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Single Amino Acid Substitutions in κ-Conotoxin PVIIA Disrupt Interaction with the *Shaker* K^+ Channel*

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κ-Conotoxin PVIIA (κ-PVIIA), a 27-amino acid peptide with three disulfide cross-links, isolated from the venom of *Conus purpurascens*, is the first conopeptide shown to inhibit the *Shaker* K^+ channel (Terlau, H., Shon, K., Grilley, M., Stocker, M., Stühmer, W., and Olivera, B. M. (1996) *Nature* 381, 148–151). Recently, two groups independently determined the solution structure for κ-PVIIA using NMR; although the structures reported were similar, two mutually exclusive models for the interaction of the peptide with the *Shaker* channel were proposed. We carried out a structure/function analysis of κ-PVIIA, with alanine substitutions for all amino acids postulated to be key residues by both groups. Our data are consistent with the critical dyad model developed by Ménez and co-workers (Dauplais, M., Lecoq, A., Song, J., Cotton, J., Jamin, N., Gilquin, B., Roumestand, C., Vita, C., de Medeiros, C., Rowan, E. G., Harvey, A. L., and Ménez, A. (1997) *J. Biol. Chem.* 272, 4802–4809) for polypeptide antagonists of K^+ channels. In the case of κ-PVIIA, Lys^7 and Phe^9 are essential for activity as predicted by Savarin *et al.* (Savarin, P., Guenneugues, M., Gilquin, B., Lamthanh, H., Gasparini, S., Zinn-Justin, S., and Ménez, A. (1998) *Biochemistry* 37, 5407–5416); these workers also correctly predicted an important role for Lys^{25}. Thus, although κ-conotoxin PVIIA has no obvious sequence homology to polypeptide toxins from other venomous animals that interact with voltage-gated K^+ channels, there may be convergent functional features in diverse K^+ channel polypeptide antagonists.

The venomous cone snails have evolved a neuropharmacological strategy largely based on highly sophisticated peptide ligands, the conotoxins (for reviews see Refs. 3 and 4). Particular molecular forms of both voltage-gated and ligand-gated ion channel families are targets of peptides belonging to specific conotoxin families. Among the ligand-gated ion channels that *Conus* peptides are known to inhibit are nicotinic acetylcholine receptors (5), *N*-methyl-D-aspartate receptors (3, 6, 7) and $5HT_3$ receptors (8). In the voltage-gated ion channel superfamily, *Conus* peptides specific for various subtypes of calcium channels (9) and sodium channels (10) have been described. κ-Conotoxin PVIIA from the venom of the Eastern Pacific fish-hunting species *Conus purpurascens* was the first conopeptide described that targets voltage-gated potassium channels (1, 11).

The role of κ-conotoxin PVIIA in prey capture has been established (1); this peptide is key to the very rapid immobilization of fish prey by *C. purpurascens*. This cone species uses the following two groups of toxins to affect different physiological programs in the prey after venom injection: a very rapid excitotoxic shock and a slower onset but irreversible neuromuscular block. κ-Conotoxin PVIIA plays a role in the initial excitotoxic shock response to venom injection; in combination with δ-conotoxin PVIA (which delays inactivation of voltage-gated sodium channels), an immediate tetanic-like immobilization is elicited upon injection into fish.

The voltage-gated potassium channel *Shaker*, cloned from *Drosophila melanogaster*, is sensitive to κ-conotoxin PVIIA (1). Electrophysiological characterization of the interaction of κ-PVIIA[1] with chimeras between the *Shaker* channel and a κ-conotoxin-insensitive potassium channel from rat brain (Kv-1.1) demonstrated that the binding determinants on *Shaker* were in the pore region of the channel, presumably at the extracellular face (11).

Recently, two different groups published structures for κ-PVIIA, determined on the basis of multidimensional NMR techniques (12, 13); the structures were generally similar. However, the interpretation of likely mechanisms of the molecular orientation of κ-conotoxin PVIIA in the channel mouth suggested by the two groups, based on the structural information, differed. Scanlon *et al.* (12) hypothesized that κ-PVIIA interaction with the *Shaker* potassium channel might be strongly analogous to the charybdotoxin-*Shaker* channel interaction (for a review of the charybdotoxin family, see Refs. 14 and 15). Although the two toxins share little primary sequence homology, it was suggested that specific amino acids in the κ-conotoxin (His^{11}, Arg^{18}, Lys^{19}, and Arg^{22}) might correspond to four key residues in charybdotoxin (Lys^{11}, Arg^{25}, Lys^{27}, and Arg^{31}). In contrast, Savarin *et al.* (13) suggested that a dyad motif of lysine and an aromatic residue (Lys^7 and Phe^9 or Phe^{23}) shared by a variety of unrelated toxins that inhibit voltage-gated potassium channels, including charybdotoxin from scorpion and BgK from sea anemone (2), would be a key determinant for κ-conotoxin PVIIA binding to the *Shaker* channel.

