A New Potassium Channel Toxin from the Sea Anemone *Heteractis magnifica*: Isolation, cDNA Cloning, and Functional Expression[†,‡]

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ABSTRACT: A new potassium channel toxin, HmK, has been isolated from the sea anemone *Heteractis magnifica*. It inhibits the binding of [^{125}I]-α-dendrotoxin (a ligand for voltage-gated K channels) to rat brain synaptosomal membranes with a K_i of about 1 nM, blocks K^+ currents through Kv 1.2 channels expressed in a mammalian cell line, and facilitates acetylcholine release at the avian neuromuscular junction. HmK comprises of 35 amino acids (M_r 4055) with the sequence R^{1}TCKDLIPVS^{10}ECTDIRCRTS^{20}-MKYRLNLCRK^{30}TCGSC^{35}. A full assignment of the disulfide linkages was made by using partial reduction with tri(2-carboxyethyl)phosphine (TCEP) at acid pH and rapid alkylation with iodoacetamide. The disulfide bridges were identified as Cys^3−Cys^{35}, Cys^{12}−Cys^{28}, and Cys^{17}−Cys^{32}. A cDNA clone encoding HmK was isolated using RT-PCR from the total RNA obtained from sea anemone tentacles, while the 5′- and 3′-flanking regions of the cDNA were amplified by RACE. The full-length cDNA was 563 bp long and contained a sequence encoding a signal peptide of 39 amino acids. The coding region for matured HmK toxin was cloned and expressed as a glutathione *S*-transferase (GST) fusion product in the cytoplasm of *Escherichia coli*. After affinity purification and cleavage, the recombinant toxin was shown to be identical to native HmK in its N-terminal sequence, chromatographic behavior, and binding to dendrotoxin binding sites on rat brain membranes.

Snake venom and venomous secretions of animals such as spiders, insects, snails, and scorpions have provided useful molecular probes for investigating the various types of K channels. The toxins include those that block voltage-dependent K channels, such as the dendrotoxins from mamba venom [reviewed by Harvey and Anderson (1991)], mast cell degranulating (MCD) peptide from the honey bee *Apis mellifera* (Bidard et al., 1987), scorpion toxins such as noxiustoxin from *Centruroides noxius* (Gurrola et al., 1989), and the phospholipase A_2 toxins [for review, see Harris (1991)]. Other toxins block Ca^{2+}-dependent K channels, such as apamin from the European honey bee (Habermann, 1984) and charybdotoxin from the Israeli scorpion *Leiurus quinquestriatus* (Miller et al., 1985; Gimenez-Gallego et al., 1988).

More recently, sea anemones have attracted attention as host to the first K channel toxins to be isolated from a marine animal. These sea anemone toxins represent a new structural class of K channel-blocking peptides and have been isolated and characterized from three species: *Bunodosoma granulifera* (toxin named BgK;[1] Aneiros et al., 1993; Dauplais et al., 1997), *Stichodactyla helianthus* (named ShK; Karlsson et al., 1992; Castaneda et al., 1995), and *Anemonia sulcata* (toxins named AsKC1, AsKC2, AsKC3, and AsKS; Schweitz et al., 1995). They are extremely basic molecules. The smaller toxins, BgK, ShK, and AsKS, have 35−37 residues, while the longer AsKCs are 58−59 amino acid peptides, all containing three disulfide bonds (Pohl et al., 1994). The three-dimensional solution structures of BgK and ShK have been determined, using 2D NMR spectroscopy (Tudor et al., 1996; Dauplais et al., 1997). All three toxins facilitate the evoked release of acetylcholine at neuromuscular junctions by the selective blockade of voltage-gated K channels (Aneiros et al., 1993; Castaneda et al., 1995). ShK has been shown to block Kv 1.3 channels in lymphocytes. Since these channels have an important role in controlling lymphocyte membrane potential and thereby T lymphocyte proliferation and lymphokine production, ShK could provide a potential candidate for a novel class of immunosuppressants (Pennington et al., 1996).

