

## A Novel *Conus* Peptide Ligand for K<sup>+</sup> Channels\*

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Voltage-gated ion channels determine the membrane excitability of cells. Although many *Conus* peptides that interact with voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels have been characterized, relatively few have been identified that interact with K<sup>+</sup> channels. We describe a novel *Conus* peptide that interacts with the *Shaker* K<sup>+</sup> channel, κM-conotoxin RIIIK from *Conus radiatus*. The peptide was chemically synthesized. Although κM-conotoxin RIIIK is structurally similar to the μ-conotoxins that are sodium channel blockers, it does not affect any of the sodium channels tested, but blocks *Shaker* K<sup>+</sup> channels. Studies using *Shaker* K<sup>+</sup> channel mutants with single residue substitutions reveal that the peptide interacts with the pore region of the channel. Introduction of a negative charge at residue 427 (K427D) greatly increases the affinity of the toxin, whereas the substitutions at two other residues, Phe<sup>425</sup> and Thr<sup>449</sup>, drastically reduced toxin affinity. Based on the *Shaker* results, a teleost homolog of the *Shaker* K<sup>+</sup> channel, TSha1 was identified as a κM-conotoxin RIIIK target. Binding of κM-conotoxin RIIIK is state-dependent, with an IC<sub>50</sub> of 20 nM for the closed state and 60 nM at 0 mV for the open state of TSha1 channels.

Basic research on voltage-gated ion channels advances on two broad fronts: first, the identification and characterization of the numerous molecular isoforms that comprise each voltage-gated ion channel family. A different stream of research focuses on a few model systems intensively to uncover basic mechanistic insights. For both of these contrasting facets of ion channel research, the small peptides made by predatory cone snails (conotoxins) (1, 2) have considerable potential. Thus, the ω-conotoxins, one family of conotoxins, have become standard reagents for discriminating among Ca<sup>2+</sup> channel subtypes (3), and characterizing the functional role of each subtype. The μ-conotoxins that block voltage-gated channels are used as probes for the outer vestibule of the channel pore. The subject of this report is a novel conotoxin that has promising properties to be an important reagent for structure/function studies of the *Shaker* K<sup>+</sup> channel, arguably the most intensively studied of

all voltage-gated ion channels. However, the characterization of the peptide also defines a new family of conotoxins, the κM family, that should provide a set of new ligands specific for different K<sup>+</sup> channel isoforms.

The first *Conus* peptide shown to target a voltage-gated ion channel was μ-conotoxin GIHA, which was discovered and characterized two decades ago (4–8). A characteristic feature of all μ-conotoxins is the arrangement of disulfide cross-links in the primary sequence, the μ-conotoxin pattern can be recognized by the following pattern of Cys residues: -CC-C-C-CC-, now defined as a class III (or M-1) conotoxin scaffold (9). After the discovery of the μ-conotoxins, other groups of *Conus* peptides with three disulfide bonds (the ω-conotoxins, δ-conotoxins, μO-conotoxins, and the spasmodic peptides) were characterized, but these had a different arrangement of Cys residues. Only one other family of conopeptides with a class III disulfide framework has been characterized, the ψ-conotoxins (10). The latter are noncompetitive antagonists of nicotinic acetylcholine receptors.

In this report, a peptide from *Conus radiatus* venom ducts that has the same class III scaffold as the μ- and the ψ-conotoxins is characterized. Despite its structural affinities, the *C. radiatus* peptide proved to have an entirely different pharmacological specificity: it affects the *Shaker* K<sup>+</sup> channel and is therefore the defining member of a new family of *Conus* peptides, the κM-conotoxins. We also identify a putative K<sup>+</sup> channel target in teleost fish, the presumed prey of *C. radiatus*. The results demonstrate that the class III framework first elucidated in the μ-conotoxins has also been exploited by the cone snails in the course of their evolution to target a diversity of voltage-gated and ligand-gated ion channels.

### EXPERIMENTAL PROCEDURES

**Synthesis**—The peptides were synthesized on Rink amide resin using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry and standard side chain protection except for the cysteine residues. For all the peptides, the cysteine side chains were trityl protected.

Peptides were removed from the resin as described previously (11, 12). Preparative purification of the linear peptides was carried out by high performance liquid chromatography with either a 5–55 or 10–50% gradient of 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid, 60% acetonitrile (buffer B60). The standard one-step oxidation protocol (13) was used to fold the peptides. Fully oxidized peptides were purified by preparative high performance liquid chromatography using either a 5–55 or 10–50% gradient of B60.

**Electrophysiological Methods**—The *Xenopus* expression system was used for investigating the potential effects of κM-conotoxin RIIIK on voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels. Oocytes from *Xenopus laevis* were prepared as described previously (14, 15). Frogs were anesthetized with 0.2% Tricaine<sup>1</sup> in ice water for surgery. Following cRNA injection, the oocytes were incubated 1–5 days to allow expression of the protein.

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<sup>1</sup> The abbreviation used is: Tricaine, 3-aminobenzoic acid ethyl ester.