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[1] The abbreviations used are: κ-PVIIA, κ-conotoxin PVIIA; HPLC, high performance liquid chromatography; WT, wild-type; Ω, ohm; mut, mutant.

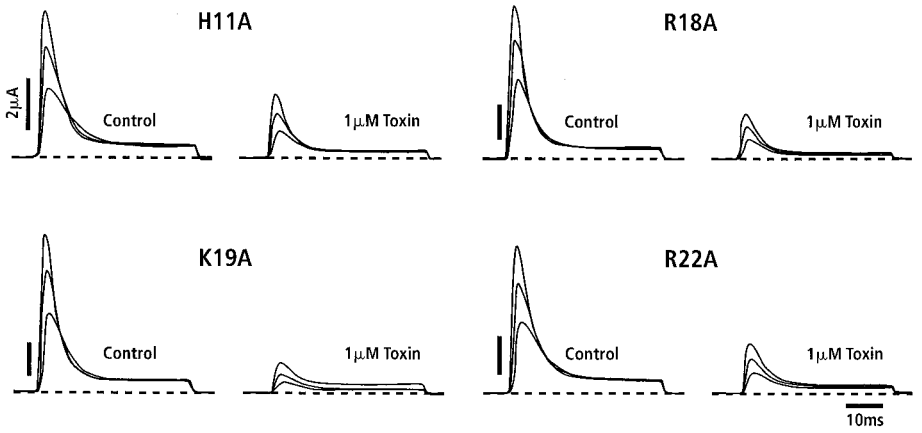

FIG. 1. **Mutation of the residues His[11], Arg[18], Lys[19], and Arg[22] to alanine results in isoforms of κ-conotoxin PVIIA that block the *Shaker* K+ channel with a potency close to the unsubstituted toxin.** Whole cell currents recorded from oocytes expressing *Shaker* H4 K+ channels upon depolarization to 0, 20, and 40 mV are shown before (*Control*) and after addition of 1 μM of the κ-conotoxin isoform indicated. The holding potential was −100 mV. Currents in the presence of toxin exhibit an apparent slowing of the activation/inactivation kinetics and an increased steady state current. This is because of the state-dependent unblocking during depolarization (see Ref. 19).

We have carried out a mutant cycle analysis of κ-conotoxin PVIIA; the results identify a binding surface on the toxin that is clearly critical for high affinity K+ channel blocking and provide functional evidence for evaluating the two hypotheses that were presented on the basis of structural information.

EXPERIMENTAL PROCEDURES

Solid-phase Peptide Synthesis—The protected peptide resin was built using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry as described previously (11) with side chains protected as follows: pentamethylchromasulfonyl (Arg), *t*-butyl (Hyp, Asp, and Ser), S-trityl (Asn, Gln, His, and Cys), and *t*-butoxycarbonyl (Lys). Protocols for the removal of peptide from resin, HPLC purification, and disulfide bridge formation have been detailed before (11). Under standard glutathione oxidation conditions, native κ-PVIIA folds into the biologically active form with >90% efficiency. Although some analogs folded with much lower efficiency, the correctly folded isomer was often well separated from other isomers during HPLC purification, making identification of the correctly folded form straightforward. Electrospray mass spectroscopy was carried out on all peptide analogs to confirm correct synthesis.

Verification by NMR and Circular Dichroism (CD)—One isoform, Asn[21]–Ala, could not be properly folded, and two other isoforms, Hyp[4]–Ala and Asn[5]–Ala, showed much reduced block but were obtained in yields that were insufficient for complete testing. Multiple substitutions for Arg[2], Lys[7], and Phe[9] were synthesized and folded (Arg[2]–Ala, Arg[2]–Gln, Arg[2]–Lys, Lys[7]–Ala, Lys[7]–Arg, Phe[9]–Ala, Phe[9]–Met, and Phe[9]–Tyr). The pattern of mixed folded isomers obtained for all analogs was very similar to wild-type κ-PVIIA. The earliest eluting major peak was collected and purified, and in each case, presumed to have the same disulfide connectivity as native PVIIA; one substitution for each of the three loci (Arg[2]–Ala, Lys[7]–Ala, and Phe[9]–Met) was analyzed by NMR and CD to confirm directly that the disulfide connectivity was native. However, Hyp[4]–Ala, Asn[5]–Ala, Leu[12]–Ala, and Asp[14]–Ala were difficult to purify in high yield, and their structures have not been confirmed. The Leu[12]–Ala analog in particular showed an unusual pattern of isomers following folding and needs to be further investigated.