Despite the structural elucidation of many K channel toxins, we know little of the regulation of biosynthesis and processing of these molecules. In this paper, we have

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[1] Abbreviations: HmK, ShK, and BgK, *H. magnifica*, *S. helianthus*, and *B. granulifera* K channel toxins, respectively; FPLC and HPLC, fast and high-performance liquid chromatography, respectively; TCEP, tri(2-carboxyethyl)phosphine; RT, reverse transcriptase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; IPTG, isopropyl β-D-thiogalactoside; GST, glutathione *S*-transferase; PTH, phenylthiohydantoin; Cys(Cam), *S*-carboxamidomethylcysteine; bp, base pair(s); kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis. Amino acids are indicated by the standard three- or one-letter abbreviations.

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isolated and sequenced a new K channel toxin (HmK) from the tropical sea anemone *Heteractis magnifica*. We have isolated the corresponding cDNA and expressed the new toxin in *Escherichia coli*. To our knowledge, this work describes for the first time the cloning and expression of a recombinant sea anemone ion-channel toxin.

EXPERIMENTAL PROCEDURES

Materials. Endoproteinase Lys-C was from Wako Pure Chemicals (Japan). Glu-C and tri(2-carboxyethyl)phosphine (TCEP) were from Boehringer Mannheim (Germany), while *Bam*HI, *Sal*I, *Taq* DNA polymerase, and [^{125}I]-α-dendrotoxin were obtained from Amersham International Inc. (U.K.). T4 DNA ligase and T4 DNA ligase buffer were purchased from New England Biolabs Inc. (U.S.A.). Protein/peptide and DNA sequencing reagents were from Perkin Elmer-Applied Biosystems Inc. (Foster City, CA). HPLC solvents, trifluoroacetic acid and acetonitrile, were from either Sigma or Pierce and Merck or J. T. Baker, respectively. The bacterial strain used for cloning and expression studies was JM109 [F′ *tra*D36 *lac*I^q Δ(*lac*Z)M15 *pro*A$^+$B$^+$I e14$^-$ (McrA$^-$) Δ-(*lac-pro*AB) *thi gyr*A96 (NaI^r) *end*AI *hsd*R17 ($r_k^-m_k^+$) *rel*AI *sup*E44 *rec*A1] (Yanisch-Perron et al., 1985). Vectors, pT7Blue and pGEX-KG were purchased from Novogen (U.S.A.) and Pharmacia Biotech (Sweden), respectively. Marathon cDNA Amplification Kit was bought from Clontech Laboratories, Inc. (U.S.A.). Iodoacetamide and glutathione-agarose were purchased from Sigma. Oligonucleotides used in this study were synthesized in Bioprocessing Technology Center (BTC), National University of Singapore, on an Applied Biosystems Inc. DNA/RNA synthesizer (model 394) and were purified by HPLC before use. All other chemicals (of analytical grades or of the highest grades available) were purchased either from Boehringer Mannheim, Pharmacia Biotech, Sigma, BDH, or Aldrich.

Heteractis magnifica. *Heteractis magnifica* specimens were obtained from a local aquarium. They were contracting sea anemones, similar to the Caribbean sea anemones (Aneiros et al., 1993). They possess a column of uniform bright red-violet color with an oral disc from 300−500 mm, densely covered with green-brownish finger-like tentacles (up to 75 mm long) with slightly swollen ends. The identity of the species was confirmed using a taxonomic guide (Fautin & Allen, 1992).

Isolation of the Toxin. Mucus secretion was obtained using the freeze−thaw technique. A single sea anemone (approximately 1 kg) was placed in a container, covered with distilled water and frozen at −20 °C. After three freeze−thaw cycles, the anemone was removed and the mucus filtered through gauze, freeze-dried, and kept at −20 °C. Lyophilized mucus was reconstituted in 0.10 M ammonium acetate (AmOAc) buffer, pH 6.7, and centrifuged, and the supernatant gel filtered on Sephadex G-50 in 0.10 M AmOAc. Fractions containing peptides were combined and further resolved by FPLC using Mono-S (Pharmacia Biotech), equilibrated with 50 mM AmOAc, pH 5.4. Bound peptides were eluted from the column with a linear gradient of NaCl in the same buffer. The last purification step for the peptides was carried out by reverse-phase HPLC using a 140B Solvent Delivery System/1000S Diode Array detector from Applied Biosystems Inc. Separation was performed in a C_8 Aquapore RP-300 column (2.1 × 100 mm).

