Action of Derivatives of μ-Conotoxin GIIIA on Sodium Channels. Single Amino Acid Substitutions in the Toxin Separately Affect Association and Dissociation Rates†

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ABSTRACT: We have studied binding and block of sodium channels by 12 derivatives of the 22-residue peptide μ-conotoxin GIIIA (μ-CTX) in which single amino acids were substituted as follows: Arg or Lys by Gln, Gln-18 by Lys, Asp by Asn, and HO-Pro by Pro. Derivatives were synthesized as described by Becker et al. [(1989) *Eur. J. Biochem. 185*, 79]. Binding was measured by displacement of labeled saxitoxin from eel electroplax membranes (100 mM choline chloride, 10 mM HEPES–NaOH, pH 7.4). Blocking kinetics were evaluated from steady-state, single-channel recordings from rat skeletal muscle sodium channels incorporated into planar, neutral phospholipid/decane bilayers (200 mM NaCl, 10 mM HEPES–NaOH, pH 7.0). Blocking events generally appeared as periods of seconds to minutes in which current through the single channel was completely eliminated. A notable exception was seen for the substitution Arg-13-Gln for which the "blocked" events showed measurable conductances of about 20–40% of the open state. The substitution of Arg-13 reduced binding to electroplax membranes to undetectable levels and increased the apparent dissociation constant determined for skeletal muscle channels by >80-fold compared with the native peptide. Other substitutions caused smaller decreases in affinity. The decreased potency of the toxin derivatives resulted both from increases in the rates of dissociation from the channel, and from decreases in association rates. Our data support the suggestion by Sato et al. [(1991) *J. Biol. Chem. 265*, 16989] that Arg-13 associates intimately with the binding site on the channel. In addition, our results suggest that certain residues affect almost exclusively the approach and docking of the toxin with its binding site, others appear to be important only to the strength of the association once binding has taken place, and yet others affect both.

The μ-conotoxins, a group of toxins from the venom of the piscivorous sea snail *Conus geographus* (Gray et al., 1988), are small peptides which consist of 22 amino acid residues and contain 3 disulfide bonds. The pattern of the 6 cysteine residues in the linear sequence of the μ-conotoxins is a constant feature, distinguishing them from other conotoxins. The μ-conotoxins block ion flux through voltage-dependent sodium channels in skeletal muscle and in electric organs, but not in brain, heart, and peripheral nerve tissue (Spence et al., 1978; Cruz et al., 1985). In the former tissues they bind competitively against the sodium channel blockers saxitoxin and tetrodotoxin (Moczydlowski et al., 1986a; Sato et al., 1983; Yanagawa et al., 1987). At the single-channel level, block by TTX,[1] STX, or the μ-conotoxins is an all-or-none event resulting from stoichiometric 1:1 binding of toxin molecules to the channel.

Beside the so-called binding site I, at which the μ-conotoxins, TTX, and STX compete, there are at least four other discrete toxin binding sites on the sodium channel (Catterall, 1988; Schweitz et al., 1985; Becker & Gordon, 1991). The identification of these binding sites has an important function for testing current models of sodium channel structure (Greenblatt et al., 1985; Guy & Seetharamulu, 1986; Guy & Conti, 1990) which have emerged from molecular biological studies (Noda et al., 1986). For example, binding site III has been traced to a 14-kDa peptide fragment of the channel protein using a photoaffinity derivative of an α-scorpion toxin (Catterall, 1980). Efforts to follow the same approach using photoaffinity derivatives of tetrodotoxin and saxitoxin have failed or yielded inconsistent results (Nakayama et al., 1986). Therefore, derivatives of μ-conotoxins may be unique and valuable tools for the identification of binding site I, and for probing the three-dimensional structure of the sodium channel. Several derivatives of μ-conotoxin GIIIA have been prepared by nonspecific insertion of a derivatizing reagent into the natural toxin, but no specific binding of these derivatives to the sodium channel protein has been observed using the products (Yanagawa et al., 1987; Cruz et al., 1989; Hatanaka et al., 1990).

Recently, the chemical synthesis of μ-conotoxin GIIIA has been worked out (Becker et al., 1989; Cruz et al., 1989). Using this synthesis procedure Becker et al. (1990) succeeded in

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[1] Abbreviations: BTX, batrachotoxin; μ-CTX, μ-conotoxin GIIIA; trityl, triphenylmethyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; Boc, *tert*-butyloxycarbonyl; tBu, *tert*-butyl-; ADPV, 5-[4′-(aminomethyl)-3′,5′-dimethoxyphenoxy]valeroyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBT, 1-hydroxybenzotriazole; DIPEA, diisopropylethylamine; FABMS, fast atom bombardment mass spectroscopy; IC_{50}, concentration of competitive inhibitor that inhibits 50% of the binding of a radioligand to a receptor; K_i, inhibition constant; STX, saxitoxin; TTX, tetrodotoxin.

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synthesizing an N-terminal-specific ^{125}I-photoaffinity derivative of μ-conotoxin GIIIA, which binds specifically to voltage-dependent sodium channels. The question then arose of where the photoaffinity reagent should be positioned within the toxin in order to target critical residues of binding site I. This binding site must comprise a very small region of the channel protein, as tetrodotoxin and saxitoxin, which bind competitively against the μ-conotoxins, are relatively small organic molecules.

Thus, we undertook a detailed structure–function examination of the μ-conotoxin GIIIA in order to identify the part(s) of the toxin which is (are) responsible for its binding and blocking activity. To this end, we synthesized analogues of μ-conotoxin GIIIA using the procedures established for the wild-type toxin. The analogues were examined using binding studies on purified membrane from eel (*Electrophorus electricus*) electric organ and single-channel current measurements on sodium channels from rat skeletal muscle incorporated into planar lipid bilayers. These studies allowed us to identify an amino acid residue (arginine-13) on μ-conotoxin GIIIA which is essential for normal blocking activity of the toxin and several other residues that contribute to binding. An abstract on this work has appeared (Prusak-Sochaczewski et al., 1992).

MATERIALS AND METHODS

Reagents were obtained from the following sources: side chain-protected amino acids were from Bachem, Heidelberg; ADPV resin was a gift from Dr. Schnorrenberg, Boehringer Ingelheim; TBTU was from FLUKA, Heidelberg; HOBT and DIPEA were from Aldrich; organic solvents were obtained from Merck, Darmstadt. [^{3}H]Saxitoxin was bought from Amersham International. Electric eels (*E. electricus*) were provided by World Wide Scientific Animals, Apoka.

All experiments using "native" toxin used synthetic peptide having the sequence of μ-conotoxin GIIIA and previously shown to be indistinguishible in properties from the native toxin isolated by purification (Becker et al., 1989).

