

Conus geographus Toxins That Discriminate between Neuronal and Muscle Sodium Channels*

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We describe the properties of a family of 22-amino acid peptides, the μ -conotoxins, which are useful probes for investigating voltage-dependent sodium channels of excitable tissues. The μ -conotoxins are present in the venom of the piscivorous marine snail, *Conus geographus* L. We have purified seven homologs of the μ -conotoxin set and determined their amino acid sequences, as follows, where Hyp = *trans*-4-hydroxyproline.



Using the major peptide (GIHA) in electrophysiological studies on nerve-muscle preparations and in single channel studies using planar lipid bilayers, we have established that the toxin blocks muscle sodium channels, while having no discernible effect on nerve or brain sodium channels. In bilayers the blocking kinetics of GIHA were derived by statistical analysis of discrete transitions between blocked and unblocked states of batrachotoxin-activated sodium channels from rat muscle. The kinetics conform to a single-site, reversible binding equilibrium with a voltage-dependent binding constant. The measured value of the equilibrium K_D for GIHA is 100 nM at 0 mV, decreasing e -fold/34 mV of hyperpolarization. This voltage dependence of blocking is similar to that of tetrodotoxin and saxitoxin as measured by the same technique. The tissue specificity and kinetic characteristics suggest that the μ -conotoxins may serve as useful ligands to distinguish sodium channel subtypes in different tissues.

While characterization of the voltage-activated sodium channel as mediator of the inward sodium current of the action potential was established by electrophysiological methods (1), recent progress has been made in the biochemical characterization of sodium channel proteins (2, 3). The basic strategy has been to use labeled toxins that bind tightly to the channel proteins to assist in their detection, assay, and purification. The major toxins used for this purpose are guanidinium toxins such as tetrodotoxin and saxitoxin, alkaloids of the vertatridine group, and a set of small proteins from scorpion venoms (for reviews, see Refs. 2-4). Using these toxins as specific ligands, purified sodium channel preparations have been described from the electric organ of the eel

Electrophorus (5-7), from chick cardiac muscle (8), rat skeletal muscle (9), and rat brain (10).

At the present time, although all preparations contain a major large subunit of molecular weight between 230,000-300,000, there are significant differences reported among the various isolated sodium channel proteins (7-10). It is not clear whether these differences stem from differences in laboratory techniques, assays used, evolutionary divergence of the channel, or different tissue types.

We show that the purified neurotoxins described in this report, the μ -conotoxins¹ from the marine snail *Conus geographus*, discriminate strongly between nerve and muscle sodium

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¹ We are attempting to be systematic in our naming of the peptides. "Conotoxin" is used to include all such molecules isolated from *Conus* snails. Capital Roman letters indicate species thus: G, *C. geographus*; M, *C. magus*; T, *C. textile*; S, *C. striatus*; etc. Roman numerals distinguish various individual toxins, with closely related forms having Roman letters appended, as conotoxin GIHA. When a physiological mode of action is known, we use a Greek letter to refer to the class as follows: α , acetylcholine receptor toxins; μ , muscle sodium channel toxins; and ω , nerve terminal toxins.

channels, lending support for the idea that there may be significant differences between the proteins. Existence of the muscle-selective μ -conotoxins could first be inferred from work on crude venom by Endean and co-workers (11, 12). In their physiological characterization of *C. geographus* venom, these investigators noted that the venom did not affect nerve action potentials, but abolished muscle action potentials even when the muscle was directly stimulated. Spence *et al.* (13) described the isolation of a toxin (Toxin III) which, by amino acid composition, is clearly a member of the μ -conotoxin family as defined in this report. They showed that Toxin III preferentially abolished muscle action potentials; however, they reported that the purified preparation would slowly and irreversibly abolish nerve action potentials and noted the discrepancy with Endean's results with the crude venom. Further biochemical characterization of this toxin was carried out in our laboratories, where the purified toxin was shown to contain hydroxyproline (14), and by a group in Japan who purified and determined the amino acid sequences of two toxins of this group (15, 16).

In this report, we provide a more comprehensive characterization of the μ -conotoxin family than has been available to date. Seven homologous μ -conotoxin peptides are described; four of the variants appear to be post-translational processing intermediates. We also provide definitive biochemical and electrophysiological evidence that one toxin in this series (μ -conotoxin GIIIA) does indeed directly block muscle Na channels. Single channel experiments on batrachotoxin-activated sodium channels incorporated into planar bilayers, from a rat muscle membrane preparation, provide the first detailed study of the kinetics of μ -conotoxin action. The kinetics of blocking are similar to those of the classic guanidinium toxins, tetrodotoxin and saxitoxin, as studied by the same method. In contrast to these latter toxins, however, μ -conotoxin exhibits at least a 1000-fold specificity for muscle *versus* nerve sodium channels.

EXPERIMENTAL PROCEDURES

Purification of μ -Conotoxins

C. geographus venom was extracted (17), and soluble proteins and peptides were fractionated on a column of Sephadex G-25 (18) as previously described. Four broad peaks were eluted from the G-25 column, the first appearing at the void volume. The μ -conotoxins were found in the second peak. This material was fractionated by reversed-phase HPLC² using a Supelco semiprep column (1.25 \times 25 cm; 5- μ m particle size; C18, not end-capped; 25 °C) at a flow rate of 3 ml/min. A gradient was applied using Solvent A, 0.1% aqueous trifluoroacetic acid, and Solvent B, 0.1% trifluoroacetic acid in 60% (v/v) acetonitrile (19). A linear gradient from 15–80% Solvent B was developed in 35 min. Under these conditions the μ -conotoxins, identified by intraperitoneal assay in mice, are the first three major peaks eluted between 15 and 30% Solvent B, with μ -conotoxin GIIIA being present in much greater amounts than the others. Each active peak was further purified by rechromatography on a VYDAC C18 column (0.46 \times 25 cm; 5- μ m particle size; not end-capped).

Amino Acid Analysis

Samples of peptides were hydrolyzed *in vacuo* with 6 N HCl, at 105 °C for 24 h. Acid was removed by drying in a Speed-Vac (Savant Instruments), and the residue was analyzed by ion-exchange chromatography on a Beckman Model 121 amino acid analyzer.

² The abbreviations used are: HPLC, high-performance liquid chromatography; Hyp, *trans*-4-hydroxyproline; PTH, phenylthiohydantoin; Cys(Cm), carboxymethylcysteine; dns, 5-dimethylaminonaphthalene-1-sulfonyl; MOPS, 4-morpholinepropanesulfonic acid.

Reduction and Carboxymethylation

Since only small amounts of conotoxins GIIIB and GIIC were available, we carried out reduction and alkylation under conditions that allowed us to put the complete reaction mixture into the sequencer cup. Approximately 5–10 nmol of peptide was dissolved in 25 μ l of *N*-ethylmorpholine/water (1:1 by volume). Fifteen microliters of a 2% solution of tributylphosphine in *n*-propyl alcohol (20) were then added, and the mixture was incubated under N₂ for 15 min at 57 °C. Twenty microliters of a 0.2 M solution of iodoacetic acid were then added. After a further 15 min (N₂, dark) excess iodoacetate was reacted by addition of 1 μ l of mercaptoethanol. These conditions had been found to be satisfactory in trial experiments with vasopressin.