SDS−PAGE. Tris-tricine SDS−PAGE was performed using 10% T polyacrylamide gels, according to the method of Schagger and Von Jagow (1987). The protein samples were denatured by boiling in SDS and dithiothreitol prior to loading onto the gel. Following electrophoresis, protein bands were stained with Coomassie Brilliant Blue R250.

Protein Assay. The protein content was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985) with bovine serum albumin as the standard. For HPLC-purified toxins, the amount of protein was determined by amino acid analysis.

Mass Spectrometric Analysis. Toxin was dissolved in 100% acetonitrile and analyzed using an ionspray mass spectra system (Perkin-Elmer SCIEX API III LC/MS/MS system). Ionspray voltage, 4000 V; orifice voltage, 75 V; mass scan range, 500−2000 amu.

Microlevel Enzymatic Digestion. HmK was reduced and modified with 4-vinylpyridine based on the procedure of Dupont et al. (1987). Pyridylethylated (PE) peptide samples were digested with endoproteinases Lys-C and Glu-C (Chung et al., 1996). PE-peptides were dissolved in 25−50 μL of digestion buffers: 0.2 M NH_4HCO_3, pH 7.8−8.1 for Glu-C digestion, and 10 mM Tris-HCl, pH 8.8 for Lys-C digestion. Denaturation and solubilization of samples were enhanced with the addition of 4 M urea followed by incubation for 2 h at room temperature prior to enzyme addition. The concentration of urea was reduced to 2 M before the addition of enzymes and incubation at 37 °C overnight. The digests were fractionated by reverse-phase HPLC on a C_{18} Vydac column (2.1 × 100 mm). Solvents A and B were 0.1% (v/v) TFA and 0.085% (v/v) TFA in 70% (v/v) acetonitrile, respectively. Elution was performed using a linear gradient of 5−65% B in 45 min at a flow rate of 200 μL/min.

Peptide Sequencing. The N-terminal amino acid sequence of HmK and its internal peptides were determined by automated Edman degradation using an Applied Biosystems 477A pulsed liquid-phase sequencer equipped with an on-line PTH-amino acid analyzer (120A). Search for homologous proteins was performed with the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) .

Assignment of Disulfide Bonds. The procedure adopted was based on that of Gray (1993a,b). Briefly, HmK (∼10 μg, in HPLC effluent) was first partially reduced by mixing with an equal volume of tri(2-carboxyethyl)phosphine (TCEP; 20 mM, in a buffer consisting of 0.17 M citric acid, adjusted to pH 3 with NaOH). Incubation was carried out for various lengths of time at either room temperature (25 °C), or at elevated temperature (65 °C). Reaction was terminated by injecting the mixture into a HPLC column (Aquapore RP-300) after appropriate dilution with 0.1% TFA to ensure adsorption of the peptide and its reduction products. Peak fractions were collected manually, and were alkylated immediately. The rapid alkylation procedure was as follows: peptide solutions, 5−10 μg in 150 μL of 0.1% TFA (pH 2), were drawn into a glass syringe having a fine-tipped needle and then forcibly squirted into iodoacetamide solution (50 mg of iodoacetamide dissolved in 100 μL of 0.5 M Tris acetate, pH 8, containing 2 mM EDTA) with vigorous mixing. Mixing was discontinued as soon as the peptide solution was completely added. After 20−30 s, the reaction was quenched by acidification with 200 μL of 0.5 M citric acid, and the mixture was desalted by reverse-phase HPLC.

The labeled Cys residues in the peptide were then detected by amino acid sequencing

Binding Assays. Samples were tested for their ability to displace a probe for voltage-dependent K channels, ^{125}I-labeled α-dendrotoxin of *Dendroaspis angusticeps* (Eastern green mamba), from synaptosomal membranes prepared from rat brain as described earlier (Harvey et al., 1989). The procedure was as follows. A membrane suspension (∼1.0 mg/mL of protein; 200 μL) was incubated at room temperature (19−21 °C), with [^{125}I]-α-dendrotoxin (10 pM) and a range of concentrations of crude mucus preparation, purified toxin, and recombinant toxin. After 30 min, the membranes were recovered by centrifugation, washed with buffer containing 0.1 mg/mL bovine serum albumin, and centrifuged, and the bound radioactivity was measured in a γ counter.