TABLE I  
Nucleic acid sequence of cDNA clone and predicted processing of peptide

---	GAA	AAG	AGA	CTA	CCA	TCG	TGT	TGC	TCC	CTT	AAC	TTG	CGG	CTT	TGC
---	E	K	R	L	P	S	C	C	S	L	N	L	R	L	C
CCA	GTA	CCA	GCA	TGC	AAA	CGT	AAC	CCT	TGT	TGC	ACA	GGA	TAA	---	
P	V	P	A	C	K	R	N	P	C	C	T	G	*	---	

---EKRLPSCCSLNLRLCPVPACKRNPCTG  
(C terminus of precursor)

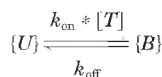
LOS CCSLNLRLCOVOACKRNOCT-NH<sub>2</sub><sup>a</sup>  
(Predicted mature peptide)

<sup>a</sup> The O represents 4-*trans*-hydroxyproline.

Prior to the electrophysiological measurements, the vitelline membranes of the oocytes were removed mechanically with fine forceps. cRNAs encoding various cloned Na<sup>+</sup> and K<sup>+</sup> channels to be tested were prepared by standard techniques. Whole cell currents were recorded under two-electrode voltage-clamp control using a Turbo-Tec amplifier (NPI Electronic, Tamm, Germany). The intracellular electrodes were filled with 2 M KCl and had a resistance between 0.6 and 1 megaohm. Current records were low-pass filtered at 1 kHz (K<sup>+</sup> channels) or 3 kHz (Na<sup>+</sup> channels) (-3dB) and sampled at 4 or 10 kHz, respectively. Leak and capacitive currents were corrected online by using a P/n method. The bath solution was normal frog Ringer's containing (in mM): 115 NaCl, 2.5 KCl, 1.8 CaCl<sub>2</sub>, 10 Hepes, pH 7.2 (NaOH). Lyophilized κM-conotoxin RIIK was dissolved in normal frog Ringer's, diluted to the final concentration, and added to the bath chamber. All electrophysiological experiments were performed at room temperature (19–22 °C).

The IC<sub>50</sub> values for the block of *Shaker* wild-type and the *Shaker* K427D channels were calculated from the peak currents at a test potential of 0 mV according to IC<sub>50</sub> = fc/(1-fc) × [Tx], where fc is the fractional current and [Tx] is the toxin concentration. For the binding of κ-conotoxin PVIIA to *Shaker* channels, it was shown that this is an approximation to obtain the affinity of the toxin to the closed state of the channel (see below). Data are given as mean ± S.D. The kinetic parameters of the state-dependent block for the different channels investigated were obtained as described in Ref. 16.

**Open Channel Binding**—From the ratio of the currents under control and toxin conditions a single exponential relaxation of the block is observed (see Fig. 5) that can be interpreted by a simple bimolecular reaction scheme,



SCHEME 1

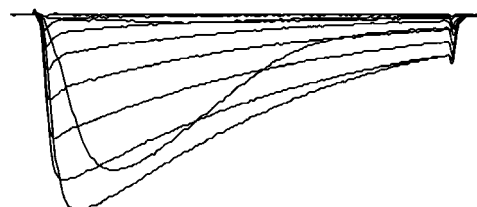
where {U} represents the toxin-free channels and {B} the channels bound to a toxin molecule. From the experimental parameters τ and U it is possible to evaluate K<sup>O</sup>, k<sub>off</sub><sup>O</sup>, k<sub>on</sub><sup>O</sup> according to the following inverse relationships:

$$K^{(O)} = \frac{[T] * U^{(O)}}{1 - U^{(O)}}; k_{\text{off}}^{(O)} = \frac{U^{(O)}}{\tau^{(O)}}; k_{\text{on}}^{(O)} = \frac{1 - U^{(O)}}{[T] * \tau^{(O)}}$$

**Closed Channel Binding**—The parameters of toxin binding to the closed state were obtained by performing a similar analysis for the currents obtained from double pulse protocols (16).

**Molecular Biology**—cDNA sequences encoding κM-RIIK were determined from a directionally cloned cDNA library prepared from poly(A)<sup>+</sup> RNA isolated from *C. radiatus* venom as described previously (17, 18). Total DNA from the library was isolated by standard methods (19), and the DNA using the polymerase chain reaction in an Air Thermo-Cycler (Idaho Technology, Salt Lake City, UT): the reaction mixture (10 μl) contained 50 ng of total DNA, 0.5 μM oligonucleotide primers corresponding to conserved nucleotide sequences at the 5'- and 3'-untranslated regions of the μ-conotoxin gene, 50 mM Tris-HCl, pH 8.5 (25 °C), 2 mM MgCl<sub>2</sub>, 250 μg/ml bovine serum albumin, 0.5 mM of each dNTP, and 0.5 units of *Taq* polymerase. PCR was carried out in capillary tubes with the thermocycler set at a denaturation temperature of 94 °C for 0 s, annealing at 54 °C for 0 s, and elongation at 72 °C for 15 s for 30 cycles. The PCR products were cloned and sequenced using standard methods. The predicted toxin sequences were compared with other known members of this family. The peptide encoded by one of the cDNAs, which was subsequently designated κM-RIIK, was synthesized and characterized using electrophysiological assays (see section above).