Electrophysiological Measurements—The coding region of *Shaker* H4 employed in this study was cloned into Bluescript vector. Point mutations in the pore region were introduced by polymerase chain reaction mutagenesis, and the amplified sequences were confirmed by sequencing. Capped cRNA was synthesized *in vitro* after linearizing the plasmid with *Hin*dIII, and the transcription with T7 RNA polymerase was performed by a standard protocol (16).

Oocytes from *Xenopus laevis* were prepared as described previously (17, 18). Frogs were anesthetized with 0.2% tricaine in ice water during surgery. cRNA coding for *Shaker* H4 was injected into oocytes, and the cells were incubated 1–7 days to allow expression of the protein. Prior to the electrophysiological measurements the vitelline membranes of the oocytes were removed mechanically with fine forceps. 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 MΩ. Current records were low-pass filtered at 1 kHz (−3 db) and sampled at 4 kHz. The bath solution was normal frog Ringer's containing (in mM) the following: 115 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 10 Hepes, pH 7.2 (NaOH).

To estimate the IC_{50} value of κ-conotoxin PVIIA and the corresponding analogs, whole cell currents of oocytes expressing either wild-type *Shaker* H4 K+ channel or mutant isoforms were recorded, and the peak currents, at a test voltage of 0 mV, were measured. IC_{50} values were calculated according to $IC_{50} = fc/(1 - fc) \cdot [Tx]$, where *fc* is the fractional current, and [Tx] is the toxin concentration applied. This is an approximation for calculating the affinity of the toxin for the closed state of the ion channel protein (19). Toxin concentrations were determined by assuming that the integrated absorbance (determined by HPLC) was equivalent for all analogs (1 μmol = 33 A_{220} units) except for Ala substitution for aromatic residues, where this value was reduced by 10%. The maximal toxin concentration tested for analogues of κ-conotoxin PVIIA that showed a low affinity to the *Shaker* K+ channel was usually 50 μM. All electrophysiological experiments were performed at room temperature (19–22 °C).

Mutant Cycle Analysis—This method provides a means of revealing interactions between given pairs of toxin and channel residues as

TABLE I

IC_{50} values for wild-type κ-conotoxin PVIIA and analogs

Data shown represent mean ± S.E. calculated from IC_{50} values independently determined for each cell (see "Experimental Procedures"); the number of independent experimental trails are specified in the table. n.d., not done.

Analog	IC_{50}	Number of cells
	nM	
PVIIA	57 ± 4	9
R2A	>50,000	5
R2Q	>50,000	3
R2K	>50,000	3
I3A	170 ± 10	5
O4A	*[a]	2
N5A	*[a]	1
Q6A	745 ± 49	5
K7A	>50,000	3
K7R	>50,000	3
F9A	>50,000	5
F9M	>50,000	3
F9Y	639 ± 101	4
Q10A	189 ± 3	4
H11A	183 ± 11	5
L12A	*[a]	3
D13A	82 ± 5	5
D14A	*[a]	3
S17A	86 ± 8	5
R18A	199 ± 10	6
K19A	69 ± 7	4
N21	n.d.	n.d.
R22A	276 ± 12	5
F23A	993 ± 107	4
N24A	1066 ± 94	3
K25A	6663 ± 393	4
V27	n.d.	n.d.

[a] These analogs gave very low yields upon folding, insufficient for an NMR analysis. The L12A analog in particular had an aberrant elution profile. All of these analogs were inactive (for O4A and N5A, IC_{50} > 10,000 nM; for L12A and D14A, IC_{50} > 50,000 nM). Although the analogs were tested in the electrophysiological assay, because we were not able to obtain independent experimental evidence that these are properly folded, the results should be interpreted with caution.

described earlier (20, 21). Values for the coupling coefficient Ω were obtained by using the formula Ω = (IC_{50}WTchannel:WTtoxin ·IC_{50} mutchannel:muttoxin)/(IC_{50}WTchannel:muttoxin · IC_{50}mutchannel:WTtoxin). Unity reflects no interaction, whereas deviation from unity indicates progressively stronger interactions. For Ω < 1 the reciprocal is given for ease of comparison of their deviation from unity (20). The change in coupling energy, ΔΔG, for a given pair of toxin-channel residues was calculated according to ΔΔG = RT ln Ω (with R = 8.314 $Jmol^{-1}K^{-1}$ and T = 295 K) (20, 22). The studies of Schreiber and Fersht (21) suggest that a pair of residues showing a change in ΔΔG > 2.1 kJ/mol^{-1} may be expected to lie within 5 Å of each other (see also Ref. 23).