Synthesis and Purification. The linear peptides were synthesized by solid-phase synthesis using (9-fluorenylmethoxy)carbonyl chemistry (Atherton et al., 1978; Meienhofer et al., 1979). The following side-chain protection groups were used: Cys, trityl; Gln, trityl; Asn, trityl; Arg, Pmc; Lys, Boc; Asp, tBu; Hyp, tBu; Thr, tBu. The protected amino acids were used with free carboxylic acid groups. The syntheses were performed on a polystyrene resin with ADPV linker (0.27 mmol/g) (Albericio et al., 1987) which delivers a peptide amide upon cleavage of the linear peptide from the resin. The coupling of the single amino acids was performed according the TBTU/HOBT/DIPEA method (Knorr, 1989). The synthesis of the analogues was performed simultaneously in a semiautomatic shaking system (Beck-Sickinger et al., 1991) using the "tea bag" method (Houghten, 1985). The primary structure of each of the linear peptides was verified by amino acid analysis and sequencing by Edman degradation.

The raw peptides were oxidized and purified as recently described (Becker et al., 1989, 1990). The molecular weight of each oxidized, purified analogue was checked by FABMS. For each analogue, the measured molecular weight corresponded to the predicted value within ±3 units, consistent with the correctness of the sequence and the complete removal of all side-chain protection groups.

Preparation of Electric Eel Membranes and Binding Assays. Eel membranes were prepared as described elsewhere (Miller et al., 1983). The competitive binding assays against [^{3}H]saxitoxin were performed as previously described (Becker et al., 1989). Binding was measured by displacement of labeled saxitoxin from eel electroplax membranes (100 mM choline chloride, 10 mM HEPES–NaOH, pH 7.4). Inhibition constants, K_i, were calculated from IC_{50}'s measured in the presence of 8 nM STX using the expression $K_i = IC_{50}/(1 + [STX]/K_{STX})$, where $K_{STX} = 0.88$ nM is the dissociation constant for STX binding to the channel.

Bilayer Methods and Single-Channel Recording. Activity of the μ-conotoxins was assayed by the ability of the peptides to block the voltage-dependent Na channel of skeletal muscle. Single voltage-activated Na channels from rat skeletal muscle tranverse tubule membranes prepared essentially as described by Guo et al. (1987) were incorporated into planar lipid bilayers formed across a hole (0.25-mm diameter) in a Teflon partition from solutions of 40 mg/mL 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine and 10 mg/mL 1-palmitoyl-2-oleoylphosphatidylcholine in decane. Planar bilayers were formed with 200 mM NaCl and 10 mM HEPES–NaOH, pH 7.0, solutions at both sides of the partition. Incorporation was induced by adding membrane vesicles (20 μg of protein/mL) to the cis side of the bilayer in the presence of 0.1 μM BTX or by using membrane vesicles (10 μg protein/mL) preincubated with BTX. In the latter case, a mixture of membrane vesicles (2 mg of protein/mL) with BTX (6 μM) was prepared and stored at −20 °C. The mixture was removed from the freezer daily and kept on ice during experiments. The success of channel incorporation tended to improve daily for up to about 6 or 7 days after addition of BTX to the vesicles. Such preparations were routinely used for up to 2 weeks.

Channel insertion generally occurred within 5 min and was detected as a step increase in the bilayer conductance. Records were collected after single-channel incorporations, or in some cases after two- or three-channel incorporations, provided that the channels were oriented in the same direction. Channel orientation was determined by noting the polarity of applied voltage required to close the channels (cytoplasmic side of the channel negative, or "hyperpolarizing" voltages in usual terminology). Block was assayed only at voltages for which the channel was open >90% of the time in the absence of toxin.

Toxin solution was added to the external side of the channel in the concentration 0.1–0.5 μM depending on the μ-CTX derivative. Subsequently, the channel current record was collected on videotape for 15–60 min, at each voltage, generally until 20–70 blocking events had occurred. Records were later analyzed by computer or by hand.

In 9 experiments, block by μ-CTX was determined in the presence of 50 μg/mL bovine serum albumin as a control against reduction of peptide concentration by nonspecific adsorption to the chamber walls. The apparent dissociation constant estimated from these experiments was not significantly different (i.e., within about 15%) from values obtained in the absence of bovine serum albumin.

Analysis of Na Channel Block by μ-CTX. The probability of the channel being blocked was measured directly from the records as the fraction of time spent in the blocked state. Because the kinetics of μ-CTX block are bimolecular, the apparent dissociation constant, K_d, for μ-CTX was calculated from the measured probability of channel block, P_b, at a given μ-CTX concentration:

$$K_d = [\mu\text{-CTX}](1 - P_b)/P_b$$

For computer analysis of slow μ-CTX blocking events, the records were filtered prior to digitization with a corner frequency of 80 Hz. To eliminate most of the brief closing

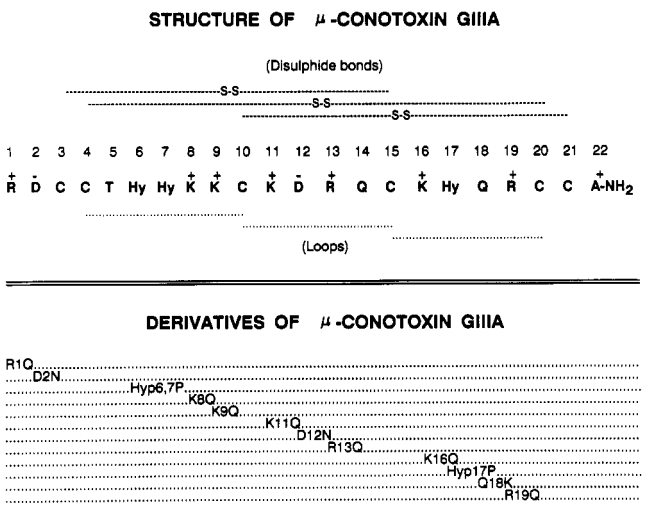

FIGURE 1: Structure of μ-conotoxin GIIIA including the primary sequence, positions of the disulfide bonds, and loops. Amino acid subsitutions used to produce the derivatives studied are indicated in the lower part of the figure.

events caused by the voltage-dependent channel gating process, the records were filtered digitally using a 10-Hz Gaussian filter and events shorter than 400 ms were excluded (Moczydlowski et al., 1984a). The mean dwell times observed were corrected for this cutoff limit, a. The true mean blocked time, t_b, is related to the observed blocked time, t_b, by (Neher & Steinbach, 1978)

$$t_b = t'_b - a$$

The true unblocked time, t_u, is related to the observed value, t'_u, by (Sachs et al., 1982)

$$t_u = t'_u \exp(-a/t_b)$$

The corrections for mean times were less than 10%.

About 50% of experiments (>100) were performed with membranes containing only one Na channel. In these cases association and dissociation rate constants were calculated from the inverse of the mean unblocked and blocked times, respectively. In analysis performed on membranes containing two or three channels, the rate constants were calculated from the multiple-level records using a birth–death analysis based on binomial statistics (Labarca et al., 1980).