Effects on Chick Biventer Cervicis Nerve−Muscle Preparations. Active fractions were also tested for effects on isolated chick biventer cervicis nerve−muscle preparations as described by Harvey and Karlsson (1980). Presynaptic facilitation increases the twitch responses to indirect stimulation with no change in the postjunctional sensitivity to acetylcholine, carbachol, or KCl. Some preparations were stimulated directly at 0.1 Hz in the presence of 10 μM tubocurarine to abolish neuromuscular transmission.

Cell Culture. Purified toxin was also tested for its ability to inhibit K^+ currents in B82 cells expressing *shaker* Kv 1.2 channels. B82 mouse fibroblasts stably transfected with rKv 1.2 (NGK1) were obtained from Prof. S. Grissmer, (Department of Applied Physiology, University of Ulm, Albert-Einstein-Allee 11, 89096 Ulm, Germany; Grissmer et al., 1994). Cells were maintained in 35 cm^2 Fulcan flask in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units/mL penicillin-G-sodium, 50 μg/mL streptomycin sulfate, and 2 mM glutamine and supplemented with 0.45 g/mL of glucose. On the day of recording the cells were treated with trypsin-EDTA to remove them from the flask. Cells were subsequently seeded on to 13 mm sterile glass cover slips for 2−3 h before use.

Electrophysiological Recordings. Voltage-clamp recordings were performed using whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Patch pipettes were prepared from borosilicate glass capillaries with inner filament (GC 120F 10, Clark Electromedical Instruments) on a Flaming/Brown micropipette puller (Model P-87, Sutter Instrument Co.) and polished to 4−6 MΩ using a Narishige MF-83 (Tokyo, Japan) micropipette polisher. The membrane currents were recorded at room temperature (22−26 °C), using a List EPC-7 patch-clamp amplifier (Adam and List Associates, Great Neck, NY), digitized and displayed on an IBM PC-compatible microcomputer, running Strathclyde Electrophysiology Software-WCP (kindly supplied by Dr. J. Dempster, Department of Physiology and Pharmacology, University of Strathclyde), equipped with a National Instruments lab PC card. Whole-cell currents were recorded by stepping from −60 mV to +50 for 250 ms every 10 s using between 70% and 80% series resistance compensation; capacitative currents were removed by analog subtraction.

Solutions. The cells were bathed in physiological salt solution containing: 10 mM glucose, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.06 mM NaH_2PO_4, 2 mM $CaCl_2$, and 10 mM HEPES, adjusted to pH 7.4 with NaOH. The pipette solution contained 10 mM glucose, 5 mM NaCl, 140 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 1 mM EGTA, adjusted to pH 7.3 with KOH.

Isolation of RNA and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from the tentacles of *H. magnifica* using a modified guanidium isothiocyanate/CsCl extraction method (Glisin et al., 1973; Ullrich et al., 1977). The integrity of the RNA was examined by denaturing agarose gel electrophoresis. Reverse transcriptase (RT) reaction was performed with 5 μg of *H. magnifica* RNA (pre-heated at 65 °C for 5 min) in a total reaction volume of 10 μL of 1× RT buffer (Promega). The downstream primer used was P2 with the sequence 5′-(A/G)CA IGA ICC (A/G)CA IGT (C/T)TT ICI (A/G)CA-3′. Other components of the RT reaction were 0.5 mM of each dNTP, 1 unit/μL RNAsin, and 50 units of M-MuLV reverse transcriptase. The mixture was incubated for 1 h at 42 °C, inactivated by heating at 95 °C for 5 min, and quickly chilled on ice. A 5 μL amount of this reaction was used in a 50 μL polymerase chain reaction. PCR was carried out as described elsewhere (Ghadessy et al., 1996). Oligonucleotide primers used were sense, P1, 5′-(A/C)GI ACI TG(C/T) AA(A/G) GA(C/T) (C/T)TI ATI CC-3′, and antisense, P2.