Sequence Analysis

Manual Methods—A sample of 182 nmol of conotoxin GIIIA was reduced with dithiothreitol and alkylated with iodoacetamide. The carboxyamidomethylated toxin was repurified by reversed-phase HPLC on the VYDAC column using conditions similar to those described above. It was then digested for 3 h at 37 °C with clostripain (50 μ l of a 1 mg/ml solution in 0.025 M potassium phosphate, pH 7.5, containing 0.2 mM calcium acetate and 2.5 mM dithiothreitol) (21). The digest was fractionated on the VYDAC column using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid, the eluate being monitored at 214 nm. Peaks were collected manually, and the peptides were subjected to amino acid analysis and to degradation by phenylisothiocyanate using Tarr's procedure (22). Phenylthiohydantoin derivatives were analyzed by reversed-phase HPLC, using the method of Hunkapiller and Hood (23).

Automated Sequencing—Samples of all μ -conotoxins were reduced, carboxymethylated, and analyzed in a Beckman 890D spinning cup sequencer (24). We used a 0.1 M Quadrol program, as supplied by the manufacturer, and a combined wash with benzene and ethyl acetate. Two milligrams of polybrene (25) were used as a carrier; before applying the toxin samples, 100 nmol of Ser-Ala was added to the Polybrene and subjected to five cycles of degradation. Conversion of the fractions was carried out using 200 μ l of 1 N HCl containing 0.1% dithiothreitol (w/v). After removal of the acid in a Speed-Vac, samples were dissolved directly in 50 μ l of 0.1% trifluoroacetic acid, containing 20% (v/v) acetonitrile. This diluent, used for sample application on HPLC, greatly improves the stability of PTH-Cys(Cm). The PTH derivatives were analyzed as described above.

Carboxyl Region of G-IIIC—A clean sequence of GIIC was obtained to residue 19, and the results beyond that point were compatible with the "expected" sequence Cys-Cys-Ala (see below). To improve our chance of obtaining definitive evidence on this from our remaining material, we first performed acid-oxidized the peptide (26). After thoroughly drying the peptide we then dissolved it in 50 μ l of 50% (v/v) *N*-ethylmorpholine and reacted it with 10 μ l of acetic anhydride. The sample was dried *in vacuo* with several additions of water, until only a thin oily film remained. It was then digested for 2 h at 37 °C with 5 μ g of trypsin in 50 μ l of 0.2 M *N*-ethylmorpholine acetate, pH 8.5. The digest was diluted with 200 μ l of water and applied directly to the sequencer cup, which contained precycled Polybrene. For this sample we used a 0.25 M Quadrol program with separate benzene and ethyl acetate washes.

Physiological Methods

Cutaneous pectoralis nerve-muscle preparations were dissected from *Rana pipiens*. Each preparation was pinned out in a rectangular trough constructed of Sylgard. Conventional intracellular recording techniques were used. The motor nerve to the muscle spanned a vaseline gap and was stimulated with platinum wires placed on either side of the gap. A thin vaseline-coated bar, constructed of Sylgard, was placed transversely over the muscle near its myocutaneous end; this partitioned the trough into two compartments spanned by the muscle. Direct stimulation of the muscle was performed by passing current through platinum electrodes placed in each compartment. Excitation-contraction coupling was blocked by formamide treatment (27). The experiments were conducted at room temperature.

Single-channel Studies in Lipid Bilayers

Single sodium channels were inserted from native plasma membrane vesicles from rat brain and rat muscle into planar phospholipid bilayers using the methods of Moczydlowski *et al.* (28) and Krueger *et al.* (29). Statistical analysis of GIIIA blocking kinetics was carried out as described previously for tetrodotoxin and saxitoxin (28, 34).

RESULTS

Purification of μ -Conotoxins—In a typical preparation 43 mg of protein, extracted from 724 mg of crude venom, was applied to the Sephadex G-25: recovery of protein was 39 mg (90%). Peptides with μ -conotoxin activity eluted earlier than did the previously described α -conotoxins (GI, GII, and GIA; Ref. 30) and ω -conotoxins (31). The pool containing the μ -conotoxins (4.4 mg, total protein) was chromatographed on reversed-phase HPLC; the toxins eluted between 9 and 18% acetonitrile, indicating a very hydrophilic nature for peptides of this size. Conotoxins GIIIA (35%), GIIIB (9%), and GIIIC (4%) accounted for approximately half of the material recovered from the column. In a later preparation, several minor peaks of activity were obtained as satellites of the major peaks. These are shown in Fig. 1, with positions of the major toxins indicated also.

Chemical Characterization of μ -Conotoxins—Table I gives the amino acid compositions of the three major toxins as measured after 24-h hydrolysis of unmodified peptides, and

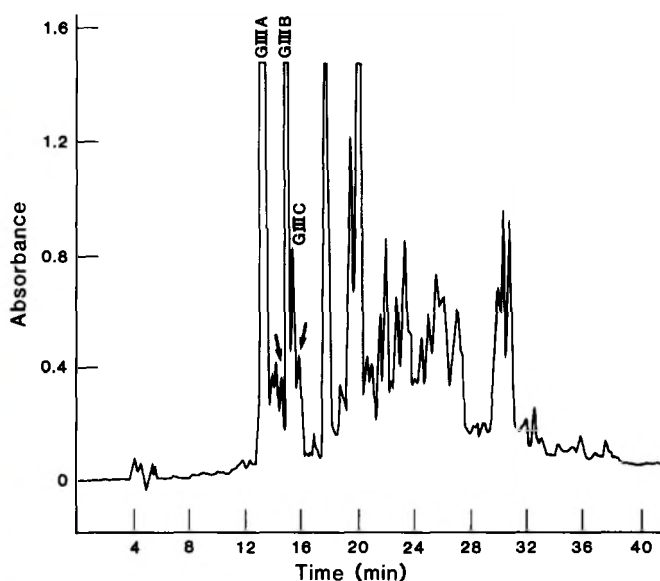


FIG. 1. Chromatographic purification of conotoxin GIIIC on VYDAC C18 semipreparative column. Solvent A, 0.1% trifluoroacetic acid; Solvent B, 0.1% trifluoroacetic acid in 60% (v/v) acetonitrile. Peptides were eluted with linear gradients, expressed as per cent Solvent B achieved at times given in parentheses (min): 0(0)/0(2)/100(52). The arrows indicate peaks corresponding to underhydroxylated forms of GIIIC.

TABLE I

Amino acid analysis of μ -conotoxins

Results are expressed as mol/mol, normalizing (Lys + Arg + Asx + Glx + Ala) to the nearest integer. Values are uncorrected for hydrolysis losses. Figures in parentheses are the numbers of residues determined from sequence analysis.