Fitting of data, derivation of confidence intervals, and preparation of figures were carried out using SigmaPlot (Jandel Scientific, Corte Madera, CA).

RESULTS

To identify the functional residues in μ-conotoxin GIIIA, 13 toxin derivatives were synthesized. The analogues differed from the native μ-CTX sequence (Figure 1) by single amino acid substitution, except for the double substitution of 4-*trans*-hydroxyproline at positions 6 and 7 by proline. The various peptides can be separated into three groups of net charges +5, +6, and +7 (Table I). To investigate the effect of different structural modifications on the μ-conotoxin function, we studied the steady-state binding to eel electroplax membranes and kinetics of block of single batrachotoxin-activated Na channels from rat skeletal muscle by synthesized derivatives.

Synthesis and Yield. The average yield of the purified oxidized mutant peptides as a percentage of the raw peptides was between 15% and 20%. Thus, the yields were in the range obtained for synthesis of the wild type (20%). The expected molecular weights were confirmed by FABMS for all peptides.

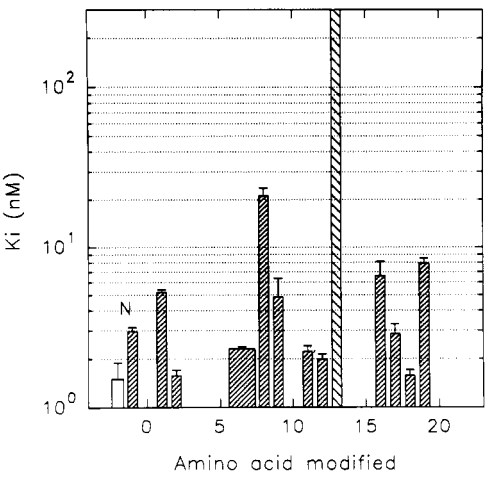

FIGURE 2: Inhibition constants for μ-CTX and derivatives measured by displacement of [^{3}H]saxitoxin from eel electroplax membranes. The constants are plotted against the position of the substituted residue as in Figure 8. For the derivative R13Q the inhibition constant could not be determined; no displacement of [^{3}H]STX was observed up to a concentration of 1 mM of R13Q. For the native peptide, determinations were done by IC_{50} analysis (hatched bar) as for the other derivatives, or by Lineweaver–Burk analysis (open bar) as described by Becker et al. (1989). Error bars are standard deviations of three measurements.

Table I: Nominal Net Charges of Derivatives and Charge Changes Produced by Amino Acid Substitutions in μ-Conotoxin GIIIA

nominal charge	derivative	Δ (charge)	charge substitution
+7	D2N, D12N	+1	− → 0
	Q18K		0 → +
+6	native	0	
	Hyp6,7P, Hyp17P		0 → 0
+5	R1Q, R13Q, R19Q	−1	+ → 0
	K9Q, K11Q, K16Q		

Table II: Binding Data for the Analogues of μ-Conotoxin GIIIA[a]

analogue	IC_{50}[b] (nM)	K_i[b] (nM)
native	26.6 ± 1.5[c]	3.0 ± 0.2[c]
		1.5 ± 0.4[d]
R1Q	47.4 ± 1.9	5.2 ± 0.2
D2N	14.2 ± 1.2	1.6 ± 0.13
Hyp6,7P	21.1 ± 0.9	2.3 ± 0.07
K8Q	190.9 ± 23.0	21.2 ± 2.58
K9Q	44.0 ± 13.4	4.9 ± 1.52
K11Q	20.5 ± 1.3	2.2 ● 0.18
D12N	17.9 ± 1.5	2.0 ± 0.16
R13Q	ND[e]	
K16Q	59.4 ± 13.9	6.6 ± 1.55
Hyp17P	25.8 ± 4.1	2.9 ± 0.45
Q18K	14.2 ± 1.2	1.6 ± 0.13
R19Q	71.2 ± 5.8	7.9 ± 0.64

[a] Competitive binding against [^{3}H]saxitoxin (8 nM) was measured using purified eel electric organ membranes. [b] Each value mean ± SEM, n = 3. [c,d] Values for native sequence from [c]IC_{50} or [d]Scatchard analysis by Becker et al. (1989). [e] ND: binding not detectable up to a concentration of 1 mM R13Q.

Arginine-13 Is Crucial for Binding to Electroplax Membranes. The data obtained by IC_{50} analysis of the inhibition of [^{3}H]saxitoxin binding by the different analogues are shown in Figure 2 and Table II. In these assays, 6 derivatives (D2N, Hyp6,7P, K11Q, D12N, Hyp17P, and Q18K) had inhibition constants (K_i's) between 1.5 and 3 nM, the range defined by two different estimates for the native sequence (Becker et al., 1989). Four others (R1Q, K9Q, K16Q, and R19Q) gave values between 4.9 and 7.9 nM, and for K8Q the value was about 21 nM. Replacement of R13 abolished measurable

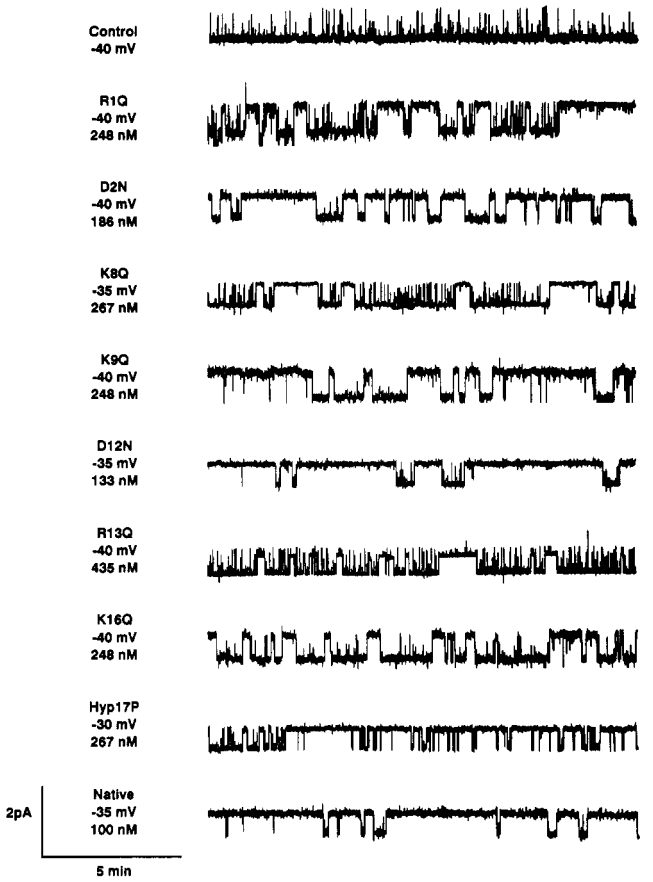

FIGURE 3: Records from single BTX-activated rat skeletal muscle sodium channels showing discrete blocking events produced by various derivatives of μ-conotoxin GIIIA. In these examples, current recordings were made at voltages of −40, −35, or −30 mV, as indicated, for single channels incorporated into planar bilayers with 200 mM sodium on each side. For plotting, records were filtered at 10 Hz. In all traces, openings are in the downward direction. In control records, taken in the absence of toxin, the channel spent >98% of the time in the open conducting state. Addition of the μ-conotoxin GIIIA or its derivative to the extracellular side of channel induced long-lived closing or blocking events. Duration of single blocked events as well as total time which the channel spent in the nonconducting state changed for different toxins and depended on toxin concentration and voltage. The peptide concentration used is indicated alongside each record.

binding to the eel membranes. Of the other residues, only substitution of K8 produced a change in K_i by substantially more than 2-fold. The minor changes observed for four additional residues will be discussed later in the paper in relation to single-channel data obtained for rat skeletal muscle channels.