Subcloning and DNA Sequencing. The PCR product was first fractionated using low melting point agarose (2%), after which the DNA band was cut out and further purified from the agarose using the GeneClean kit (Bio101). The amplified product (50 ng) was then ligated with 50 ng of pT7Blue T-vectors in a volume of 10 μL containing 2−3 Weiss units of T4 DNA ligase buffered with T4 DNA ligase buffer. The recombinant plasmids were transformed into competent *E. coli* JM109 cells by electroporation. Prior to growing colonies for plasmid isolation, the presence of the insert, as well as its orientation, was determined using direct colony PCR. Individual clones were grown in LB broth containing 0.1 mg/mL ampicillin and their plasmids were isolated using the mini plasmid preparation method (alkaline lysis) (Sambrook et al., 1988). Inserts in the pT7Blue T-vectors were sequenced on both strands with the forward and reverse M13 primers using the dideoxy chain termination method of Sanger et al. (1977) on an automated DNA sequencer (ABI model 373A) using the manufacturer's own protocol and reagents. Analysis of sequencing data was carried out using the SeqEd v1.03 program (ABI).

Rapid Amplification of cDNA Ends (RACE). RACE was used to amplify DNA sequences from a messenger RNA (mRNA) template between a defined internal site and unknown sequences of either the 3′- or 5′-ends of the mRNA. A Marathon cDNA Amplification Kit (Clontech) was used, and the protocol was based on the manufacturer's specification. In brief, first strand cDNA was synthesized from total RNA using a cDNA synthesis primer (10 μM), M-MuLV reverse transcriptase (100 units/μL), and the deoxynucleotide mixture. Second-strand synthesis was performed with a cocktail of *E. coli* DNA polymerase 1, RNase H, and *E. coli* DNA ligase. Following the creation of blunt-ends with T4 DNA polymerase, the ds cDNA was ligated to the cDNA adaptors (supplied in the cDNA Amplification Kit) to give an uncloned library of adaptor-ligated ds cDNA. The 5′- and 3′-RACE reactions were performed using PCR. Both reactions were primed with an internal gene-specific primer (GSP1 for 5′-RACE and GSP2 for 3′-RACE) and the adaptor primer 1, AP1. The RACE products were cloned into pT7Blue T-vector and sequenced.

Reconstruction of Recombinant HmK Gene and Its Expression as a Fusion Protein. HmK sequence harbored in the pT7Blue T-vector was amplified by PCR and cloned into the expression vector pGEX-KG (Pharmacia Biotech) to produce a glutathione *S*-transferase−HmK fusion protein in *E. coli* JM109. PCR primers with restriction enzyme sites flanking the structural region of HmK were designed from the RACE sequence data. *Sal*I site was included into the 3′ PCR primer, while the 5′ primer incorporated a *Bam*HI site. The reconstructed DNA fragment was cleaved with restriction endonucleases *Bam*HI and *Sal*I, gel purified and ligated to similarly prepared pGEX-KG DNA. The ligated product was then transformed into competent *E. coli* JM109 cells. Individual clones were first screened for the presence of HmK sequence by PCR. Clones carrying inserts were grown at 37 °C in LB broth containing 100 μg/mL ampicillin until $A_{600} \sim 0.5$ (a cell density of 5×10^8 cells/mL). Cells were induced with isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.3 mM at 25 °C, harvested after 2 h, resuspended in ice-cold PBS and frozen at −70 °C.

Purification and Characterization of Recombinant HmK. Induced frozen cells were thawed, and lysozyme was added to a final concentration of 2 mg/mL. After 20 min on ice, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM, and the cells were lysed by sonication. Triton X-100 was added (final concentration of 1%) to minimize association of fusion proteins with bacterial proteins. After centrifugation, the clarified crude cell extract was applied to a glutathione-agarose affinity column. Unbound proteins were eluted from the glutathione column with PBS. The bound GST-HmK fusion protein was eluted with 50 mM Tris-HCl, pH 8.0 containing 50 mM reduced glutathione. The purified fusion proteins were cleaved at 25 °C for 5−10 h using thrombin (3000 units/mg) at a w/w ratio of approximately 1% the amount of fusion protein. Digestion buffer was the same elution buffer after the addition of NaCl to 150 mM and $CaCl_2$ to 2.5 mM, and adjustment of protein concentration to 1 mg/mL. Recombinant HmK was purified from the digest by cation-exchange (Mono-S, FPLC) and reverse-phase (C_8 Aquapore RP-300, HPLC) chromatography, before analysis by N-terminal sequencing and analysis of its ability to displace [^{125}I]-α-dendrotoxin from rat brain synaptosomal membranes.