	GIIIA	GIIB	GIIC
Lys	4.0 (4)	3.9 (4)	4.9 (5)
Arg	3.0 (3)	4.2 (4)	3.1 (3)
Asx	2.1 (2)	1.9 (2)	2.0 (2)
Thr	0.93 (1)	0.87 (1)	0.93 (1)
Glx	1.8 (2)	0 (0)	0 (0)
Hyp ^a	3.1 (3)	3.4 (3)	3.1 (3)
Ala	1.1 (1)	1.0 (1)	1.0 (1)
$\frac{1}{2}$ Cys ^b	5.5 (6)	5.1 (6)	4.7 (6)
Met	0 (0)	0.86 (1)	0 (0)
Leu	0 (0)	0 (0)	1.0 (1)

^a Hydroxyproline values are subject to $\pm 10\%$ error.

^b Measured as cystine, from unmodified peptides.

as deduced from sequence studies. As we had previously described for conotoxin GIIIA, all of the peptides contain 3 mol/mol of *trans*-4-hydroxyproline (14).

The results of the sequencer analyses are shown on Table II, which gives the yields of the amino acids obtained during sequencer runs on reduced and carboxymethylated toxins. Stepwise repetitive yields were 89–90%. About half of the losses were due to tailover, which has been a persistent problem with these peptides. It is improved somewhat, but not eliminated, by using a double cleavage at hydroxyproline residues.

We used a novel procedure for reduction and carboxymethylation, based on that of Rüegg and Rudinger (20) which uses tributylphosphine. The aim was to avoid purifying small amounts of alkylated peptide from large excesses of the usual reactants. These peptides are so hydrophilic that their carboxymethyl derivatives elute among the reagent peaks on reversed-phase HPLC (data not shown). Although the reaction proceeded smoothly for vasopressin, we found that a side reaction can occur with the excess tributylphosphine. It appears that on addition of iodoacetate a quaternary phosphonium compound is produced, which can alkylate methionine and carboxymethylcysteine, giving derivatives whose phenylthiohydantoin migrate close to that of Ala. We presume that these represent transfer of a butyl group to form sulphonium compounds. The amount formed was variable: about 50% of Met-18 in GIIIB was modified, while the Cys(Cm) was unaffected; in the run shown for GIIIC, the Cys(Cm) was modified about 20%.

With GIIIA we experienced very heavy losses of the carboxy-terminal Ala. Its presence was inferred from amino acid analysis (Table I), but at step 22 a net increase of only 9 pmol above background was obtained. No other amino acid increased, and the rise was statistically significant (previous 9 steps gave 32 ± 2.5 pmol of Ala; step 22 gave 41). With GIIIB an improved yield of Ala-22 was obtained by decreasing the chlorobutane wash in the previous three steps, albeit at the expense of a lower extraction efficiency for all PTH derivatives in those steps.

For GIIIC the side reaction on Cys(Cm) (see above) precluded identification, and a separate analysis was made. Approximately 3 nmol of toxin was performed acid-oxidized, acetylated, and trypsin-digested. The results of a sequencer analysis are given in Table III. The main cleavage by trypsin was between Arg-14 and Cys-15, as evidenced by the preponderance of amino acids corresponding to the sequence 15–22. Minor cleavages were also apparent between Lys-8 and Lys-9, and between Arg-1 and Asp-2, but these did not interfere with identification of the major peptide.

Manual sequencing of conotoxin GIIIA was also carried out. Clostripain digestion of reduced and carboxamidomethylated GIIIA gave a much more complex pattern of splits than had been expected. Cleavage after Arg-13 and Arg-19 was complete, as far as we could judge: not all peaks were analyzed, since our main object was to identify the carboxyl-terminal fragment. However, "ragged" cleavage occurred also at Lys-8 and Lys-9, and possibly at Lys-11, giving several peptide fragments of similar compositions. Fragments for which good compositional and/or sequence data were obtained are indicated in Fig. 2.

Because the α -conotoxins all have amidated carboxy termini, we examined the status of this group in the μ -conotoxins. The terminal tripeptide fragment of GIIIA was isolated from the clostripain digest of carboxamidomethylated toxin and had the composition (Cys_{2,2}, Ala_{1,0}) after acid hydrolysis. It had a net positive charge on electrophoresis at pH 6.5,

TABLE II
Sequencer analyses of μ -conotoxins

Yields are expressed in pmol, uncorrected for extraction losses. * indicates differences from GIIIA.

		GIIIA				GIIIB				GIIIC		
		Yield	Back-ground	Net pmol		Yield	Back-ground	Net pmol		Yield	Back-ground	Net pmol
1	Arg	6440	0	6440	Arg	4440	0	4440	Arg	2565	0	2565
2	Asp	7040	120	6920	Asp	6910	40	6870	Asp	2920	150	2770
3	Cys	5820	760	5060	Cys	6170	480	5690	Cys	1800	380	1420
4	Cys	6040	760	5280	Cys	5640	480	5160	Cys	1780	380	1400
5	Thr	2060	0	2060	Thr	6790	130	6660	Thr	2625	20	2605
6	Hyp	4180	65	4120	Hyp	4880	230	4650	Hyp	2035	250	1785
7	Hyp	4180	65	4120	Hyp	4820	230	4590	Hyp	1965	250	1715
8	Lys	2030	200	1830	*Arg	1910	200	1710	Lys	1105	145	960
9	Lys	2570	200	2370	Lys	3110	340	2770	Lys	1720	(705) ^a	1015
10	Cys	2960	310	2650	Cys	3210	610	2600	Cys	1400	610	790
11	Lys	2200	200	2000	Lys	4570	340	4230	Lys	1495	150	1345
12	Asp	2540	140	2400	Asp	2790	290	2500	Asp	1075	240	835
13	Arg	1645	130	1515	Arg	1940	330	1610	Arg	855	265	590
14	Gln	1870	165	1705	*Arg	2760	(1265) ^a	1495	*Arg	1840	(750) ^a	1090
15	Cys	2215	375	1840	Cys	2325	635	1690	Cys	965	635	330
16	Lys	1240	170	1070	Lys	2305	490	1815	Lys	735	380	355
17	Hyp	1420	160	1260	Hyp	1680	340	1340	Hyp	750	270	480
18	Gln	1430	200	1230	*Met	780	80	700 ^b	*Leu	625	125	500
19	Arg	920	130	790	*Lys	735	245	490 ^c	*Lys	735	365	370
20	Cys	1240	340	900	Cys	620	200	420 ^c				
21	Cys	1460	(680) ^a	780	Cys	840	510	330 ^c				
22	(Ala)	41	32	9	Ala	90	32	58 ^c				
	Repetitive Yield ^d (R1-R19; D2-D12; C3-C15; Hyp-6- Hyp-17)			90%	Repetitive Yield (R1-R14; D2-D12; C3-C14; Hyp-6- Hyp-17)			90%	Repetitive Yield (R1-R13; D2-D12; C3-C15; Hyp-6-Hyp- 17)			89%

^a Parentheses indicate that background includes a large contribution from tailover of preceding residue.

^b Additional peak was present due to alkylation via tributylphosphine (see text).

^c All yields and background peaks dropped about 50%, due to shorter extraction times.