All Derivatives Block Single-Channel Currents through Skeletal Muscle Channels. Figure 3 shows examples of single Na channel records obtained in the presence of various analogues at a negative voltage (−40 to −30 mV). At these voltages and in the absence of toxin the channels are essentially open all the time. Control records show only occasional brief closing events which are generally <500 ms in duration. Addition of μ-CTX or one of the derivatives to the extracellular side of the channel induces the appearance of long-lived blocked events (many seconds in duration). With the exception of R13Q, the size of the unitary conductance steps before and after toxin addition was identical. In the examples shown, even though R13Q was added at about 4-fold higher concentration, the channel was blocked less than 20% of the time compared to the native peptide and D12N for which the blocked periods account for more than 90% of the record.

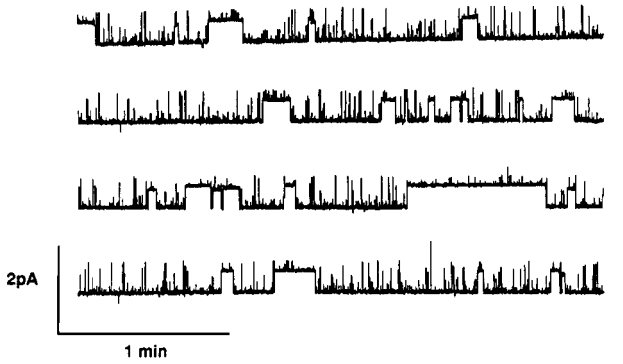

FIGURE 4: Single-channel current records (approximately 5 min per sweep) taken in the presence of 435 nM R13Q. Distinct blocked states lasting up to several minutes in duration are visible, but the "blocked" state shows a clear conductance that is 20–40% of that in the unblocked state. $V = -40$ mV.

Thus, it is immediately evident from the records that the various toxins differ widely in their affinities and the kinetics of their action, with native peptide and D12N having the longest-lived blocked events (mean ≃400 s) and R13Q having the shortest (mean ≃9 s).

R13Q Uniquely Blocks to a Nonzero Subconductance State. One striking point in Figure 3, reemphasized by the additional records on an expanded time scale in Figure 4, is that the seconds long interruptions in current flow induced by R13Q do not result in complete occlusion of current flow through the channel but, rather, appear as abrupt steps to a reduced, but nonzero, current. The conductance level histogram in Figure 5 indicates that, while brief spontaneous gating closures still defined a fully closed level, the toxin-induced blocked states, which account for a much larger fraction of the record, show a finite, somewhat variable conductance of about 20–40% of the open state. All other toxins, including saxitoxin (Krueger et al., 1983; French et al., 1984), tetrodotoxin, and a variety of saxitoxin derivatives (Moczydlowski et al., 1984a,b), that have been shown to act at toxin site I on the sodium channel cause an all-or-none block. Thus, R13Q is unique in that it binds and inhibits, but only partially occludes the individual channel.

Membrane Hyperpolarization Favors Na Channel Block. The quantitative effect of analogues on the muscle channels was analyzed as previously described for the guanidinium toxins and μ-CTX (French et al., 1984; Moczydlowski et al., 1984a,b). The kinetics of μ-CTX block at the single-channel level have been shown to conform to that of reversible binding equilibrium to a single site (Cruz et al., 1985). Therefore, the association and dissociation rates for the blocking reaction were obtained as the reciprocals of the mean dwell times in the unblocked and blocked states, respectively. The apparent dissociation constants calculated directly from the mean probability of channel block, P_b, were confirmed to be virtually identical with those obtained from the ratio of the dissociation rate constant to the association rate constant.

Figure 6 shows an example of binding rates measured for derivative Hyp6,7P. The absolute magnitudes of the rate constants and apparent dissociation constants varied widely from derivative to derivative, and Figure 7 shows the least squares fits to the measured constants for all of the derivatives, except Q18K, as a function of voltage. Qualitatively, the voltage dependencies are similar for all derivatives. Hyperpolarization (making the cytoplasmic side more negative) enhanced block by decreasing the dissociation rate and increasing the association rate. These trends were seen for all derivatives except Q18K, although our data are not

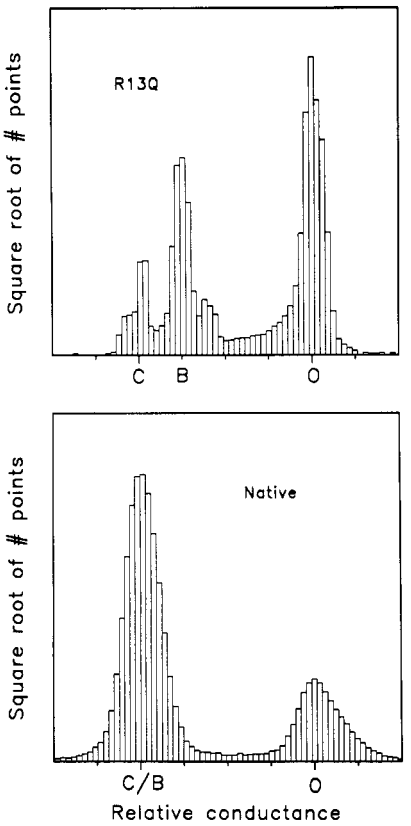

FIGURE 5: All points histograms showing the frequency of different conductance levels in records taken in the presence of synthetic μ-CTX, or the derivative R13Q. In the presence of the toxin with the native sequence, there are only two levels, the open conductance (O) and the closed or blocked (nonconducting) level (C/B), at which the only conductance is the background leakage of the bilayer. In the presence of R13Q, "blocked" (B) states occur in a range of conductances of about 20–40% of the open state. Single-channel conductance was calculated as $\gamma = i/(V - V_{rev})$, where i = single-channel current, V = voltage, and V_{rev} = reversal potential, and the conductance for the open channel was normalized to 1. For this example, concentrations and voltages were as follows: [R13Q], 435 nM, –40 mV; [μ-CTX], 100 nM, –35 mV.

extensive enough to determine whether there are significant differences in voltage sensitivity among the different derivatives (see Table III).