### Control



### 10 μM-κMRIIK

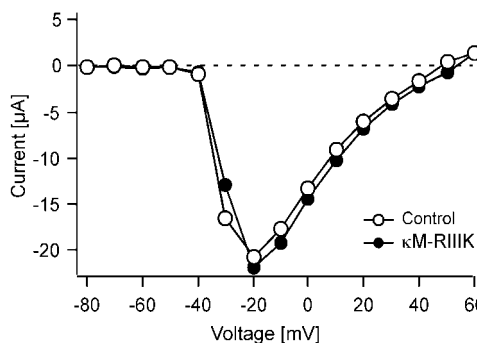
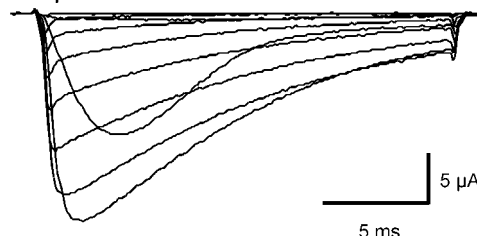


FIG. 1. κM-conotoxin RIIK does not block Na<sub>v</sub>1.4-mediated currents. Upper panel, whole cell currents recorded from an oocyte expressing Na<sub>v</sub>1.4 Na<sup>+</sup> channels evoked by test potentials from -80 to +60 mV in steps of 10 mV. Addition of 10 μM κM-conotoxin RIIK results in no effect on the evoked currents (middle panel), which is also demonstrated by the current-voltage relationships (lower panel). The dashed line corresponds to zero current.

The wild-type, Δ6-46, and substitution mutant clones of the *Shaker* K<sup>+</sup> channel were a generous gift of Dr. Martin Stocker.

## RESULTS

**Cloning and Synthesis of the κM-conotoxin**—A cDNA clone from a *C. radiatus* venom duct library was sequenced and the predicted mature peptide sequence deduced from rules now standard for conopeptide precursors. The predicted sequence, one including the expected post-translational processing of the original ribosomally translated polypeptide, is shown in Table I. Because in all known μ-conotoxins and ψ-conotoxins (the groups with amino acid sequences most related to that of the mature κM peptide), proline residues are always found to be hydroxylated, the peptide is inferred to have a hydroxyproline residue at all loci encoded by a proline codon. The sequence

N-terminal to the mature toxin contains a canonical dibasic signal for proteolytic cleavage (underlined in Table I), whereas the C-terminal amino acid sequence predicted by the clone would be expected to be post-translationally processed to an amidated C-terminal threonine residue.

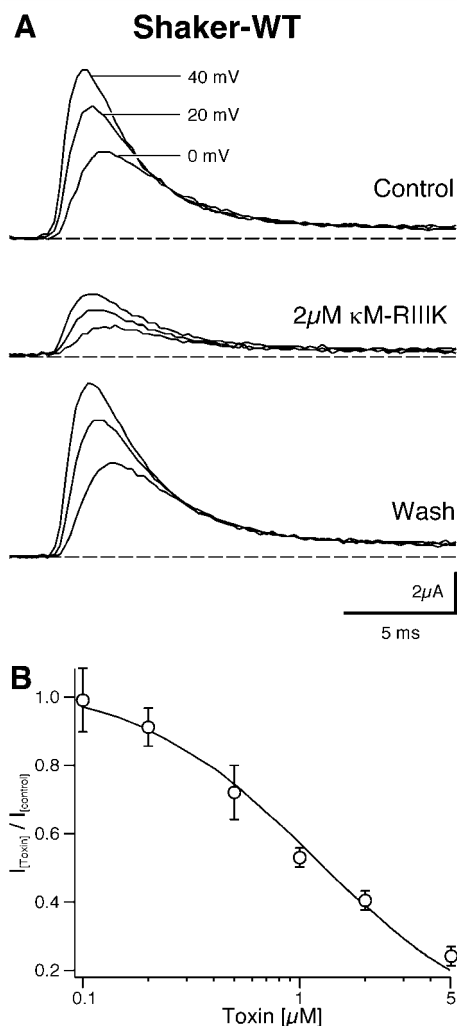
The predicted mature peptide that was chemically synthesized is shown in the bottom of Table I. Procedures used in the chemical synthesis and folding of the peptide are detailed under "Experimental Procedures."

**Biological Activity and Electrophysiological Characterization**—The peptide elicited obvious symptomatology upon injection

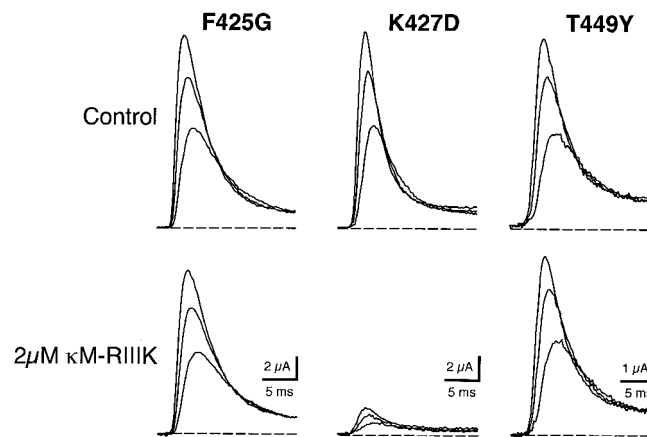
into mice both intracerebrovascular and intrathecal. When 4 nmol of the synthetic peptide were injected by the intracerebrovascular route, seizures were observed. However, when the peptide was injected intraperitoneal into mice, there were no visible effects. Electrophysiological experiments using amphibian nerve-muscle preparations were similarly unaffected by 10  $\mu\text{M}$  of the peptide.