RESULTS AND DISCUSSION

Analogs of κ-PVIIA containing alanine substitutions in each amino acid position of the peptide were synthesized and folded (see "Experimental Procedures"), and the affinity of these isoforms was functionally assayed by electrophysiological measurements using the *Xenopus* oocyte expression system. Fig. 1 shows whole cell currents from oocytes expressing *Shaker* H4 K^+ channels measured at different test potentials. Addition of 1 μM of four isoforms, His^{11}–Ala, Arg^{18}–Ala, Arg^{19}–Ala, and Arg^{22}–Ala, of κ-conotoxin PVIIA leads to a block of the evoked currents of about 60 to 80%. Alanine substitutions of Ile^3, Gln^{10}, Asp^{13}, and Ser^{17} gave similar results. For these eight isoforms, the IC_{50} values were all within 5-fold of wild-type κ-conotoxin PVIIA (see Table I). We note that some substitutions (Ile^3–Ala and Gln^{10}–Ala, for example) cause a bigger change in the IC_{50} value than do others (such as Asp^{13}–Ala, Ser^{17}–Ala, and Lys^{19}–Ala, which were all within 2-fold of wild-type PVIIA). Thus, residues such as Ile^3 or Gln^{10} could well make some contribution to the interaction of PVIIA with the *Shaker* channel, but this would be relatively minor compared with the residues discussed below.

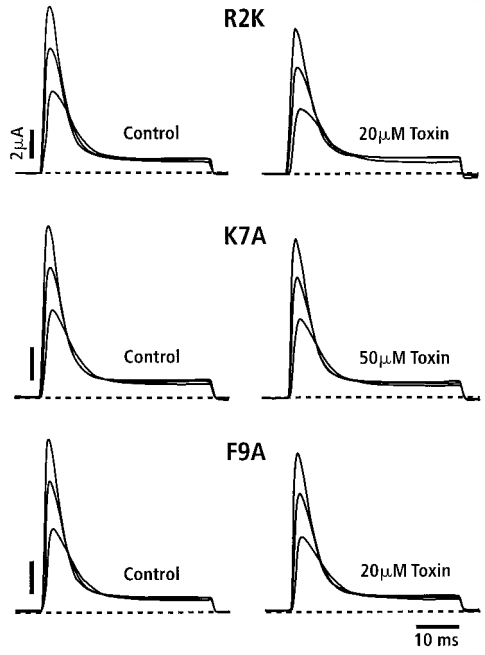

FIG. 2. **Mutation of residues Arg^2, Lys^7, and Phe^9 results in isoforms of κ-conotoxin PVIIA that do not block *Shaker* K^+ channels.** Whole cell currents recorded from oocytes expressing *Shaker* His^4 K^+ channels are shown before (*Control*) and after addition of 20 or 50 μM of the κ-conotoxin PVIIA isoform indicated. The pulse protocol was as in Fig. 1.

In contrast to the mutations of the eight amino acid residues of κ-conotoxin PVIIA described above, mutations of other residues resulted in a major reduction of the affinity for *Shaker* K^+ channels; the IC_{50} values of the different isoforms are summarized in Table I. Examples of κ-conotoxin PVIIA analogs that dramatically diminished activity are presented in Fig. 2. Because the alanine mutation at position 2 (Arg^2–Ala) resulted in an isoform of κ-conotoxin PVIIA that did not block the evoked *Shaker* K^+ currents, this residue was also mutated to a lysine (Arg^2–Lys), and the affinity of this isoform was investigated. The upper panel of Fig. 2 shows that even 20 μM of the isoform Arg^2–Lys of κ-conotoxin PVIIA has almost no effect on the evoked *Shaker* K^+ currents. This demonstrates that even a charge-conserving substitution at residue 2 of κ-conotoxin PVIIA results in a profound reduction in toxin affinity, suggesting that the arginine at this position is critically important for the interaction of the toxin with the channel protein. Similar results were obtained with the alanine mutation of the lysine at position 7. The middle panel of Fig. 2 shows that even at a concentration of 50 μM the Lys^7–Ala isoform had almost no blocking effect on the evoked currents. The same results were obtained with mutations of the phenylalanine at position 9 (Fig. 2, *lower panel*). Interestingly, even a conservative substitution of phenylalanine to a tyrosine reduced the block by an order of magnitude (see Table I).