The rate constants (k_{on} in M^{-1} s^{-1}, association; k_{off} in s^{-1}, dissociation) and the equilibrium dissociation constant (K_d in M) (see Figure 6) can be described as exponential functions of voltage, V, as follows. The sensitivity of each parameter to voltage is represented by an "effective valence", z_{on}, z_{off}, or z, which is proportional to the slope of the semilogarithmic plot of the rate or equilibrium constant vs voltage, V. The factor RT/F = 25 mV at room temperature (≃22 °C).

$$k_{on}(V) = k_{on}(0)\exp(-z_{on}VF/RT)$$

$$k_{off}(V) = k_{off}(0)\exp(z_{off}VF/RT)$$

$$K_d(V) = k_{off}/k_{on} = K_d(0)\exp(zVF/RT)$$

$$z = z_{off} + z_{on}$$

With the derivative Q18K, although some blocking and unblocking fluctuations were seen, experiments at ≥100 nM peptide consistently terminated by the channel permanently "disappearing" while the bilayer remained intact (9 of 9 experiments terminated within 5–20 min). For comparison, with other derivatives, only about 10% of experiments ended

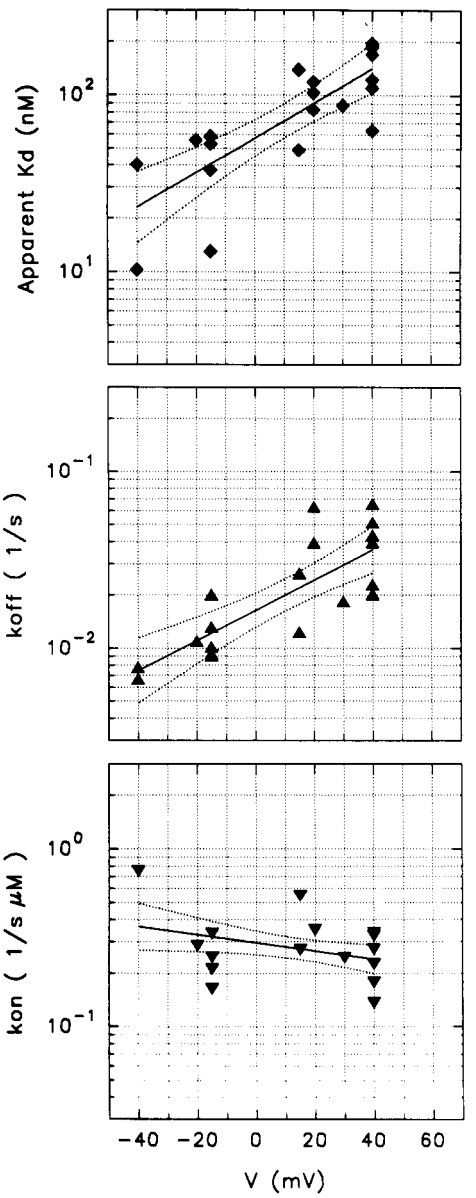

FIGURE 6: Voltage dependence of the apparent dissociation constants and the dissociation and association rate constants of μ-CTX derivative Hyp6,7P. Each data point represents an estimate from a single recording with 11–75 blocked events (mean ± SD, 40.1 ± 18.3) with record durations from 15 to 58 min (mean ± SD, 33.8 ± 13.4). The data are from 8 experiments (6 with single channels, and 2 with two or three channels). Solid lines are least squares fits to the data. Dotted lines indicate 95% confidence intervals.

with channel disappearance. At [Q18K] ≃ 10 nM, premature disappearances of channels were not such a problem, but we have not obtained sufficient long, stationary recordings to enable steady-state kinetic analysis of block by this peptide. This behavior seems to reflect that it is possible for Q18K to bind almost irreversibly to the rat skeletal muscle channel and that there may be more than one bound state. It is worth noting that Q18K ranked with D2N as being the most potent peptide in the eel membrane binding assay described above.

Amino Acid Substitutions Generally Decrease Efficacy of Block of Rat Skeletal Muscle Channels. All but two of the substitutions appeared to significantly decrease the ability of the peptides to block the rat skeletal muscle channels (see Figure 8). The apparent dissociation constants, compared at V = 0 mV, for the double substitution Hyp6,7P, and for D12N, were changed by less than a factor of 2 from the native peptide. Next in the ranking came D2N, with a K_d about 3 times

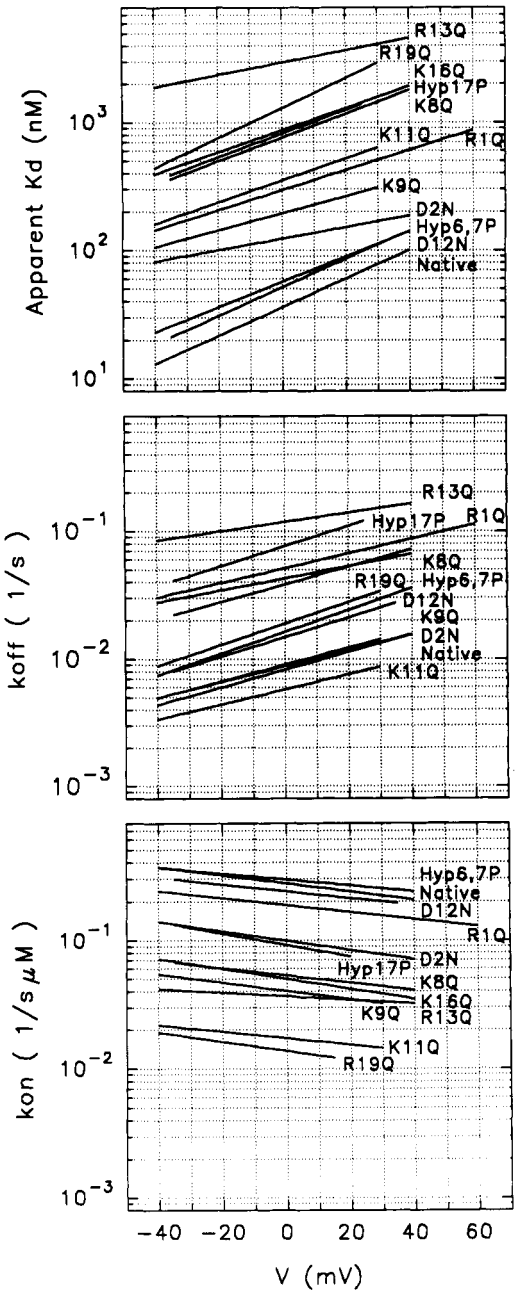

FIGURE 7: Voltage dependence of the apparent dissociation constants and the dissociation and association rate constants of μ-CTX and derivatives. Solid lines are least squares fits to data such as those shown in Figure 6 for Hyp6,7P.

greater than the native. Three positive-to-neutral substitutions (K9Q, R1Q, and K11Q) gave K_d's 7–8 times the native value; for K8Q, K16Q, and Hyp17P the factor was 20–30 times; for R19Q, 40 times; and for R13Q, 80–100 times, depending on the method of determining the K_d. Thus, in common with the eel binding studies described above, and the rat diaphragm ED_{50} measurements of Sato et al. (1991), the single-channel data clearly point to R13 as the most important single residue for μ-CTX action. Beyond this, however, the interaction appears to be complex, with a number of residues significantly contributing to the potency of the native toxin.