RESULTS AND DISCUSSION

Isolation of K Channel Toxin. Reconstituted mucus of *H. magnifica* was fractionated into four distinguishable peaks by gel filtration. Peaks were labeled I, II, III, and IV (Figure 1a). Selected fractions from each peak were analyzed by SDS−PAGE (Figure 1a, inset). The first peak contained high molecular weight proteins (20 000−100 000) while peak II consisted solely of cytolysins with an M_r of 19 000 as reported earlier (Khoo et al., 1993). The Tris-tricine SDS−PAGE system of Schagger and Von Jagow (1987) which we employed was able to resolve low molecular weight peptides of peak III. The last eluting peak, peak IV, consisted of nonproteineous materials which absorbed at 280 nm; it has been suggested to contain mainly low molecular weight substances, such as inhibitors of acetylcholinesterase and chloride ions (Aneiros et al., 1993; Castaneda et al., 1995). Peak III was separated into 12 subfractions by cation-exchange (Mono-S, FPLC) chromatography (Figure 1b). Fractions 1−4 contained contaminating cytolysins, and were therefore not examined further. Fractions 5−12 were further fractionated by reverse-phase HPLC, and the resulting peptide peaks were identified by partial N-terminal amino acid sequencing. The last eluting peak from the Mono-S chromatography (Figure 1b, peak 12) was resolved into three distinct peaks by rpHPLC (Figure 1c), the first of which was found to have an N-terminal sequence that was homologous to ShK (using the BLAST algorithm; Altschul et al., 1990), a K channel toxin isolated from the Caribbean sea anemone *Stichodactyla helianthus* (Castaneda et al., 1995). The new peptide toxin was named HmK (*H. magnifica* K channel toxin) in accordance with the nomenclature used to name BgK and ShK. The yield of the HPLC-purified HmK was about 50 μg per gram of the lyophilized mucus.

Primary Structure of HmK. The complete amino acid sequence of the HmK was determined from the N-terminal sequence and the sequences of the peptides derived from the proteolytic digestion. It consists of 35 amino acid residues (Figure 2). The calculated molecular weight of the peptide was 4055, and this is in agreement with the mass of 4054.65 kDa obtained by mass spectrometry. HmK is highly homologous (overall identity of 60%) to ShK, which also contains 35 amino acids with an identical molecular weight. HmK, however, is only about 40% identical to BgK (37 amino acids) and AsKS (36 amino acids). The amino acid sequence data indicated the presence of six cysteinyl residues in HmK.

Disulfide Bonds of HmK. Short peptides with a high density of disulfides have often proved difficult to analyze using the conventional strategy of breaking the peptide chain with proteases, and analyzing the bridged fragments generated. Consequently, we have adopted the elegant chemical approach of Gray (1993a), using partial reduction of our native toxin with TCEP at acid pH, to elucidate the disulfide linkages of HmK. HmK was first partially reduced at room temperature to give a series of peaks by rpHPLC (Figure 3a). The four major peaks were assigned N and R1−R3. R1, R2, and R3 were alkylated separately with iodoacetamide. Sequence analysis of the alkylated product R1 showed clean labeling of Cys^3 (Figure 4A) and Cys^{35} (Figure 4F). Cys^{12}, Cys^{17}, Cys^{28}, and Cys^{32} showed insignificant labeling (Figures 4B−E). This result indicates a Cys^3−Cys^{35} linkage. Similarly, sequence analysis of the main alkylation product of R2 was performed. In addition to the expected labeling at position 3 and 35, there was a significant increase in PTH-Cys(Cam) at cycles 17 (Figure 4G) and 32 (Figure 4H), but not at cycles 12 and 28 (data not shown). Taking the earlier results into account, it can be concluded that peptide R2 was reduced at disulfides Cys^3−Cys^{35} and Cys^{17}−Cys^{32}. R3, as expected, was fully reduced as all the Cys residues were labeled by iodoacetamide. Thus, the three disulfide bonds can be unambiguously identified as Cys^3−Cys^{35}, Cys^{12}−Cys^{28}, and Cys^{17}−Cys^{32} (Figure 2). Our results with *native* HmK toxin indicate a similar pattern of Cys bridges as found in *synthetic* ShK toxin by earlier workers, using either NMR (Tudor et al., 1996) or analysis of protease digested products by HPLC and mass spectrometry (Pohl et al., 1994).