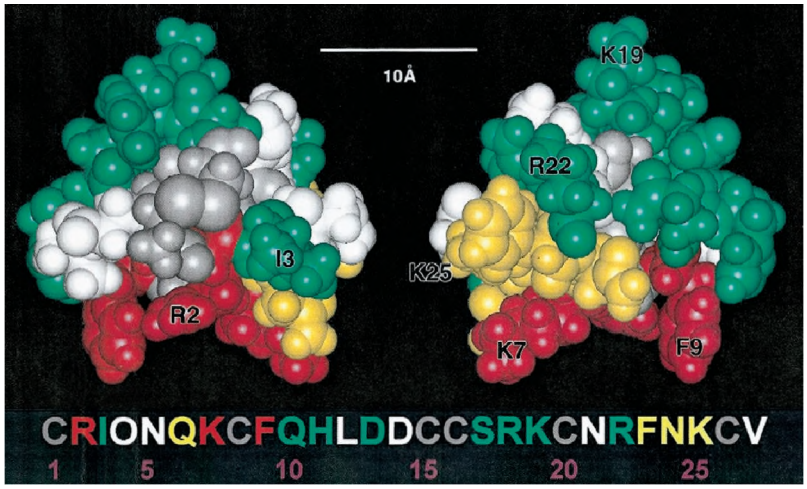

FIG. 3. **A model of the interaction surface of κ-conotoxin PVIIA.** *Red* indicates that the IC_{50} value following alanine substitution in this position was >10 μM, *yellow* labels residues where alanine substitution resulted in an intermediate IC_{50} value (500 nM to 10 μM), and *green* labels residues where analogs had an IC_{50} value within 10-fold of the native peptide (<500 nM). *White* residues were not substituted, could not be properly folded, or the relevant analogs were not available in sufficient quantity to verify proper folding (see the legend to Table I). Cysteines are shown in *gray*. The coordinates for the structure shown in the figure were based on the NMR structure of κ-conotoxin PVIIA (Protein Data Bank number 1KCP) (13).

Alanine mutations of the amino acid residues Gln[6], Phe[23], Asn[24], and Lys[25] exhibited an intermediate behavior in that the IC_{50} values of these isoforms were higher than 500 nM but less than or near 10 μM. A summary of the IC_{50} values of all isoforms tested is given in Table I.

Thus, the alanine walk mutagenesis of κ-conotoxin PVIIA has identified several residues critical for functional block of the *Shaker* channel. A diagrammatic representation of the most important residues is shown in Fig. 3. The critical residues are colored red, and it is immediately apparent that these are adjacent to each other in the three-dimensional structure, though not in the primary amino acid sequence. The toxin surface, which presumably interacts with the outer vestibule of the *Shaker* pore to cause a channel block, is strongly suggested by our study to contain Arg[2], Lys[7], and Phe[9] as key amino acids.

Two other positions, Leu[12] and Asp[14], may also play a critical role in κ-PVIIA-*Shaker* interaction. As shown in Table I, the Leu[12]–Ala and Asp[14]–Ala analogs were inactive; however, the yields upon folding these analogs were very poor, and we were unable to obtain sufficient amounts to do an NMR analysis. For this reason, we have been conservative in our interpretation and have not colored these residues red in the model shown in Fig. 3. We note however that these are adjacent to the three confirmed critical residues on the toxin surface.

The differing predictions of Savarin *et al.* (13) and Scanlon *et al.* (12) can be compared using these results. Scanlon *et al.* (12) suggested that four positively charged residues in κ-PVIIA were analogous to certain residues in charybdotoxin for interaction with the *Shaker* channel. A key residue in charybdotoxin is Lys[27], which is postulated to directly occlude the pore of the channel (24); Scanlon *et al.* (12) suggested that the analogous position would be Lys[19] of κ-conotoxin PVIIA. Other charged residues that have been shown to be important in the charybdotoxin-*Shaker* interaction are Arg[25], Lys[11], and Lys[31] (25); the suggested analogous residues were Arg[18], His[11], and Arg[22] of κ-conotoxin.

