonyautoxins are stereochemically placed much like the glycine moiety of CqTX (Fig. 7). Perhaps their minimal influence on potency is also to be attributed to their projecting out of the entrance to the binding site pocket and being removed from nearby reactive site points. On the other hand, the sulfate groups on the 21-sulfocarbamoyl toxins extend the carbamoyl side-chain so much that steric hinderance interferes with the entry of the toxin into the binding site pocket. Understandably, when those sulfate groups are cleaved through acid hydrolysis (Shimizu, Kobayashi, Genenah, and Oshima, 1984), the product toxins become considerably more active because they are now freed of the steric interference and can productively occupy the binding site to block the sodium channel.

Observations on mutated sodium channels of rat brain suggest that negatively charged amino acid residues in short segments 2 between S5 and S6, at positions 384 and 387 of repeat I, and equivalently placed residues in repeats II, III, and IV strongly influence the actions of TTX and STX (Noda, Suzuki, Numa, and Stühmer, 1989; Kontis and Goldin, 1991; Terlau, Heinemann, Stühmer, Pusch, Conti, Imoto, and Numa, 1991). Mutating the anionic glutamate or aspartate to the uncharged glutamine or asparagine raises the ED_{50} for blocking I_{Na} by more than three orders of magnitude. We speculated that if glutamate 387 corresponded to our binding site *a*, then the carbonyl oxygen of asparagine 388 could represent our binding sites *b* and *c*
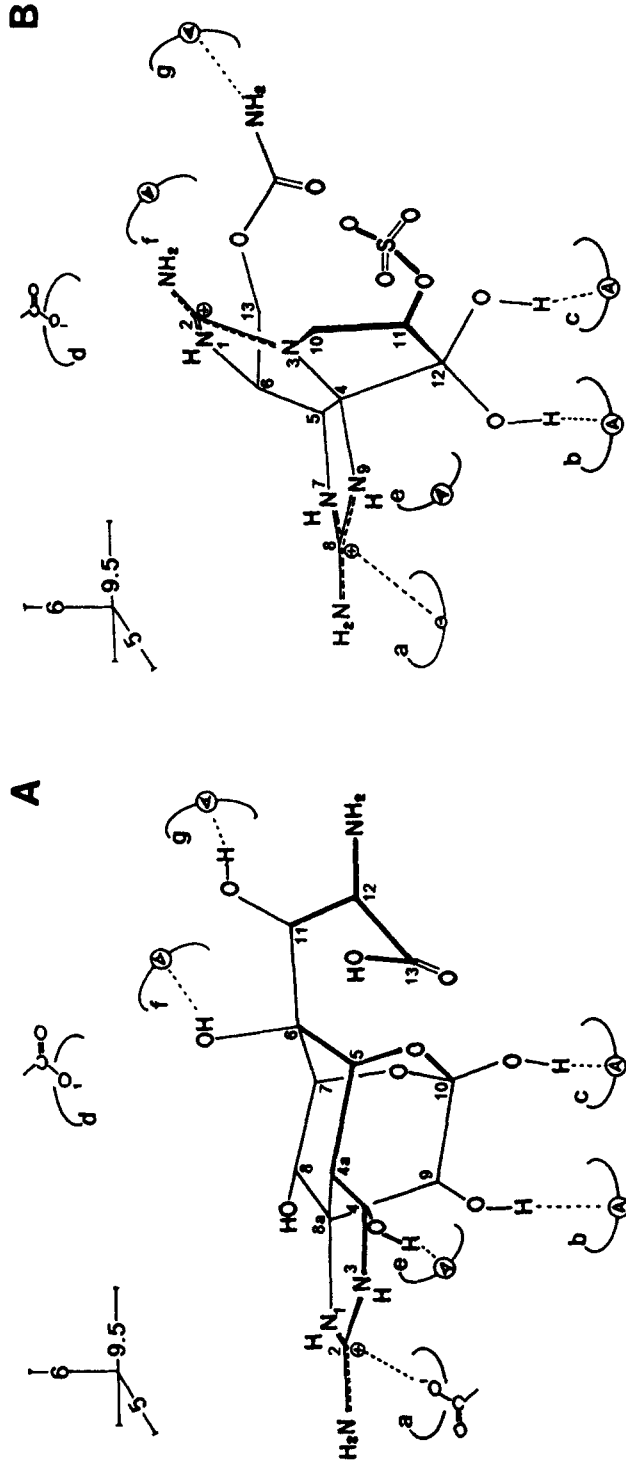
FIGURE 7. Possible dimensions and anchoring site points in the TTX/STX binding site. The binding site is in a pocket, being viewed head-on through the entrance of the pocket. Identified anchoring points in the binding site (sites a–g) have been shown to be complementary to specific groups in TTX, STX, and neoSTX molecules (see references in text). TTX and STX share site points a, b, c, f, and g; neoSTX has an additional site point d. Site point e is complementary to the C-4 —OH of TTX. (A) A molecule of CqTX is shown in perspective view in presumed correct docking orientation, with the glycine moiety protruding out toward the entrance. In spite of the large steric volume and additional charges in glycine, CqTX shares all the structural features of TTX and hence all the complementary site points. Its equipotency with TTX is due to glycine projecting out of the pocket of reactive sites. (B) A molecule of a gonyautoxin, sulfated on the C-11 of STX, is shown in perspective view, occupying the same binding sites. The sulfate group also projects out of the pocket, accounting for the relatively minor effect it has on the potency of STX. In 21-sulfocarbamoylSTX (not shown), the sulfate group on the end of the carbamoyl side-chain would cause steric hinderance for the toxin molecule to occupy the binding site; hence its weakness. When such end sulfate groups are hydrolyzed off in acid conditions, the steric hinderance is removed and toxin potency increases, sometimes many times.

(Yang et al., 1992a, b). However, the situation is complicated by the contradictory observations that mutants containing arginine 388 showed no significant difference in sensitivity to TTX and STX (Terlau et al., 1991), whereas in the sodium channel of rat cardiac myocyte TTX/STX insensitivity is associated with the presence of arginine at 388 (Rogart, Cribbs, Muglia, Kephart, and Kaiser, 1989). Not unexpectedly, molecular models of short segments of amino acid residues from 383 to 390 do not show any folds that could envelop the TTX or the STX molecule. However, in the concentric model proposed by Guy and Conti (1991), the TTX/STX binding site could straddle two repeats, which could contain the appropriate residues to provide the crevice that serves as the toxin binding site. Experimental identification of the binding site would require specific marker compounds that need to be made. However, a successful identification of the binding site could add much to a better understanding of the structure and function of the sodium channel.

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