^d Repetitive yields are conservative, based on net pmol.

TABLE III
Carboxyl region of GIIIC

		Yield	Background	Net pmol
1	Cys ^a	335	0	335
2	Lys ^b	1770	310	1460
3	Hyp	1400	200	1200
4	Leu	1015	20	995
5	Lys ^b	1030	55	975
6	Cys ^a	162	81	81
7	Cys ^a	197	81	116
8	Ala	61	10	51

^a Analyzed as PTH-cysteic acid, which extracts poorly from the sequencer cup.

^b Analyzed as ϵ -N-acetyl-PTH-lysine, which elutes just after PTH-Ala on HPLC.

indicating a blocked α -carboxyl. After removal of the two residues of carboxamidomethylcysteine by Edman degradation, the remaining material was dansylated but not hydrolyzed. Electrophoresis at pH 4.4 (35) showed no dns-Ala, but there was fluorescent material running with the same mobility as that of authentic dns-Ala-NH₂. This material was eluted, hydrolyzed (6 N HCl, 20 h, 105 °C), and rerun at pH 4.4. dns-Ala was obtained, confirming the presence of amidated Ala at the carboxyl terminus.

Minor Toxin Species: Underhydroxylation—Amino acid analysis of the minor active fractions (indicated by arrows in Fig. 1) suggested that they might contain peptides related to GIIIA and GIIIB, but with 2 Hyp and 1 Pro. They were repurified by HPLC to remove possible contamination by GIIIB and GIIIC, respectively. Several components were isolated that had approximately the same biological activity on

a molar basis, as judged by peak area from HPLC.

Insufficient material was available for accurate estimation of Hyp and Pro by amino acid analysis, but three minor variants (approximately 3–4 nmol each) were performic acid-oxidized and analyzed in the sequencer.

The earlier peak, after repurification, was found to be a mixture of [Pro⁷]conotoxin GIIIA (approximately 80%) and [Pro⁶]conotoxin GIIIA (approximately 20%). There was no detectable contamination by any form of GIIIB or GIIIC, as shown by lack of Met and Leu at step 18 of the sequencer run: detection limit was 2%.

The later peak contained predominantly GIIIB underhydroxylated at positions 6 and 7. The isomers [Pro⁶]conotoxin GIIIB and [Pro⁷]conotoxin GIIIB were present in two overlapping peaks from rechromatography. Though they were not fully resolved, the equal potency of fractions containing different ratios of the two (3:1 and 1:1) suggests strongly that both isomers are active. Minor amounts (~6%) of some form of GIIIC were present, but no specific structure can be assigned.

In these fractions there was no more than 2% of isomers that lacked the hydroxyl on Hyp¹⁷. We cannot exclude these as being present elsewhere in the chromatogram.

Physiological Studies Using Purified μ -Conotoxin GIIIA—Application of μ M concentrations of μ -conotoxin GIIIA paralyzed an isolated skeletal muscle preparation from frog (cutaneous pectoralis) within a few minutes. Intracellular recordings from muscle fibers show that the toxin causes paralysis by blocking action potentials in the muscle. After exposure to 3 μ M toxin for 8 min, muscle action potentials were not elicited by direct or indirect (nerve) stimulation. In contrast, the toxin

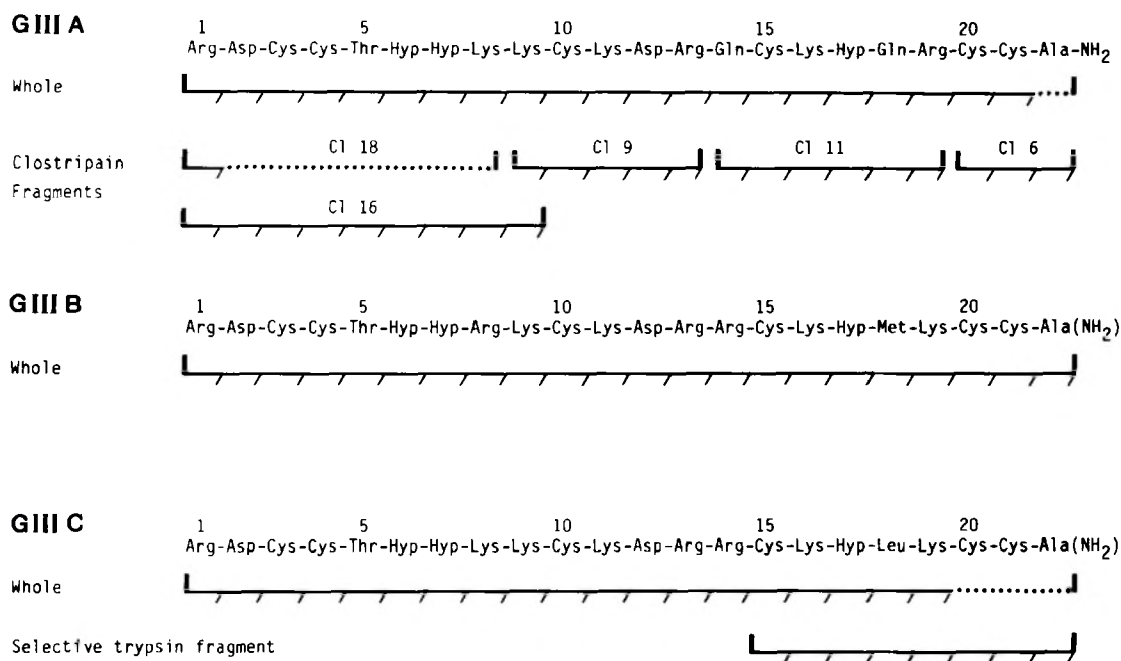


FIG. 2. Primary structures of μ -conotoxins, deduced from sequence analysis and compositions of whole toxins and fragments. Whole toxins, reduced and carboxymethylated, were analyzed in the sequencer (see text and Table II). Clostripain fragments of GIII were separated by reversed-phase HPLC, and analyzed manually and/or in the sequencer. The *selective trypsin fragment* of GIIC was produced by performic acid oxidation, acetylation, and trypsin digestion (see text and Table III). Amino acids positioned by phenylisothiocyanate degradation are indicated by bars (\rightarrow) on the appropriate lines; those whose presence, but not order, is suggested by amino acid analysis are indicated by dotted lines. Presence of the carboxy-terminal amide is positively established only for GIIIA.