The results of the alanine walk mutagenesis study described above do not support the postulated charybdotoxin analogy. In particular, the Lys[19]–Ala analog has an IC_{50} value for the *Shaker* channel close to that of the wild-type toxin. None of the other proposed residues show much change in IC_{50} when substituted by alanine; all gave values within a factor of 5 of the wild-type value and are among the residues represented in green in Fig. 3. The analogous placement of positive charges at the three-dimensional level between charybdotoxin and κ-conotoxin PVIIA appears not to be functionally significant.

There were prior indications from electrophysiological work that some of the specific interactions of κ-conotoxin PVIIA residues with outer vestibule amino acids of the *Shaker* channel pore were probably significantly different from charybdotoxin-*Shaker* interactions. Most notably, the mutation Phe[425]–Gly on *Shaker* increases the affinity of charybdotoxin by ~2,000-fold (26) but abolishes the binding of κ-PVIIA at the concentrations tested (11). The conservative mutation Phe[425]–Tyr has close to wild-type κ-PVIIA affinity, suggesting an important role for an aromatic residue at this position in the PVIIA-*Shaker* interaction.

Savarin *et al.* (13) postulated that, like other polypeptide toxins that are able to inhibit K^+ channels, there may have been convergent evolution in one key feature of otherwise structurally unrelated toxins, the presence of a dyad consisting of a lysine residue (postulated to directly interact with the pore) and a hydrophobic amino acid (2). Upon considering the various choices in κ-conotoxin PVIIA, Savarin *et al.* (13) predicted that for κ-conotoxin PVIIA the likely key residues were Lys[7] and either Phe[9] or Phe[23] of κ-PVIIA. Our results confirm that Lys[7] and Phe[9] are indeed important for binding of κ-conotoxin to the *Shaker* potassium channel (and to a much lesser extent, so is Phe[23]). Another notable feature of the predictions of Savarin *et al.* (13) is a potential role for Lys[25]. This residue makes Van der Waals contact with Lys[7] (mean distance of αC atoms derived from NMR structure is 4.4 Å), and an arrangement of two proximal lysine residues was compared with a similar motif in dendrotoxin that was found to have functional significance (27). We find that the Lys[25] mutant has the most reduced affinity of the analogs in the intermediate group (those residues colored yellow in Fig. 3), with an IC_{50} value ~100-fold higher than the wild-type peptide.

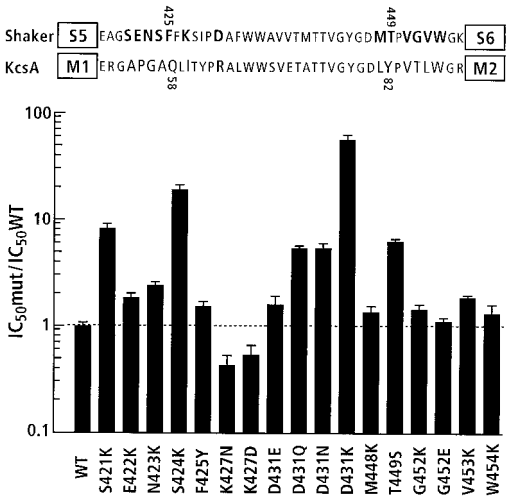

FIG. 4. **Influence of single point mutations in the pore region of *Shaker* on κ-PVIIA binding.** The *upper panel* depicts the amino acid alignment of the pore region of the *Shaker* K^+ channel (S5–S6 linker) and the corresponding region of the bacterial K^+ channel KcsA (28). Residues where point mutations in the *Shaker* K^+ channel have been introduced are marked in *bold*. The *bar diagram* shows the affinity of κ-PVIIA to the individual channel mutants normalized to wild-type IC_{50}. Data represent mean ± S.E. (n = 3–9).

A number of pore mutations of the *Shaker* channel were generated, and affinities of PVIIA and PVIIA mutants were measured. The effects of these single amino acid substitutions on the affinity of wild-type PVIIA are shown in Fig. 4. The mutations Lys[427]–Asn and Lys[427]–Asp resulted in an increased affinity of PVIIA of a factor of ~2. Similar effects have been reported for Lys[427]–Glu (12). Several other mutations (Phe[425]–Gly, Thr[449]–Lys, Thr[449]–Tyr, Thr[449]–Gln (11), and Val[451]–Lys) were not sensitive to PVIIA and therefore were not useful for the cycling analysis. All other channel mutations led to a slight reduction of PVIIA affinity or had no measurable effect (see Fig. 4, *lower panel*).