Because the peptide has a Class III framework similar to the  $\mu$ -conotoxins (which are sodium channel ligands), the effects of the synthetic  $\kappa\text{M}$  peptide on three cloned Na<sup>+</sup> channel subtypes (*i.e.* Na<sub>v</sub>1.2 (rat brain type II), Na<sub>v</sub>1.4 (rat skeletal muscle), and Na<sub>v</sub>1.5 (mouse cardiac channel) subtypes) expressed in *Xenopus* oocyte were examined (see "Experimental Procedures"). At a concentration of 2 or 10  $\mu\text{M}$ , the peptide did not show any detectable effect on the currents produced by these cloned sodium channel subtypes. This is shown in Fig. 1 for Na<sub>v</sub>1.4, which is a high affinity target of  $\mu$ -conotoxins GIIIA and PIIIA.

The peptide has been tested on nine different cloned potassium channels. No activity (with 10  $\mu\text{M}$  peptide) was detected on Kv1.1, Kv1.3, Kv1.4, Kv2.1, Kv3.4, Kv4.2, hERG, and r-eag K<sup>+</sup> channel clones expressed in oocytes. However, when the peptide was tested on the *Shaker* K<sup>+</sup> channel, an inhibition of channel conductance was observed as shown in Fig. 2. The inhibition is readily reversible as shown in the bottom panel of Fig. 2A. The IC<sub>50</sub> for the *Shaker* channel obtained from measuring the peak currents is  $1.21 \pm 0.25 \mu\text{M}$  ( $n = 5$  dose-response experiments, see Fig. 2B). The Hill coefficient is  $\sim 1$ , suggesting that binding of a single toxin molecule is sufficient to inhibit the *Shaker* channel.



**FIG. 2.  $\kappa\text{M}$ -conotoxin R11IK blocks *Shaker*-mediated currents.** A, whole cell currents recorded from an oocyte expressing *Shaker* K<sup>+</sup> channels evoked by test potentials to 0, 20, and 40 mV (upper panel). Addition of 2  $\mu\text{M}$   $\kappa\text{M}$ -conotoxin R11IK results in a block of the currents (middle panel), which is reversible (lower panel). The dashed line corresponds to zero current. B, dose-response curve for the block by  $\kappa\text{M}$ -conotoxin at a test potential of 0 mV ( $n = 5$ ).



**FIG. 3. Mutations in the pore do affect the binding of  $\kappa\text{M}$ -conotoxin R11IK to *Shaker* channels.** Upper panel, whole cell currents from oocytes expressing the mutated channel of *Shaker* H4. Mutating the phenylalanine to glycine (F425G) results in a channel that is insensitive to 2  $\mu\text{M}$   $\kappa\text{M}$ -conotoxin R11IK (left panel). Mutating the lysine 427 to an aspartate (K427D) results in a channel with increased sensitivity to this toxin (middle panel). Mutating the threonine at position 449 to tyrosine (T449Y) results in a channel that is insensitive to 2  $\mu\text{M}$   $\kappa\text{M}$ -conotoxin R11IK. Voltage steps are as described in the legend to Fig. 2.

TABLE II

Comparison of *Shaker* and *TShal* K<sup>+</sup> channel sequences

Shown is the TShal amino acid sequence in the P-loop region between S5 and S6 (Nguyen *et al.*, (20)) aligned with the corresponding *Shaker* sequence.

		425	427			449	
<i>Shaker</i>	S5	E	A	G	S	E	N
		S	F	F	K	S	I
		P	D	A	F	W	W
		V	V	T	M	T	T
		V	G	Y	G	D	M
		T	P	V	G	V	W
		G	K				
<i>Tshal</i>		E	A	D	E	F	E
		S	Q	F	E	S	I
		P	D	A	F	W	W
		V	V	S	M	T	T
		V	G	Y	G	D	M
		V	P	T	T	I	G
		G	K				
		352	354			376	
		S6				S6	

**Studies Using Shaker  $K^+$  Channel Mutants with Single Residue Substitutions**—The interaction of *C. radiatus* peptide with a number of mutants of the *Shaker* potassium channel were assessed. Many ligands that decrease the conductance of the *Shaker*  $K^+$  channel bind to the outer vestibule of the *Shaker* channel. Among the key amino acids in this general region found to affect the affinity of other *Shaker*  $K^+$  channel ligands are Phe<sup>425</sup>, Lys<sup>427</sup>, and Thr<sup>449</sup>. We therefore determined whether single amino acid substitutions at these loci might affect the affinity of the *C. radiatus* peptide for the *Shaker* channel.

The results for three different amino acid substitutions (F425G, K427D, and T449Y) are shown in Fig. 3. The different substitutions show strikingly different effects; two of the mutant channels, F425G and T449Y (the latter affects the tetraethyl ammonium sensitivity of the *Shaker* channel), were found to be much more resistant to the *C. radiatus* peptide. In contrast, the K427D mutant exhibited about a 10-fold greater affinity for the toxin ( $IC_{50} = 109 \pm 61$  nM,  $n = 5$ ) than was observed for the wild-type *Shaker* channel. A smaller increase is observed when Lys<sup>427</sup> is substituted with a neutral amino acid leading to an  $IC_{50}$  of  $180 \pm 27$  nM ( $n = 3$ ) for the K427N substitution. The results in Fig. 3 reveal that substitution of any of the three residues, believed to be near the extracellular opening of the channel pore, significantly affects toxin affinity. Thus, the three AAs appear to be significant determinants for the peptide to bind to the *Shaker*  $K^+$  channel, with either increases or decreases in affinity observed.

The data are consistent with the *C. radiatus* peptide blocking the conductance of the *Shaker*  $K^+$  channel by interactions with the outer vestibule region. Presumably, toxin binding would block *Shaker* channel conductance by impeding transit of  $K^+$  through the extracellular opening of the pore.