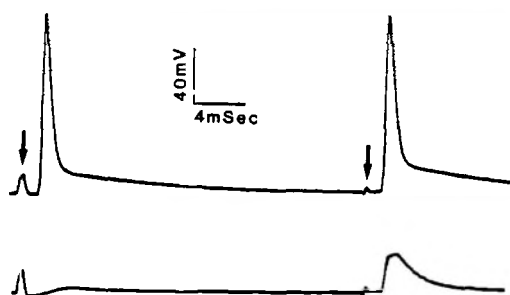


FIG. 3. The intracellular responses of a muscle fiber were monitored while the fiber was stimulated directly (left) or indirectly via its motor nerve (right). Arrows point to stimulus artifacts. Top trace, control action potentials obtained by direct and indirect stimulation in absence of toxin. Bottom trace, response after 8 min in μ -conotoxin GIIIA (3 μ M). In the bottom trace, action potentials in response to both direct and indirect stimulation are essentially completely blocked, whereas the end plate potential in response to indirect stimulation persists. Control responses were obtained after toxin was washed out, showing reversibility of toxin effects. To prevent muscle movement during stimulation, excitation contraction coupling was blocked by treatment with formamide (27).

did not block synaptic transmission, and end plate potentials could still be evoked by stimulating the motor nerve (Fig. 3). since action potentials in the nerve terminal are normally essential for neuromuscular transmission in frog (32), this observation indicates that action potentials in nerve are not blocked by the toxin under these conditions, although action potentials in muscle are. Similar results were obtained with 50 μ M toxin, with exposures of up to 15 min, showing that the action is highly selective for muscle.

One way in which the μ -conotoxin could affect muscle action potentials is by blocking sodium channels that are responsible for the action potential. To test this possibility,

the effect of μ -conotoxin on the veratridine-induced depolarization of muscle fibers was examined. Veratridine depolarizes by opening sodium action potential channels, and tetrodotoxin, the classical sodium channel inhibitor, can block this effect of veratridine. Like tetrodotoxin, μ -conotoxin repolarizes veratridine-treated muscle fibers. These results indicate that the μ -conotoxin acts by blocking the muscle sodium channel. Unlike tetrodotoxin, however, μ -conotoxin does not act on sodium channels of nerves at concentrations of at least 50 μ M. The toxin has also been tested on mouse phrenic nerve diaphragm preparations with results similar to those described above for frog muscles.

Studies on Single Sodium Channels in Planar Lipid Bilayers—In order to further explore the biochemical basis of μ -conotoxin action, planar bilayer methods were used to study this toxin's effect on single sodium channel currents from rat muscle (28) or rat brain synaptosomes (29, 33) measured at constant voltage. In these experiments, sodium channels from native membrane vesicles are incorporated into planar phospholipid bilayers in the presence of batrachotoxin, an agent belonging to the class of lipophilic activators along with veratridine (4). Batrachotoxin has been previously shown to specifically eliminate the voltage-dependent inactivation process of the sodium channel and also shift the voltage dependence of activation by about 50 mV more negative than normal channels (28, 33, 36, 37). In the voltage range of -50 to $+50$ mV, the batrachotoxin-activated channel from muscle is open 95% of the time, with the remaining 5% composed of short-lived closing events on the order of 100 ms in duration (28). This behavior is illustrated in Fig. 4A, which shows a typical current record at $+50$ mV from a bilayer containing two muscle Na^+ channels in the presence of 0.2 μ M batrachotoxin and 0.2 M NaCl on both sides of the bilayer. In this control record, when the voltage is changed from 0 to $+50$

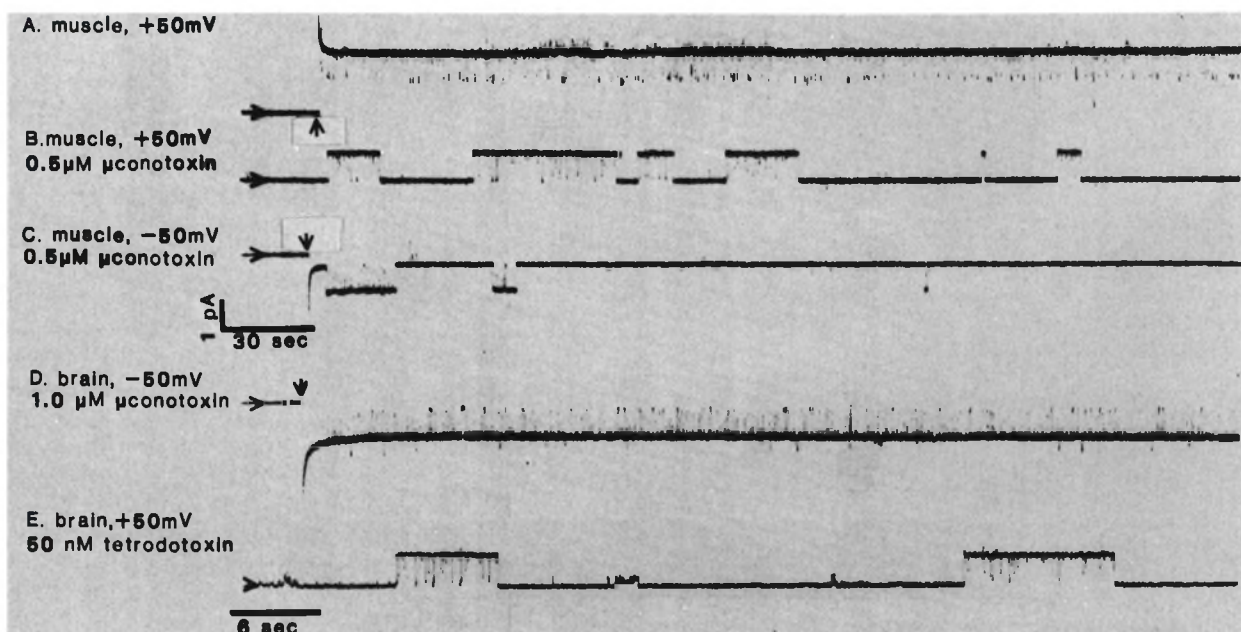


FIG. 4. μ -Conotoxin block specific for muscle Na^+ channels observed at the single channel level. Batrachotoxin-activated Na^+ channels were incorporated into planar lipid bilayers from plasma membrane preparations of adult rat skeletal muscle or brain as described previously (28, 29). Lipid bilayers were cast from a solution of 20 mg/ml of phosphatidylethanolamine and 5 mg/ml of phosphatidylcholine in decane. The bath on both sides of the bilayer contained 10 mM MOPS-NaOH, pH 7.4, 0.2 M NaCl, 0.1 mM EDTA, 0.2 μM batrachotoxin at 22 °C. The indicated concentration of μ -conotoxin GIIIA and 0.2 mg/ml of bovine serum albumin was present on the side corresponding to the extracellular face of the channel. The sign of the voltage is referred to using the physiological intracellular convention. The horizontal arrow at the left of each trace corresponds to the zero current level. In A, B, and E, opening is in the upward direction at positive voltage; in C and D, opening is in the downward direction at negative voltage. In B, C, and D, the vertical arrow marks when the holding voltage was changed from 0 mV to the indicated value as noted by the capacitive transient. In B and E, representative segments of long records are shown. A, example of a bilayer containing two muscle Na^+ channels at +50 mV; virtually identical behavior is observed at -50 mV as previously described (28). B, a different bilayer than A containing one muscle Na^+ channel at +50 mV in the presence of 0.5 μM GIIIA. Silent periods of no channel activity correspond to blocked states. C, the same bilayer as in B at -50 mV. D, a bilayer containing one brain Na^+ channel at -50 mV in the presence of 1 μM GIIIA on the extracellular side of the channel. E, a record from the same bilayer as in D after addition of 50 nM tetrodotoxin to the same side as that containing GIIIA. The time scale here is five times faster than for traces A-D.

mV, the current is observed to relax to a steady level of 2 pA after the capacitive transient. Occasional brief closing events of 1-pA amplitude (20 pS conductance) are observed, which correspond to the closing of either of the two channels in the bilayer by an inherent gating process. The control behavior at -50 mV is essentially identical to that at +50 mV (28).