With the important exception of Hyp17P, substitutions that significantly reduced the potency of the toxin were substitutions of positive residues by glutamine. Complementing this point are the observations mentioned above suggesting that Q18K may greatly enhance the strength of binding and perhaps complicate the mechanism. On the other hand, charge changes produced by replacing the negative aspartate residues had little effect on potency.

Certain Substitutions Change either Association Rate or Dissociation Rate but Not Both. Both association rate constants and dissociation rate constants estimated, at V = 0 mV, from the single-channel data vary over about a 20-fold range. The native sequence peptide showed nearly the slowest dissociation and nearly the fastest association, making it, in terms of equilibrium block, the most potent peptide tested—a tribute to millions of years of evolution. At the other end of the scale, R13Q showed nearly the fastest dissociation and nearly the slowest association, making it the least potent derivative tested with an apparent dissociation constant 80–100 times greater than that of the native peptide. This is very close to the result of Sato et al. (1991) who found that the ED_{50} for the rat diaphragm preparation increased by a factor of about 200 when alanine was substituted for arginine-13. Theoretically, if both rate constants could be optimized for binding in a single peptide, an even wider range of potencies would result—20 × 20 = a factor of 400. Noting this, we examine directly, in Figure 9, the effect of substitutions on the individual rate constants at V = 0 mV.

In Figure 9, for the various derivatives, the dissociation rate is plotted against the *reciprocal* of the association rate in order to depict, on each axis, the relative contribution of the individual rate constants to the apparent equilibrium dissociation constant. Thus, derivatives appearing near the "origin" of the graph are the most potent, and displacement upward or to the right reflects a decrease in potency. It is clear from these data that for certain derivatives large changes (10–40 times) in potency can be attributed almost exclusively to either the dissociation rate constant (Hyp17P) or to the association rate constant (R19Q, K11Q). Thus, the removal of an OH group from HYP17 has little effect on the rate at which the peptide gains access to the binding site, but does lead to a 9-fold decrease in the mean residence time once the peptide is bound. Conversely, replacement of the positive residues, K11 or R19, by glutamine leads to little change in block time—the mean time the peptide is bound—but dramatically (≃20 times) decreases the rate of binding.

DISCUSSION

Choice of Substitutions. Because it seems clear that μ-conotoxins bind to the sodium channel at least in part by ionic forces (Kao, 1986), the charged residues were replaced, one by one, in separate derivatives (R1Q, D2N, K8Q, K9Q, K11Q, D12N, R13Q, K16Q, R19Q). Lysine and arginine were substituted by glutamine, and aspartate was substituted by asparagine. Thereby, the size of the respective amino acid side chain was maintained as constant as possible while neutralizing the charge. In the cases of D2N, D12N, and Q18K the global positive charge of the toxin was increased in order to test whether the toxin potency could be increased in this way. In addition, the hydroxyprolines were changed to prolines, Hyp6 and Hyp7 being changed in one single step, because μ-conotoxins with proline in each of these positions have been found in the venom of *C. geographus* (Cruz et al., 1985). Despite the occurrence of nonhydroxylated residues in some natural μ-conotoxins, it seemed worthwhile to examine the role of hydroxyproline, this relatively uncommon amino acid being conserved in the μ-conotoxins GIIIA, GIIIB, and GIIIC (Cruz et al., 1985).

Comparison of Eel Electroplax and Rat Skeletal Channels. The competitive binding experiments on eel electroplax membranes delivered the surprising result that most of the

Table III: Correlation Coefficients (r) for the Dependence on Voltage of Apparent Dissociation Constants and Rate Constants for Block of Skeletal Muscle Sodium Channels[a]

analogue	r (K_d)	probability, P	r (k_{on})	probability, P	r (k_{off})	probability, P
R1Q	0.720	$0.01 < P < 0.05$	0.330	>0.05	0.650	>0.05
D2N	0.363	>0.05	0.325	>0.05	0.836	<0.01
Hyp6,7P	0.804	<0.01	0.353	>0.05	0.795	<0.01
K8Q	0.947	<0.01	0.819	$0.01 < P < 0.05$	0.894	<0.01
K9Q	0.663	<0.01	0.543	>0.05	0.689	$0.01 < P < 0.05$
K11Q	0.743	$0.01 < P < 0.05$	0.690	>0.05	0.525	>0.05
D12N	0.936	<0.01	0.535	$0.01 < P < 0.05$	0.899	<0.01
R13Q	0.470	>0.05	0.653	<0.01	0.447	>0.05
K16Q	0.746	<0.01	0.726	$0.01 < P < 0.05$	0.707	$0.01 < P < 0.05$
Hyp17P	0.860	<0.01	0.537	$0.01 < P < 0.05$	0.832	<0.01
R19Q	0.906	<0.01	0.390	>0.05	0.763	>0.05
native	0.731	<0.01	0.443	>0.05	0.551	$0.01 < P < 0.05$

[a] P is the probability that a larger r value would occur due to the random scatter of the data.

individual amino acids could be exchanged with little change in binding activity. Except for K8Q and R13Q, the K_i's fell in the range 0.5–3 times the value for the native peptide. A similar observation was reported for another conotoxin, the "King Kong Peptide" (Woodward et al., 1990). Mutants of this peptide showed an unchanged action as long as the cysteines were not replaced and as long as the position of these cysteines in the sequence remained unchanged. By contrast, for the rat skeletal single channels, all of the substitutions in μ-conotoxin GIIIA, except Hyp6,7P and D12N, increased the apparent K_d's by more than a factor of 3, with D2N, of the remaining derivatives, showing the smallest change from the native affinity.

Thus, the common threads that emerge are the following: (1) R13 is the most critical residue; (2) the hydroxyl groups on hydroxyprolines-6 and -7 and the charges on the aspartate residues have little importance to toxin activity. Looking more closely at the data, it appears that all the remaining positive charges contribute measurably, even if to a minor degree, to toxin action, with the possible exception of K11 for binding to electroplax membranes.