**Effects on a Fish  $K^+$  Channel: The Sha1  $K^+$  Channel from Trout**—Because *C. radiatus* is believed to be a fish-hunting cone snail, the results above suggest that the presumptive physiologically relevant molecular target is a voltage-gated  $K^+$  channel in fish. We tested one teleost  $K^+$  channel available as a cDNA clone, the Sha1 channel from trout (20). We chose the Sha1 channel because the results with *Shaker* showed that the K427D *Shaker* mutant has a higher affinity than wild-type, and in the trout sequence, the homologous position to Lys<sup>427</sup> in *Shaker* has a Glu residue (see Table II).

The results are shown in Fig. 4. The Sha1 channel, which is a noninactivating voltage-gated  $K^+$  channel, is more potently inhibited by the *C. radiatus* peptide than is the *Shaker* channel. At a concentration of  $1 \mu\text{M}$   $\kappa\text{M}$ -conotoxin RIIIK the evoked currents are almost completely inhibited. The inhibition is reversible, as well as voltage-dependent. The effects of the toxin as a function of test potential are shown in the *bottom panel* of Fig. 4. These results directly establish that the toxin is able to block the conductance of a vertebrate voltage-gated  $K^+$  channel.

**State Dependence of Shaker  $K^+$  Channel Inhibition**—We evaluated whether the affinity of the peptide changed as a function of the state of the *Shaker* channel. The open channel properties were investigated by relaxation of partial block during step depolarizations. For this work,  $\Delta 6-46$  channels of *Shaker* lacking the N-type inactivation were used: this made it easier to evaluate unblocking of open channels. A sample of results obtained are shown in Fig. 5; the unblock follows an exponential time course and is voltage-dependent. Similar results were obtained for *Shaker*- $\Delta 6-46$  K427D and the TSha1 (Fig. 5).

Double-pulse protocols were used to characterize the re-equilibration of closed channel binding (see Terlau *et al.*, Ref. 16). The data derived from the two types of experiments were

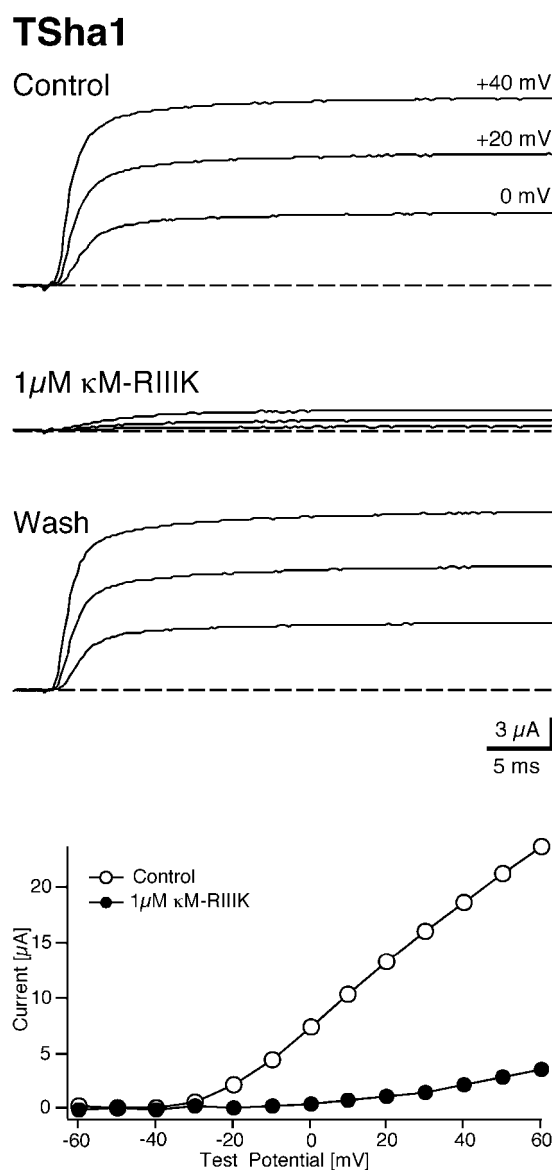


FIG. 4. The trout homolog of *Shaker* channels, TSha1, is blocked by  $\kappa\text{M}$ -conotoxin RIIIK. *A*, whole cell currents recorded from an oocyte expressing TSha1  $K^+$  channels evoked by test potentials to 0, 20, and 40 mV (*upper panel*). Addition of  $1 \mu\text{M}$   $\kappa\text{M}$ -conotoxin RIIIK almost completely blocks the currents (*middle panel*) in a reversible manner (*lower panel*). The dashed line corresponds to zero current. *B*, I-V-relationship of the evoked current in the absence and presence of  $1 \mu\text{M}$   $\kappa\text{M}$ -conotoxin RIIIK.

used to calculate kinetic parameters ( $K_{on}$ ,  $K_{off}$ , and  $IC_{50}$ ) for both the open and the closed states of the *Shaker*- $\Delta 6-46$  channel, *Shaker*- $\Delta 6-46$  K427D, and the TSha1 from trout (see Table III). The calculations demonstrate that binding of this toxin to open *versus* closed channels is very different, *i.e.* that the toxin interactions with the *Shaker* channel are state-dependent.