In the presence of μ -conotoxin GIIIA, a new phenomenon is observed as shown in Fig. 4B. GIIIA induces the appearance of long-lived discrete blocked states in the current records. This behavior is similar to that of channels blocked by tetrodotoxin and a series of natural saxitoxin derivatives that have been documented for the rat brain (33, 38) and rat muscle (28, 34) Na^+ channel in bilayers. Two additional characteristics of tetrodotoxin and saxitoxin action are also exhibited by GIIIA: block is only observed when toxin is present on the extracellular side of the channel (not shown),³ and the blocking activity is voltage-dependent, increasing with hyperpolarization. The latter effect is shown qualitatively by the typical record of Fig. 4C, where the channel is blocked for longer periods at -50 than +50 mV (Fig. 4B). Aside from these similarities, the duration of the blocked periods induced by GIIIA is longer than that of any saxitoxin derivative tested

³ The orientation of the channel in the bilayer can be independently verified by examining its behavior at -100 mV, *i.e.* hyperpolarizing voltage closes the channel (28, 33, 38).

to date. For example, the average duration of blocking events at -25 mV is 250 s for GIIIA, while that of the most potent saxitoxin derivative (neosaxitoxin) is 120 s at this voltage (34).

In contrast to the immediate and routinely observed effect of GIIIA on muscle sodium channels, no effect was observed when sodium channels from rat brain synaptosomes were studied. An example of this result is shown in Fig. 4D, where a brain channel in the presence of 1 μM GIIIA is shown at -50 mV. Under these conditions, the rat muscle channel was blocked greater than 90% of the time. To verify that the channel in Fig. 4D is indeed a Na^+ channel, 50 nM tetrodotoxin was added to the same side of the bilayer as the 1 μM GIIIA. In Fig. 4E, a segment of a record at +50 mV shows that this μ -conotoxin-insensitive rat brain Na^+ channel is blocked by tetrodotoxin, as previously documented (33, 39).

In order to quantitate the effect of GIIIA on the muscle channel, the stochastic blocking kinetics of single channels were analyzed as previously described for tetrodotoxin (28). As predicted by a single-site, reversible binding equilibrium, the probability distributions of the blocked and unblocked dwell time events conform to exponential distribution as shown in Fig. 5A for data taken at +25 mV. Also, as shown in Fig. 5B, the reciprocal of the mean unblocked time is directly proportional to GIIIA concentration, while the mean blocked time is independent of toxin concentration. The

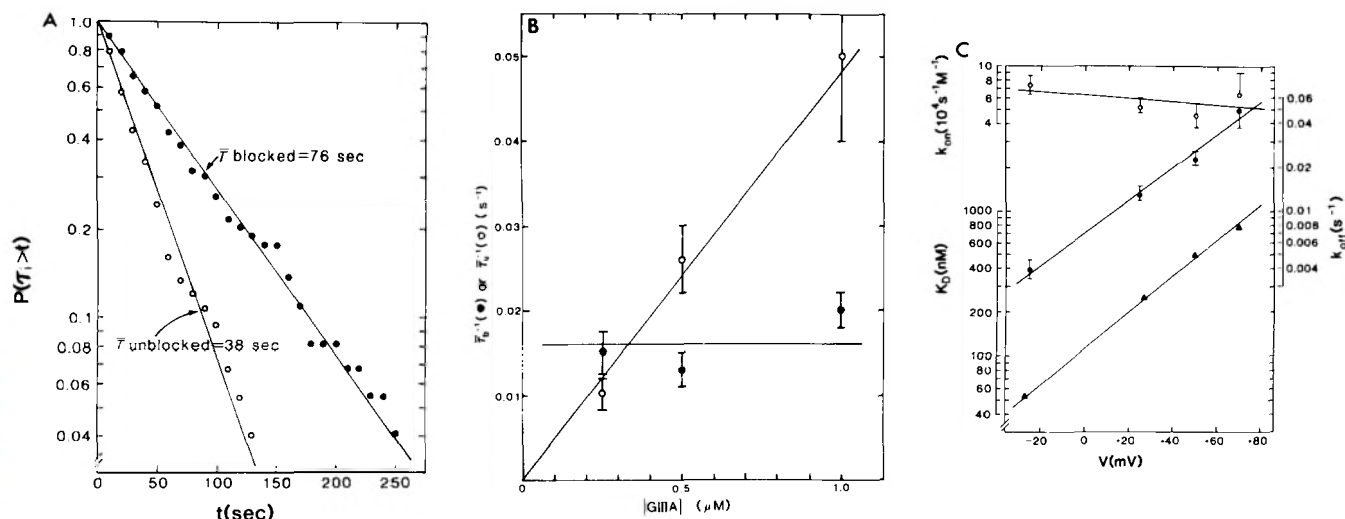


FIG. 5. Kinetics of GIIIA blocking of single batrachotoxin-activated Na⁺ channels from rat muscle in planar bilayers. *A*, cumulative probability distribution histogram compiled from 78 blocked (●) and unblocked (○) events measured at +25 mV and 0.25 μM GIIIA. The ordinate corresponds to the probability that an event is greater than a given time on the abscissa axis. The data were fitted to exponential distributions with time constants as indicated. *B*, dependence of the reciprocal mean blocked time, $\bar{\tau}_b^{-1}$ (●) or reciprocal mean unblocked time, $\bar{\tau}_u^{-1}$ (○) on GIIIA concentration. Mean values at different concentrations were measured from populations of 70–100 events. The horizontal line corresponds to the mean dissociation rate constant, 0.016 s⁻¹, of the three values of $\bar{\tau}_b^{-1}$. The slope of the linear fit of $\bar{\tau}_u^{-1}$ versus [GIIIA] corresponds to a bimolecular association rate constant of 4.8×10^4 s⁻¹ M⁻¹. *C*, voltage dependence of association (○) and dissociation (●) rate constants measured from mean dwell times in the presence of 0.5 μM GIIIA. The equilibrium dissociation constant, K_D (▲) was calculated as the ratio, k_{off}/k_{on} . Fits to exponential functions of voltage are given in the text.

results of Fig. 5, *A* and *B*, identify the blocked periods as the dwell times of individual GIIIA molecules bound to the channel and the unblocked periods as the individual waiting times for a GIIIA molecule to bind (39). For such a simple exponential binding process, the reciprocals of mean blocked and unblocked times at any voltage give the rate constants for dissociation and association, respectively. The bimolecular association rate constant can be obtained from the slope of the pseudo-first order rate constant versus concentration (Fig. 5*B*). In Fig. 5*C*, the derived dissociation and association rate constants for experiments at 0.5 μM GIIIA are plotted versus applied voltage. The dissociation rate constant was found to vary exponentially with voltage as previously documented for tetrodotoxin and saxitoxin (28, 33, 34). However, the association rate constant of GIIIA exhibits little or no dependence on voltage. The ratio of the two rate constants, or the equilibrium K_D , is also plotted in Fig. 5*C*. The data in Fig. 5*C* were fitted to exponential functions of voltage to obtain the following relationships, where the voltage, V , is in mV.

$$\begin{aligned} k_{off}(V) &= 7 \times 10^{-3} \exp(0.026 V) & \text{s}^{-1} \\ k_{on}(V) &= 6 \times 10^4 \exp(-0.003 V) & \text{s}^{-1} \text{M}^{-1} \\ K_D(V) &= 1.1 \times 10^{-7} \exp(0.029 V) & \text{M} \end{aligned}$$

GIIIA exhibits an e -fold increase in $K_D/34$ mV of depolarization, a voltage dependence similar to that previously reported for tetrodotoxin blocking of rat muscle or rat brain channels in planar bilayers (28, 33, 34, 38).