Considering the guanidinium toxins, the OH groups at C4, C9, and C10 of tetrodotoxin and the OH group at C12 of saxitoxin are important for binding to the receptor (Kao, 1986). These OH groups are presumed to form hydrogen bonds. It is possible that the OH group of Hyp17 plays a similar role in binding of μ-conotoxins to skeletal muscle channels. The essential guanidinium group on each toxin should form an ionic bond with a carboxylate group on the receptor (Kao, 1986), a hypothesis that is supported by Noda et al. (1989), who identified by site-directed mutagenesis a specific glutamate residue, in the sequence of a rat brain Na channel, as an essential part of the tetrodotoxin binding site on the channel. The guanidinium group of the R13 side chain of μ-conotoxin GIIIA, situated on the second of three loops (see Figure 1)—an exposed, flexible part of the peptide structure (Lancelin et al., 1991; Ott et al., 1991)—may well bind to the homologous glutamate residue on the skeletal muscle channel.

Comparison with the Other Observations. Sato et al. (1991) assayed the ability of a variety of μ-conotoxin GIIIA derivatives to inhibit twitch of isolated rat diaphragm following direct electrical stimulation. In their study, neutral substitutions were made with alanine, as opposed to glutamine or asparagine in our own work. The range and relative values of their ED_{50} values parallel closely the apparent dissociation constants we determined from studies of block of single rate skeletal muscle Na channels. The ED_{50}'s ranked as follows: D12A < D2A < Q14A < K9A ≃ native ≃ T5A ≃ Hyp7A < Q18A < K11A < K8A < R1A < K16A ≃ Hyp17A < R19A < R13A. The K_d's from the single-channel measurements are ordered: native < D12Q ≃ Hyp6,7P < D2N < K9Q < R1Q < K11Q < K8Q < Hyp17P < K16Q < R19Q < R13Q. Only for K9 does there appear to be a large quantitative difference between the two studies, K9A having about the same ED_{50} as the native, and K9Q, in the single-channel studies, showing about 7-fold weaker affinity. The similarity of the results of two groups using very different assays on rat muscle suggests that the quantitative differences from the eel binding data may be providing clues to real, though subtle, differences between the structures of toxin site I in these preparations.

Regarding the difference in absolute values of the K_i's from our eel membrane binding studies and the apparent K_d's from the studies of single rat skeletal muscle channels, we prefer not to draw strong conclusions at present. Moczydlowski et al. (1986a) obtained K_i's in the range 25–56 nM for both eel electric organ and rat skeletal muscle, with their bilayer studies on rat skeletal channels giving a slightly higher apparent K_d [110 nM, from Cruz et al. (1985)]. There is, however, the precedent of a different sodium channel-specific conotoxin, conotoxin GS, showing about 30-fold higher affinity for eel membranes than for rat skeletal muscle (Yanagawa et al., 1988), providing further evidence of functionally important differences between the conotoxin-binding domains on eel and rat skeletal channels.

Net Charge Is Not Necessarily Important. In contrast to the study of saxitoxin derivatives by Moczydlowski et al. (1984b), none of the conotoxin derivatives thus far studied have possessed a net charge changed by more than about 15% from the native toxin (±1e). Despite this, it seems clear that two charged residues are not particularly important—the aspartate residues D2 and D12—whereas others are. The idea that electrostatic interactions of individual residues have a role subordinate to such factors ad detailed fit to the receptor is suggested by two observations. First, substitutions of D12, which lead to minor changes in activity, involve a charge change of the same magnitude as the substitutions R13Q or R13A which show arginine-13 to be critical. Second, the charge-conserving adjacent substitution, R13K, results in a substantial change in ED_{50}—a 6-fold increment over the native (Sato et al., 1991).

Our results also provide some preliminary support for another qualitative resemblance between the mechanism of action of the guanidinium toxins and the peptide μ-conotoxins. Our data to date reveal no significant dependence of z, the apparent valence for equilibrium block, on nominal net charge of the toxin ($P > 0.1$). Likewise, there is no significant correlation between dissociation rate constant and net charge among the derivatives tested ($P > 0.1$). On the other hand,

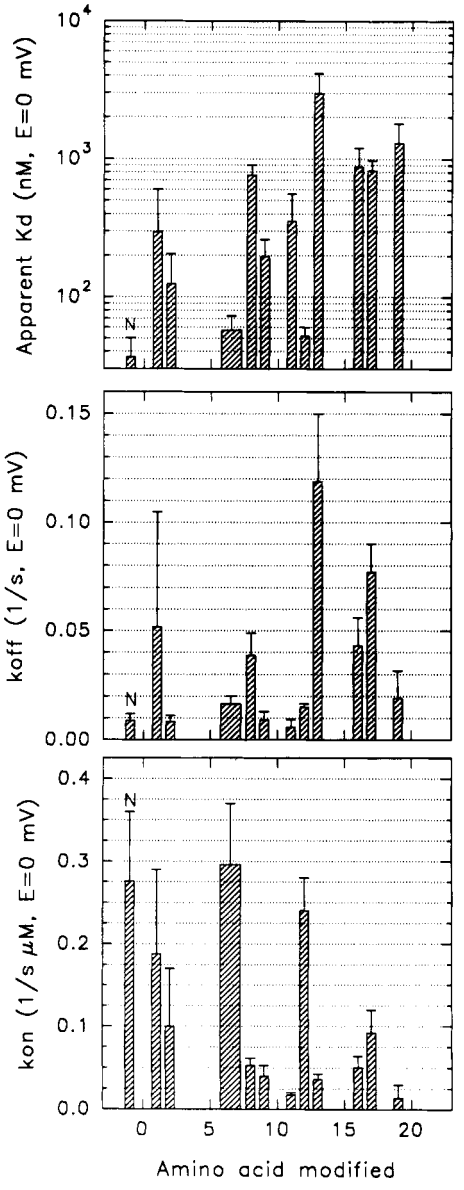

FIGURE 8: Apparent dissociation constants and the dissociation and association rate constants of μ-CTX and derivatives determined at $V = 0$ mV. Bars representing constants for native toxin are marked with letter N. The derivative constants are plotted against the position of the substituted residue. Substitutions examined were the following: R1Q, D2N, Hyp6,7P, K8Q, K9Q, K11Q, D12N, R13Q, K16Q, Hyp17P, R19Q. Error bars are 95% confidence intervals from least squares fits to data such as those shown in Figure 6 for Hyp6,7P.

the association rate constants showed a weak, but statistically significant ($P < 0.05$), dependence on nominal net charge (Moczydlowski et al., 1986b). This latter observation is consistent with an electrostatic contribution to orientation or local concentration of the toxin in the vicinity of the receptor. More extensive studies should be performed to rigorously confirm this point. Furthermore, we emphasize that we would expect, at least in some residues, a correlation between charge and dissociation rate to emerge if several different charge changes could be made at the same location.