The results of the mutant cycling analysis are shown in Table II and Fig. 5. The mutant PVIIA peptides (with single amino acid substitutions) were tested against twelve different mutant *Shaker* channel isoforms. In most cases, strong interactions were not detected (the Ω values were close to 1, and the coupling energy (ΔΔG) was close to 0). However, some Ω values obtained provide at least suggestive data for the orientation of the peptide in the postulated outer vestibule of the channel. The Ω value for the Lys[25]–Ala (toxin)-Met[448]–Lys (channel) pair of 7.3, which is equivalent to a ΔΔG value of 4.86 kJ/mol^{-1}, indicates an interaction between these two residues. Lys[25] is close to Phe[9] on the toxin molecule (distance of the αC atoms is ~8 Å). The interaction between Lys[25] and Met[448] makes the relatively modest Ω value of 2.7 for the Phe[9]–Tyr (toxin)-Thr[449]–Ser (channel) pair potentially significant. Any other substitution for Phe[9], except Tyr, decreased the IC_{50} value

TABLE II
Mutant Cycling Analysis

ΔΔG values are given in kJ/mol^{-1} and were calculated as described under "Experimental Procedures." Number of experiments is given in parentheses. n.d., not done.

κ-PVIIA mutants	*Shaker* mutants											
	S421K	N423K	S424K	F425Y	K427N	K427D	D431E	D431N	M448K	T449S	G452K	G452E
I3A	0.07 (2)	0.18 (2)	2.73 (4)	0.24 (2)	n.d.	0.27 (3)	n.d.	0.71 (2)	0.22 (3)	1.70 (3)	n.d.	n.d.
Q6A	1.58 (1)	0.04 (2)	2.06 (2)	0.19 (1)	n.d.	0.20 (2)	n.d.	1.03 (2)	1.11 (2)	0.97 (2)	n.d.	n.d.
F9Y	0.26 (1)	0.89 (2)	1.58 (3)	0.82 (2)	n.d.	0.33 (2)	n.d.	0.14 (2)	0.41 (3)	2.41 (4)	n.d.	n.d.
Q10A	0.07 (2)	0.24 (3)	0.03 (1)	1.18 (2)	0.23 (1)	0.01 (2)	n.d.	0.41 (3)	0.24 (2)	2.25 (3)	n.d.	n.d.
D13A	0.02 (4)	1.17 (4)	0.14 (2)	0.90 (1)	1.23 (3)	0.65 (3)	0.05 (3)	0.37 (6)	1.13 (3)	0.38 (2)	0.67 (2)	0.45 (1)
R18A	0.37 (3)	1.02 (3)	1.12 (2)	0.51 (1)	0.47 (2)	0.37 (3)	0.47 (2)	0.41 (5)	0.37 (2)	0.36 (2)	0.29 (1)	0.71 (1)
K19A	0.14 (2)	0.47 (2)	0.21 (2)	1.16 (2)	1.44 (2)	0.07 (1)	0.36 (2)	0.41 (3)	0.06 (2)	0.14 (1)	0.16 (1)	1.29 (1)
R22A	2.06 (4)	3.13 (4)	2.65 (4)	0.47 (2)	n.d.	0.76 (3)	0.08 (1)	0.27 (2)	0.47 (2)	0.76 (2)	n.d.	1.15 (1)
F23A	2.18 (2)	0.20 (2)	2.53 (3)	1.50 (2)	n.d.	0.77 (3)	n.d.	0.14 (2)	0.84 (2)	1.50 (3)	n.d.	n.d.
N24A	0.81 (1)	0.39 (1)	0.14 (1)	0.82 (1)	n.d.	0.20 (1)	n.d.	0.54 (1)	0.35 (2)	0.40 (1)	n.d.	n.d.
K25A	1.57 (1)	1.43 (1)	2.41 (1)	0.77 (1)	n.d.	1.02 (1)	n.d.	3.18 (3)	4.86 (6)	2.05 (1)	n.d.	n.d.