DISCUSSION

The family of μ -conotoxins described above is one of several functionally distinct classes of peptide toxins isolated from the venom of the marine snail *C. geographus*. Other peptide toxins in the venom include the ω -conotoxins, which inhibit Ca²⁺ channels at the presynaptic terminus, and the α -conotoxins, which block acetylcholine receptors at the postsynaptic terminus. We have directly demonstrated in this report

that one μ -conotoxin, μ -conotoxin GIIIA, blocks muscle Na⁺ channels. Given the tight homology of these peptides, it seems likely that the muscle Na⁺ channel is the primary physiological target of all μ -conotoxins. Thus the venom of *C. geographus* has 3 toxin families, the α -, ω -, and μ -conotoxins, which block successive steps in neuromuscular signal transmission.

Chemically, we have isolated and characterized seven active peptides of this series. All μ -conotoxins isolated to date are highly basic, 22-amino acid peptides rich in Cys residues (Table II). Purification to homogeneity has allowed us to demonstrate that one member of this particular group is highly selective in blocking the voltage-activated sodium channels of muscle membrane, while having no effect on those of nerve membranes.

Three of the μ -conotoxins, GIIIA, GIIIB, and GIIC, are different in primary structure and presumably coded by different genes. The first of these most closely corresponds to toxin III described by Spence *et al.* (13), and geographutoxin I of Nakamura *et al.* (15); GIIIB corresponds to geographutoxin II of these authors (15). Previously, we showed that GIIIA contained hydroxyproline (14), and Sato *et al.* (16) reported the amino acid sequences of GIIIA and GIIIB. In all three primary forms of the toxins, there are 3 residues of hydroxyproline, which is present in sequences different from those found in collagen. Presumably the hydroxylation enzymes are quite distinct.

The role of hydroxyproline in the toxins is unclear. Four underhydroxylated variants were isolated in low yield, and we suspect that other minor forms are also present. Although quantities were very limited, it was clear that the underhydroxylated forms of GIIIA and GIIIB are biologically active, as detected both by injection *in vivo* and by electrophysiological studies on nerve-muscle preparations. We obtained 6-Pro and 7-Pro variants of these peptides but did not detect any 17-Pro. This might reflect a somewhat less efficient activity of the enzyme on the -Pro-Pro- sequence at position 6–7 but

could also be explained by failure to separate the 17-Pro variants from the major forms. Hydroxylation is thus very efficient at all three positions in the μ -conotoxins. *Conus* venom prolyl hydroxylase does show specificity, however, since the α -conotoxins in the same venom contain exclusively the unmodified Pro, while the ω -conotoxins have hydroxyproline (31). Chemical synthesis of proline-containing variants may be simpler than that of the fully hydroxylated forms and should allow a much more thorough study of the role of hydroxylation in biological function.

The major significance of the μ -conotoxins resides in their unique physiological activity. Both electrophysiological experiments and studies using single channels in lipid bilayers indicate that μ -conotoxin GIIIA blocks muscle Na^+ channels but has little or no activity on those from nerve and brain. Even at concentrations >15-fold higher than those which completely abolish muscle action potentials, the toxin did not affect propagation of nerve action potentials. These results with the purified μ -conotoxin GIIIA are sufficient to account for the selective effects of the crude venom on muscle action potential.

The tentative conclusion drawn from early studies using crude venom was that the Na^+ channel was unlikely to be the toxin target (12). Spence *et al.* (13) were the first to suggest that the toxin might "block the inward movement of Na^+ ions during activity," although the hypothesis was not further explored. Their toxin preparation caused an inactivation of nerve action potentials as well. In order to explain the discrepancy with Edean's results with crude venom (12), it was suggested that the failure of crude venom to inactivate nerve action potentials was "most likely to be the result of poor penetration of the active peptide into the nerve bundle combined with inadequate dosing" (13). This implied that their toxin might not really discriminate between nerve and muscle Na^+ channels.

The results presented above strongly indicate that μ -conotoxin GIIIA can intrinsically distinguish between nerve and muscle Na^+ channels. Arguments that the discrimination observed may be due to selective access can be discounted for two reasons: first, the discrimination is found even in reconstituted lipid bilayers, where access should be unrestricted; and second, the much larger scorpion toxins interact with both nerve and muscle sodium channels (4), implying that access is not a critical problem even in intact nerve and muscle. We are unable to explain the results of Spence *et al.* (13) on nerve action potentials; in some of the studies above, higher toxin concentrations were used than in their work. One note of caution: all our results on discrimination to date have been obtained with the major variant, GIIIA. Although it seems likely that all μ -conotoxins inhibit muscle Na^+ channels, it remains to be established whether other variants and underhydroxylated forms discriminate as effectively against neuronal channels.

In addition to confirming the selective action of GIIIA on muscle Na^+ channels, the planar bilayer method was used to analyze the kinetics of μ -conotoxin block of single batrachotoxin-activated channels. The results of this study reveal essential similarities between the effect of GIIIA and that of tetrodotoxin or saxitoxin studied by the same method: an effect is only observed on the extracellular side of the channel, discrete blocked states of zero conductance are induced, the kinetics follow a reversible equilibrium of binding to a single site, and binding increases with hyperpolarizing voltage. On the other hand, certain aspects of GIIIA action contrast with that of tetrodotoxin and saxitoxin: both the association and dissociation rate constants of GIIIA are slower than that of

any saxitoxin derivatives examined to date (34), and most of the voltage dependence of GIIIA binding resides in the dissociation step (e -fold/40 mV), while both the association and dissociation reactions of saxitoxin derivatives have similar voltage dependence (e -fold/80 mV) (34). The similarities of GIIIA action on muscle channels, compared to the guanidinium toxins, leads us to suspect that GIIIA may share part of the tetrodotoxin/saxitoxin receptor site. To address this question, binding competition between the μ -conotoxins and saxitoxin will be the subject of future investigations using radio-labeled toxins. Also, once the μ -conotoxins are available in radioactive form, it may be possible to directly quantitate the proportion of binding sites homologous to skeletal muscle Na^+ channels in various tissues. While the present bilayer results demonstrate that GIIIA-insensitive Na^+ channels exist in brain synaptosomes, this method is inappropriate for measuring the proportion of such channels.