Does R13 Occlude the Pore? Although the idea has been challenged [see Miller and Garber (1988) for a recent account of arguments], there is much precedent for the notion that tetrodotoxin and saxitoxin block sodium channel currents by physically occluding the ion-conducting pore with a projecting guanidinium group. On the basis of structural data, Lancelin et al. (1991) suggested that one of the cationic residues of μ-conotoxin GIIIA may compete for the same site. Our single-

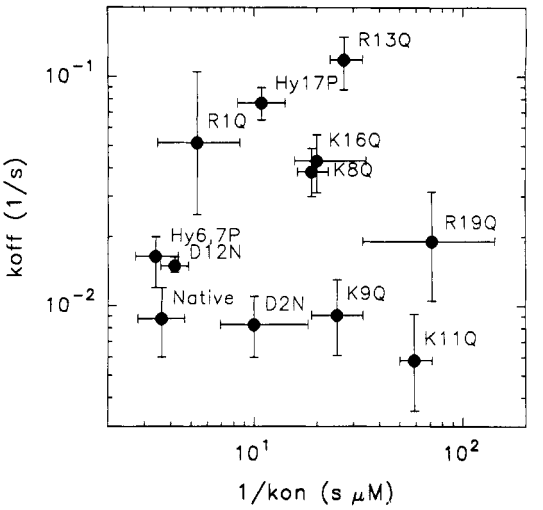

FIGURE 9: Plot of the dissociation rate constant against the reciprocal of the association rate constant designed to show the relative contributions of strength of binding (small dissociation rate indicates strong binding) and rate of association (small reciprocal association rate produces a strong equilibrium binding) on the apparent dissociation constant for μ-CTX binding. Thus, derivatives represented near the origin are the most potent blockers. Error bars indicate 95% confidence limits as in Figure 6.

channel studies shed new light on this issue and prompt our hypothesis that R13 not only may play a central role in enabling high-affinity binding but also may be critical to complete occlusion of the conducting pathway. The derivative R13Q, once attached, binds quite strongly to the rat skeletal muscle channel (mean dwell time about 9 s). This compares with 8 s for TTX and 16 s for STX (Guo et al., 1987). Nonetheless, block of single-channel current by R13Q is incomplete—the "blocked" state is an easily measurable subconductance (Figures 3–5). This suggests the following hypothesis: that the μ-conotoxin binding "site" encompasses a substantial area on the surface of the channel protein, allowing multiple ionic and nonionic interactions between the peptide toxin and the channel. Functionally central among these interactions would be bonding between R13 and a critical anionic residue in the mouth of the pore. With R13Q, there would be no cationic residue to lock into the pore and completely prevent current flow, but there would be sufficient ancillary interactions to hold the peptide to a large part of the binding site. In that position, it could sufficiently inhibit access of ions to the pore to substantially reduce the single-channel conductance while the peptide is bound.

Relative Importance of Access vs Strength of Binding to the Channel Site. One striking point in the first report (Cruz et al., 1985) of single-channel observations of μ-CTX block of skeletal muscle sodium channels was that, even though the affinity for the channels was of the same order as those for TTX and STX, the kinetics of block were about an order of magnitude *slower* (blocked times ≃10 s for the guanidinium toxins and ≃100 s for μ-conotoxin GIIIA). This implied that association of the peptide was more than 10-fold slower than for the smaller toxins, as verified by calculations of the rate constant. This use of single-channel recordings to directly measure unidirectional rate constants in conjunction with systematic variations in the peptide structure provides a powerful approach to unraveling the detailed mechanism of interaction between the toxin and the channel.

The data of Figure 9 suggest that certain residues are important primarily in the approach and docking of the peptides with the channel site (e.g., K11 and R19) while others

are important mostly for their contribution to the strength of binding once the toxin has attached (e.g., Hyp17 and R1). Although, given a sufficient number of events, there can be little ambiguity in the interpretation of the mean blocked times and hence the dissociation rate, a change in the apparent association rate could arise from an erroneously low concentration of the active peptide, due perhaps to imperfect folding of some fraction of the peptide molecules. We consider that this is unlikely to account for the large changes in apparent association rate that we observed for the following reasons: (1) yields for all derivatives were comparable to those for synthesis of the native sequence, whose properties were indistinguishible from the toxin obtained by purification from *C. geographus* tissue; (2) all peptides ran as a single peak on HPLC; (3) comparable derivatives synthesized by Sato et al. showed no changes in NMR spectra except those associated with side-chain substitution.

A simplistic hypothesis regarding roles of different residues would be that residues that exclusively affect the association rate are involved only in long-range electrostatic interactions. This notion would be tested most stringently by varying ionic strength in experiments with a series of new derivatives having multiple charge changes at a single site [cf. MacKinnon and Miller (1989)]. Support for this hypothesis is already available in two observations of Sato et al. (1991). They reported that the charge-conserving substitution R1K produced a small increment in ED_{50} similar to that seen for the neutral substitution R1A, consistent with a requirement for detailed fit. On the other hand, R19K showed wild-type toxic activity, as if charge conservation at this position is sufficient for normal activity. Furthermore, R13K, in their study, showed somewhat decreased activity, but this substitution had much less effect than either R13A or R13Q in our own work. These observations are consistent with R13 participating in long- and short-range interactions, both of which are important. Clearly, more mutations and more single-channel analysis will be extremely valuable in investigating these issues further. In conjunction with such studies, work is in progress to determine the three-dimensional structure of R13Q using NMR methods.

What Comprises the "Macrosite" for μ-Conotoxin Binding? The μ-conotoxin macrosite (Olivera et al., 1991) presumably includes several microsites on the channel protein with which the different residues that influence docking and binding interact. Of the factors mentioned by Janin and Chothia (1990), at least electrostatic and hydrogen bond interactions, and residue packing, appear important for the tight binding of the peptides to the channel protein. Probably, a minority of the microsites, including residues homologous to Glu387 of the rat brain type II channel (Glu364, eel, and Glu403, rat skeletal; Trimmer et al., 1989), make up the TTX and STX sites. The μ-CTX macrosite almost certainly involves the extracellular ends of proposed pore-forming short transmembrane segments in one or more of the homologous repeat domains (Guy & Conti, 1990). The size of μ-CTX (Lancelin et al., 1991) and the broad distribution of residues that contribute to the toxin–channel interaction raise the possibility of additional, significant interactions with other domains of the channel. Such interactions would provide important constraints on structural models of the channel.

CONCLUSIONS

The new information about functional roles of different residues suggests novel approaches to the design of Na channel labels. For example, the strategic location for a photoaffinity label for the center of toxin binding site I would be in the vicinity of R13. Becker et al. (1990) already demonstrated that the channel protein can be labelled by a N-terminal photoaffinity derivative of μ-conotoxin GIIIA. Furthermore, even the R13Q substitution yields a peptide which binds to a rat skeletal muscle channel, on average, for several seconds. This would provide ample opportunity for a photoaffinity label to react with the channel protein. On the other hand, a radioligand which relies on the peptide's innate binding affinity might be more aptly labeled at some residue that has little role in toxin binding. Finally, the availability of mutants, such as R13Q, which disrupt part of the toxin's binding mechanism without abolishing the specificity of its action, raises the possibility of making a minute functional dissection of the interaction of this highly specific family of toxins with an important channel protein.

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