In addition to defining the disulfide bonds, a definite pattern in the reduction process was apparent from our results. At 25 °C, reduction with TCEP for 10 min yielded three peptide peaks, R1-R3 (Figure 3a), with R1, which has the Cys^3−Cys^{35} bond being reduced, as the predominant product. Prolonging the reduction time shifted the equilib-

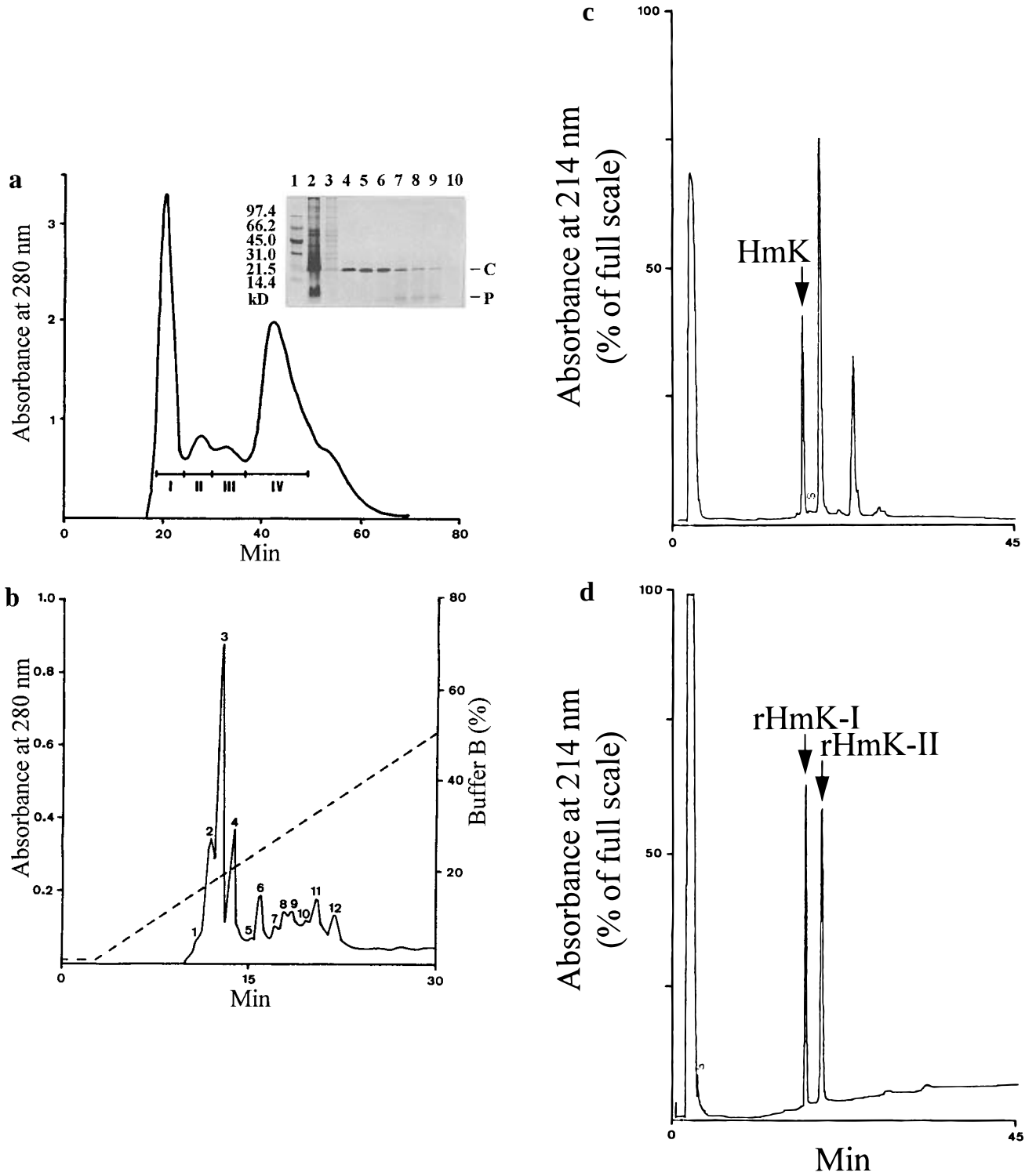

FIGURE 1: Purification of HmK. (a) Gel filtration of 1 g of lyophilized mucus on Sephadex G-50 (45 × 2.5 cm). The sample was dissolved in 5 mL of 0.1 M AmOAc (pH 6.7) and clarified by centrifugation (18 000 rpm, 30 min). Flow rate was 20 mL/h, and fractions of 5 mL were collected. Inset: SDS−PAGE of fractions from Sephadex G-50 run. Lane 1, low molecular weight markers; lane 2, crude mucus of *H. magnifica*; lane 3, peak I fraction; lanes 4−6, peak II fraction; lanes 7−9, peak III fraction; and lane 10, peak IV fraction; C, cytolysin; P, peptides. (b) FPLC ion-exchange chromatography of fraction III (see panel a) on Mono-S (5/5 HR) column. The pH of fraction III was first adjusted to 5.4. Equilibrating buffer; buffer A, 50 mM AmOAc, pH 5.4; buffer B, buffer A with 1 M NaCl. Elution of bound peptides was achieved with