For all three channel types the affinity of the toxin for the open state measured at 0 mV is about three to four times lower compared with the closed state. The results show that the affinity of  $\kappa\text{M}$ -conotoxin RIIIK to TSha1 from trout is about 20 nM for the closed and 60 nM for the open state measured at 0 mV. This demonstrates that TSha1 is a high affinity target for this peptide. The other data are in accordance with the results obtained with *Shaker* wild type and K427D channels (with inactivation; Figs. 2 and 3) demonstrating that for fast inactivating channels the calculation of the  $IC_{50}$  from the peak currents is an approximation for the affinity to the closed state of

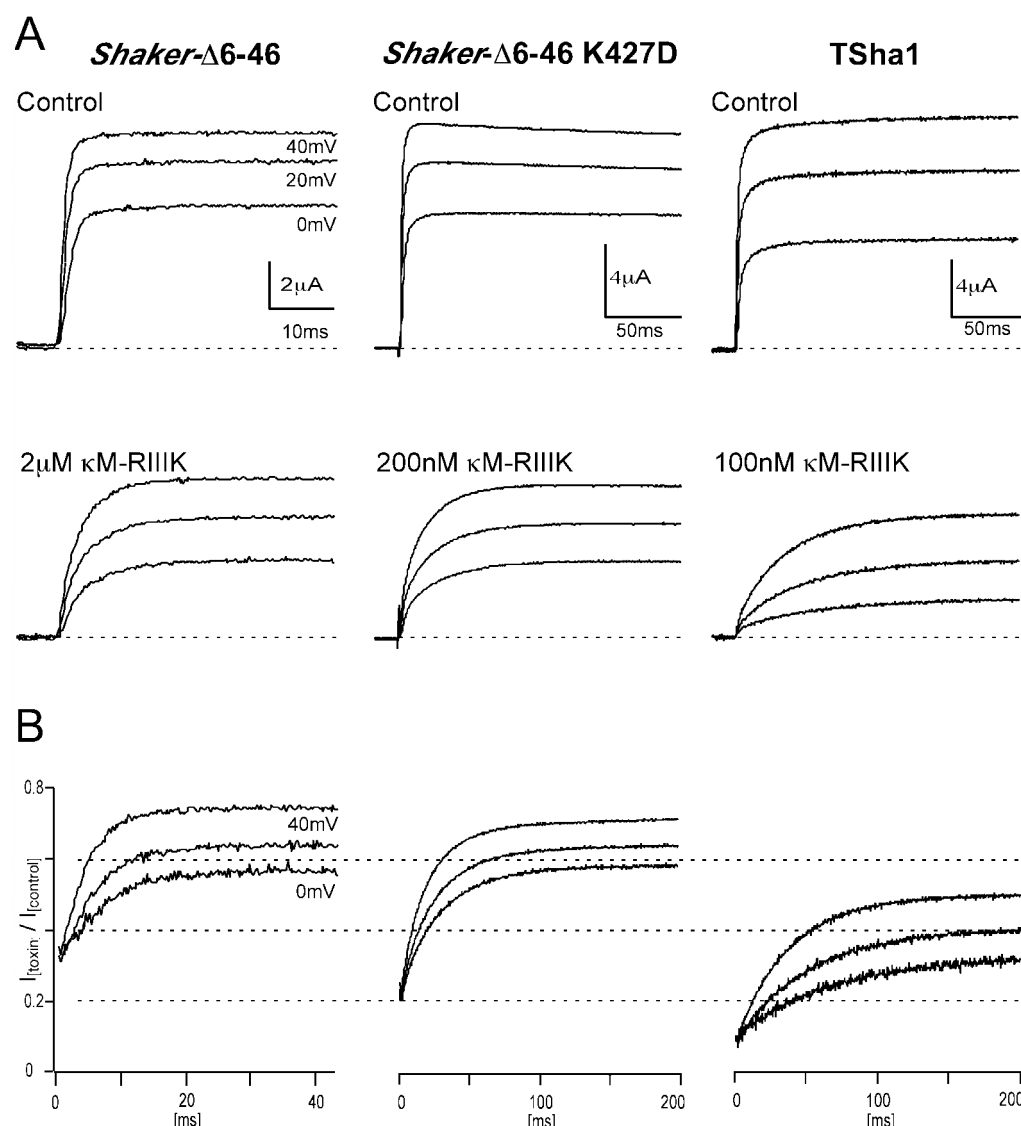


FIG. 5. The block of  $\kappa$ M-conotoxin RIIIK of *Shaker-Δ6-46*, *Shaker-Δ6-46 K427D*, and *TSha1* is state-dependent. A, whole cell currents recorded from oocytes expressing *Shaker-Δ6-46*, *Shaker-Δ6-46 K427D*, or *TSha1*  $K^+$  channels evoked by test potentials to 0, 20, and 40 mV under control conditions (upper panel) and after addition of the indicated amounts of  $\kappa$ M-conotoxin RIIIK (lower panel). B, current ratios obtained for the three test potentials showing a single exponential relaxation of the probability of the channels to be unblocked obtained by calculating  $I_{\text{Toxin}}/I_{\text{Control}}$ .

those channels (see Terlau *et al.*, Ref. 16).

Furthermore, Table III shows that the major reason for the lower affinities of the  $\kappa$ M-peptide to the open state of the channels is an increase in  $k_{\text{off}}^{(O)}$  but also the  $k_{\text{on}}^{(O)}$  is affected. The comparison of the kinetic parameters of the binding for the three different channel reveals that the  $k_{\text{off}}^{(O)}$  for open channel binding for *TSha1* is about 20 times smaller than in the *Shaker-Δ6-46* channel. In contrast, the  $k_{\text{on}}^{(O)}$  in *TSha1* is only three times higher than in the *Shaker-Δ6-46* channel. The binding kinetics of  $\kappa$ M-RIIIK to the closed state of the three channel types investigated is slower than the binding to the open state.