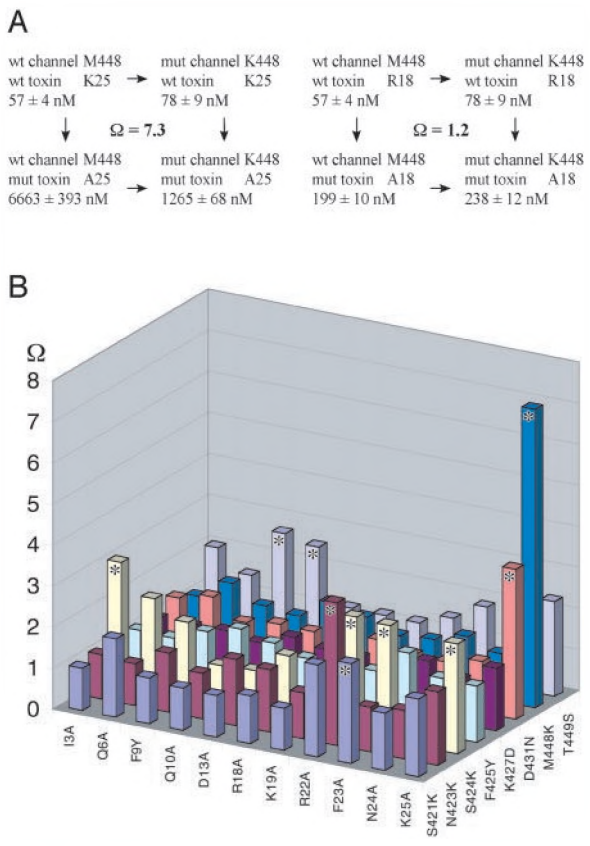

FIG. 5. **Mutant cycling analysis of *Shaker* channel and κ-PVIIA interaction.** *A*, examples for mutant cycles of *Shaker* Met^{448} with κ-PVIIA-Lys^{25} (indicating an interaction; Ω = 7.3) and κ-PVIIA-Arg^{18} (indicating no interaction; Ω = 1.2). IC_{50} values are given as mean ± S.E. *B*, *bar diagram* showing a plot of the Ω values for different toxin (*x* axis) and channel (*z* axis) mutant combinations (calculated as described under "Experimental Procedures"). Pairs of residues showing a change in ΔΔG >2.1 kJ/mol^{-1} are highlighted by an *asterisk*.

below detectable limits, and we were therefore constrained to using this conservative substitution (even the Phe^9–Met substitution abolished detectable activity). This is suggestive evidence that the key Thr^{449} residue, known to play a role in charybdotoxin binding, may be in close proximity to Phe^9 of PVIIA. Thus, Phe^9 of κ-conotoxin PVIIA may be at a homologous locus in the vestibule to Tyr^{36} in charybdotoxin, *i.e.* the aromatic residue of the dyad postulated by Ménez and co-workers (2). This would therefore also make Phe^9 analogous to Phe^{25} in KTX/AgTX and Tyr^{23} in Shk and Tyr^{26} in BgK (2, 22). The mean distance between the αC atom of Lys^7 and the center of the benzene ring of Phe^9 derived from the NMR structure (13) is 8.3 Å (range, 7.4–10.1 Å). There also seems to be some interaction of Lys^{25} with Asp^{431} (Ω 3.7).

The Ω values obtained for the Arg^{22}–Ala (toxin)-Asn^{423}–Lys (channel) pair of 3.6 and the Ile^3–Ala (toxin)-Ser^{424}–Lys (channel) pair of 3.0 might indicate that there is also some interaction of these residues. Unfortunately, because no substitution for Arg^2 and Lys^7 has been found that gives measurable affinity, we were unable to carry out a mutant cycling analysis for these important loci. By using the spatial constraints that can be derived from the mutant cycle experiments, a docking of PVIIA into the KcsA crystal structure reveals that the docking configuration may fit with the mutant cycling data. An alignment of the residues that make up the pore regions of *Shaker* and KcsA is shown in the upper panel of Fig. 4. By assuming that Lys^{25} of κ-PVIIA is in the vicinity of Leu^{81} (Met^{448} in *Shaker*), Phe^9 of the toxin can be positioned close to Tyr^{82} (Thr^{449}) of the adjacent subunit of the channel pore vestibule (not shown). In this orientation, Lys^7 of κ-PVIIA is facing toward the ion channel pore. Furthermore, Arg^{22} of κ-PVIIA is close to Gly^{56} (Asn^{423}) and Ala^{57} (Ser^{424}) of the same channel subunit with which Lys^{25} interacts, whereas Ile^3 of κ-PVIIA is in the vicinity of Ala^{57} (Ser^{424}) of the channel opposite to that with which Phe^9 interacts.

In summary, the data in this report do not support the predictions of Scanlon *et al.* (12) that there are positive residue homologies between PVIIA and charybdotoxin but are consistent with the critical dyad hypothesis developed by Ménez and co-workers (2) for polypeptide antagonists of voltage-gated K$^+$ channels.

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