The strong specificity of μ -conotoxin for muscle *versus* nerve is surprising, given the functional and electrophysiological similarities of nerve and muscle sodium channel currents (40). A comparison of published data on selectivity, conductance, gating, and pharmacology for the batrachotoxin-activated channel from muscle (28, 34) and brain (33, 38) in planar bilayers reveals few differences between the single channels from these tissues as dramatic as the GIIIA results. However, it was previously suspected that the two channels might not be identical, since the brain channel has a distinctly larger conductance, about 20% greater than that of the muscle channel (28).

We can only estimate a lower limit for the selectivity of GIIIA for muscle *versus* nerve, since no effect was observed at 1 μM in the bilayer system and at 50 μM for intact nerve experiments. Assuming that the K_D of GIIIA for nerve is greater than 100 μM at 0 mV, the minimum value of the K_D ratio for nerve/muscle is 10^3 , using the muscle K_D of 0.1 μM at 0 mV, measured here. To temper our emphasis of the difference between the nerve and muscle channels, it should be pointed out that such differences in the free energy of binding can be achieved by a minor structural modification in the toxin receptor site, perhaps an alteration of only one amino acid. Although the nerve/muscle discrimination of μ -conotoxin is novel, it has been previously proposed that tissue-specific variants of Na^+ channels may exist as demonstrated by differences in sensitivity or binding of saxitoxin, tetrodotoxin, their derivatives, and other Na^+ channel toxins (41–45). The availability of μ -conotoxin provides an additional, highly tissue-specific probe of Na^+ channel structure.

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REFERENCES

- Hodgkin, A. L. & Huxley, A. F. (1952) *J. Physiol. (Lond.)* **117**, 500–544
- Catterall, W. A. (1984) *Science* **233**, 653–661
- Agnew, W. S. (1984) *Annu. Rev. Physiol.* **46**, 517–530
- Catterall, W. A. (1980) *Annu. Rev. Pharmacol. Toxicol.* **20**, 15–43
- Agnew, W. S., Levinson, S. R., Brabson, J. S. & Raftery, M. A. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2606–2610
- Nakayama, H., Withy, R. M. & Raftery, M. A. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 7575–7579
- Miller, J. A., Agnew, W. S. & Levinson, S. R. (1983) *Biochemistry* **22**, 462–470

8. Lombet, A. & Lazdunski, M. (1984) *Eur. J. Biochem.* **141**, 651-660
9. Barchi, R. L. (1983) *J. Neurochem.* **40**, 1377-1385
10. Hartshorne, R. P. & Catterall, W. A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 4620-4624
11. Whyte, J. M. & Endean, R. (1962) *Toxicon* **1**, 25-31
12. Endean, R., Parish, G. & Gyr, P. (1974) *Toxicon* **12**, 131-138
13. Spence, I., Gillissen, D., Gregson, R. P. & Quinn, R. J. (1977) *Life Sci.* **21**, 1759-1770
14. Stone, B. L. & Gray, W. R. (1982) *Arch. Biochem. Biophys.* **216**, 765-767
15. Nakamura, H., Kobayashi, J., Ohizumi, Y. & Hirata, Y. (1983) *Experientia (Basel)* **39**, 590-591
16. Sato, S., Nakamura, H., Ohizumi, Y., Kobayashi, J. & Hirata, Y. (1983) *FEBS Lett.* **155**, 277-280
17. Cruz, L. J., Gray, W. R. & Olivera, B. M. (1978) *Arch. Biochem. Biophys.* **190**, 539-548
18. McIntosh, M., Cruz, L. J., Hunkapiller, M. W., Gray, W. R. & Olivera, B. M. (1982) *Arch. Biochem. Biophys.* **218**, 329-334
19. Rivier, J. E. (1978) *J. Liq. Chromatogr.* **1**, 343-366
20. Rüegg, U. T. & Rudinger, J. (1977) *Methods Enzymol.* **47**, 111-116
21. Mitchell, W. M. (1977) *Methods Enzymol.* **47**, 165-170
22. Tarr, G. E. (1977) *Methods Enzymol.* **47**, 335-357
23. Hunkapiller, M. W. & Hood, L. E. (1978) *Biochemistry* **17**, 2124-2133
24. Edman, P. & Begg, G. (1967) *Eur. J. Biochem.* **1**, 80-91
25. Tarr, G. E., Beecher, J. F., Bell, M. & McKean, D. J. (1978) *Anal. Biochem.* **84**, 622-627
26. Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197-199
27. de Motta, G. E., Córdoba, F., de León, M. & del Castillo, J. (1982) *J. Neurosci. Res.* **7**, 163-178
28. Moczydlowski, E., Garber, S. S. & Miller, C. (1984) *J. Gen. Physiol.* **84**, 665-686
29. Krueger, B. K., Worley, J. F. & French, R. J. (1983) *Nature* **303**, 172-175
30. Gray, W. R., Luque, A., Olivera, B. M., Barrett, J. & Cruz, L. J. (1981) *J. Biol. Chem.* **256**, 4734-4740
31. Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A. & Gray, W. R. (1984) *Biochemistry* **23**, 5090-5095
32. Katz, B. & Miledi, R. (1968) *J. Physiol.* **199**, 729-741
33. French, R. J., Worley, J. F. & Krueger, B. K. (1984) *Biophys. J.* **45**, 301-310
34. Moczydlowski, E., Hall, S., Garber, S. S., Strichartz, G. S. & Miller, C. (1984) *J. Gen. Physiol.* **84**, 687-704
35. Gray, W. R. (1967) *Methods Enzymol.* **11**, 469-475
36. Huang, L. M., Moran, N. & Ehrenstein, G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 2082-2085
37. Dubois, J. M., Schneider, M. F. & Khodorov, B. I. (1983) *J. Gen. Physiol.* **81**, 829-844
38. Hartshorne, R. P., Keller, B. U., Talvenheimo, J. A., Catterall, W. A. & Montal, M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 240-244
39. Colquhoun, D. & Hawkes, A. G. (1984) in *Single-Channel Recording* (Sakmann, B. & Neher, E., eds) pp. 135-175, Plenum Press, New York
40. Campbell, D. T. & Hille, B. (1976) *J. Gen. Physiol.* **67**, 309-323
41. Rogart, R. B., Regan, L. J., Dziekan, L. C. & Galper, J. B. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 1106-1110
42. Jaimovich, E., Chicheportiche, R., Lombet, A., Lazdunski, M., Ildefonse, M. & Rougier, O. (1983) *Pflügers Arch. Eur. J. Physiol.* **397**, 1-5
43. Frelin, C., Vigne, P. & Lazdunski, M. (1983) *J. Biol. Chem.* **258**, 7256-7259
44. Pappone, P. A. (1980) *J. Physiol.* **306**, 377-410
45. Chang, C. C. & Tseng, K. H. (1978) *Br. J. Pharmacol.* **63**, 551-559