The data shown in Table III indicate that the negatively charged residues in *Shaker-Δ6-46 K427D* and the *TSha1* channel strongly affect  $k_{\text{on}}^{(C)}$ , which is almost identical in these two channels, but about four times higher than in the *Shaker-Δ6-46* channel. In contrast, the  $k_{\text{off}}^{(C)}$  differs by a factor of three between *Shaker-Δ6-46* and *Shaker-Δ6-46 K427D* channels, but by a factor of 10 between the *Shaker-Δ6-46 K427D* channel and *TSha1*. This indicates that a negatively charged amino acid at residue 427 is a key determinant for rapid association, whereas  $k_{\text{off}}^{(C)}$  is primarily influenced by other residues.

## DISCUSSION

The peptide characterized above, although structurally related to the  $\mu$ -conotoxins that block voltage-gated  $Na^+$  channel conductance, clearly inhibits the *Shaker*  $K^+$  channel. We designate this peptide as  $\kappa$ M-conotoxin RIIIK, the first member of a new family of conotoxins targeted to  $K^+$  channels.

The characterization of  $\kappa$ M-RIIIK is noteworthy in several respects. First, the discovery that  $\kappa$ M-RIIIK targets the *Shaker* potassium channel provides a new scaffold for a *Shaker* channel ligand, the smallest disulfide cross-linked framework so far characterized for any polypeptide antagonist of  $K^+$  channels. Because this is the most intensively studied voltage-gated ion channel both from a molecular and functional perspective, the availability of a novel framework in a small polypeptide ligand provides a new platform for examining the topology of this and related channels. We have also established that the interaction of  $\kappa$ M-conotoxin RIIIK with the *Shaker* channel is state-dependent. Thus, the peptide is a potentially useful probe for conformational changes that occur in the transition of a voltage-gated ion channel from a closed to an open state.

TABLE III  
 Summary of  $K_D$ ,  $k_{on}$ , and  $k_{off}$  values

Data are given as mean  $\pm$  S.D.;  $n$  = number of independent experiments.  $K_D$ ,  $k_{on}$ , and  $k_{off}$  values for the binding of  $\kappa$ M-conotoxin RIIIK to the open and closed state of the different channels are calculated as described under "Experimental Procedures" (see also Ref. 16).

	Open state				Closed state			
	$k^{(O)}$ (0 mV)	$k_{on}^{(O)}$	$k_{off}^{(O)}$	$n$	$K^{(C)}$	$k_{on}^{(C)}$	$k_{off}^{(C)}$	$n$
	nM	$\mu M^{-1} s^{-1}$	$s^{-1}$		nM	$\mu M^{-1} s^{-1}$	$s^{-1}$	
<i>Shaker</i> - $\Delta$ 6-46	3330 $\pm$ 1960	30 $\pm$ 7	90 $\pm$ 30	3	1260 $\pm$ 360	4.0 $\pm$ 0.7	6.0 $\pm$ 3.0	4
<i>Shaker</i> - $\Delta$ 6-46K427D	392 $\pm$ 194	57 $\pm$ 26	19 $\pm$ 2.5	3	146 $\pm$ 54	16.7 $\pm$ 8.6	2.2 $\pm$ 0.9	6
Tsha1	65 $\pm$ 31	88 $\pm$ 25	5.2 $\pm$ 1.8	4	18 $\pm$ 12	16.9 $\pm$ 6.9	0.25 $\pm$ 0.04	3

 TABLE IV  
 Comparison of  $\kappa$ M-conotoxin RIIIK to other M-superfamily Peptides and  $\kappa$ -PVIIA

O = 4-*trans*-hydroxyproline; Z = pyroglutamate, # denotes an amidated C-terminal amino acid. The arrow indicates the Arg residue known to be critical for  $\mu$ -conotoxin function, which is absent in  $\kappa$ M-RIIIK. Note that the  $\kappa$ M-,  $\mu$ -, and  $\psi$ -conotoxins all have the same pattern of Cys residues, whereas  $\kappa$ -PVIIA, the only other peptide that also blocks  $K^+$  channels, has an entirely different arrangement of Cys.

$\kappa$ M-RIIIK	LOS <b>CC</b> SLNLR <b>LC</b> OV <b>OA</b> <b>CK</b> RN <b>O</b> <b>CC</b> T#
$\mu$ -GIIIA	RD <b>CC</b> TO <b>OK</b> K- <b>CKD</b> R <b>Q</b> <b>CK</b> O <b>Q</b> R <b>CC</b> A#
$\mu$ -PIIIA	ZR <b>CC</b> GF <b>OK</b> S- <b>CRS</b> R <b>Q</b> <b>CK</b> O <b>H</b> R <b>CC</b> #
	↑
$\psi$ -PIIIE	HO <b>CC</b> LYG <b>K</b> RRYOG <b>CS</b> SAS <b>CC</b> QR#
$\kappa$ -PVIIA	<b>CR</b> ION <b>Q</b> <b>K</b> CF <b>Q</b> HLDD <b>CS</b> SR <b>K</b> CNRFN <b>K</b> CV

It was unexpected that a scaffold well known for sodium channel-targeted ligands would also be used by a *Conus* species to target potassium channels. In retrospect, because these ion channels belong to the same superfamily, the observation can be rationalized ex post-facto. As will be detailed elsewhere, the  $\mu$ -conotoxins and  $\kappa$ M-conotoxin RIIIK belong to the same gene superfamily of conopeptides.

The sequences of two  $\mu$ -conotoxins and a  $\psi$ -conotoxin are compared with the  $\kappa$ M-conotoxin in Table IV. The  $\kappa$ M-conotoxin is distinctive in having a longer first loop, between the second and third cysteine residues. Structure/function studies on the  $\mu$ -conotoxins have established that the arginine residue indicated by the arrow (Arg<sup>13</sup> in  $\mu$ -conotoxin GIIIA) is a critical residue for blocking voltage-gated sodium channels, because the charged guanidino group of the arginine residue is believed to functionally block the pore. In contrast, both the  $\psi$ -conotoxins and  $\kappa$ M-conotoxin RIIIK lack this critical arginine residue. These differential biochemical characteristics provide a guide in the search for, and identification of, additional members of the  $\kappa$ M-conotoxin family. Considerable work has been done on  $\mu$ -conotoxin/sodium channel interactions. Whether the orientation of  $\kappa$ M-conotoxin within the potassium channel is analogous to the orientation of  $\mu$ -conotoxins in the outer vestibule region of sodium channels remains to be determined.

$\kappa$ M-Conotoxin RIIIK is not the only conopeptide known to inhibit the *Shaker*  $K^+$  channel by binding to the outer vestibule. This was first demonstrated for  $\kappa$ -conotoxin PVIIA from *Conus purpurascens* (21–23). Although both *C. radiatus* (the source of  $\kappa$ M-RIIIK) and *C. purpurascens* are probably fish-hunting, they are not closely related species as judged by available molecular phylogeny data for the genus *Conus* (24, 25). Because of the accelerated evolution of venom peptides during speciation through focal mutation (1, 2, 26–28), the different groups of *Conus* species use a different spectrum of conotoxin families as major ligands in their venoms. The results pre-

sented here establish that two different species of cone snails have evolved structurally and genetically unrelated peptides, both of which block the *Shaker*  $K^+$  channel. The two peptides have entirely different structural scaffolds;  $\kappa$ M-RIIIK is most closely related to the  $\mu$ -conotoxins, whereas  $\kappa$ -PVIIA has the greatest structural similarity to the  $\omega$ -conotoxins, which target voltage-gated  $Ca^{2+}$  channels. Even more conopeptides have been found that target the *Shaker*  $K^+$  channel,<sup>2</sup> and which are genetically and structurally unrelated to either PVIIA or  $\kappa$ M-RIIIK. Thus, screening a broad range of *Conus* venoms makes it possible to identify a structurally diverse set of ligands that target a given ion channel subtype.

The effect of *Shaker*  $K^+$  channel amino acid substitutions on the interaction with  $\kappa$ M-conotoxin RIIIK has provided insight into the physiologically relevant channel target of the peptide. The discovery that the K427D *Shaker* mutant had a higher affinity for the toxin suggested that the physiological target of this peptide might have a negatively charged residue at the homologous locus. Because *C. radiatus* is believed to be a piscivorous *Conus* species, we examined the sequences of recently cloned teleost channels (20) related to the *Shaker* potassium channel. Because the Sha1 channel from trout had a negative residue at this position, we tested the peptide on the trout channel expressed in oocytes. The trout Sha1 channel was potentially inhibited by  $\kappa$ M-conotoxin RIIIK with an  $IC_{50}$  of 20 nM for the closed state. Thus, this teleost voltage-gated  $K^+$  channel subtype is a better target for  $\kappa$ M-conotoxin RIIIK than *Shaker*, exhibiting an almost 50-fold higher affinity. Although little is known about the true teleost prey of *Conus radiatus*, we postulate that the actual high affinity target of  $\kappa$ M-conotoxin RIIIK is a voltage-gated  $K^+$  channel related to the trout Sha1 channel. However, the full spectrum of  $K^+$  channels in teleost fish has not yet been elucidated.

Previously, it has been suggested that positively charged toxins have an accelerated dissociation from open channels because of the voltage-dependent occupancy of a site at the outer end of the conducting pore by a  $K^+$  ion (16). The site has been postulated to also be occupied by external cations in closed channels, thereby antagonizing the association rate. Our results with the *C. radiatus* peptide, which is positively charged, are generally consistent with this model and suggest that electrostatic interactions between the peptide and  $K^+$  ions in the pore are a major factor in state-dependent binding.

Finally, we speculate on the endogenous biological role of  $\kappa$ M-conotoxin RIIIK. One potential function for  $K^+$  channel-targeted *Conus* peptides was previously proposed: that they serve as components of the "lightning-strike cabal" of toxins that cause excitotoxic shock, a physiological strategy to instantaneously immobilize prey (2, 21). Thus, a reasonable hypothesis is that  $\kappa$ M-conotoxin RIIIK targets a  $K^+$  channel subtype in peripheral axons similar to the trout Sha1 channel, and in combination with other excitatory peptides (such as the  $\delta$ -conotoxins that inhibit Na channel inactivation), causes a massive

<sup>2</sup> J. Imperial, H. Terlau, and B. Olivera, unpublished data.

depolarization of peripheral axons near the venom injection site. This elicits trains of action potentials propagated bidirectionally, in effect, the action potentials directed centrally allow the toxins to functionally bridge the blood-brain barrier; the end point is equivalent to a tonic/clonic seizure, resulting in a very rapid tetanic paralysis of the fish prey.

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