a gradient that increased the concentration of B by 50% in 30 min (dotted line) at a flow-rate of 1 mL/min. Eluting peaks were pooled manually. (c) Reverse-phase HPLC of Peak 12 from Mono-S FPLC (see panel b) on an Aquapore RP-300 column (2.1 × 100 mm) at 200 μL/min. Solvent A, 0.1% (v/v) trifluoroacetic acid (TFA); solvent B, 0.085% (v/v) TFA in 70% (v/v) acetonitrile. Elution was performed using a linear gradient of 5−100% from 0−45 min. Full scale absorbance, 0.5. (d) Reverse-phase HPLC of recombinant HmK from Mono-S FPLC on an Aquapore RP-300 column. Conditions were as described for panel c.

rium to the right with more of R2 and R3 being formed (Figure 3b), indicating that bridges Cys^{17}−Cys^{32} and Cys^{12}−Cys^{28} became increasingly accessible to the TCEP. R3 probably represents the completely reduced toxin, being the only product after further treatment of the toxin at 65 °C (data not shown). The kinetics of reduction suggest that the Cys^{3}−Cys^{35} bond is very accessible (highly exposed) and is present on the surface of the molecule while the Cys^{17}−

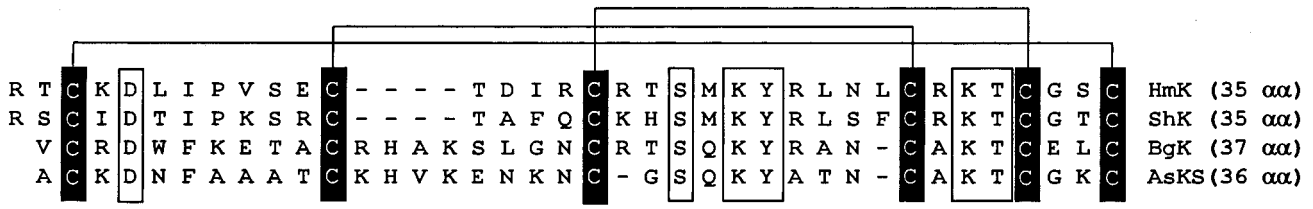

FIGURE 2: Complete amino acid sequence of HmK, and sequence homology between HmK and other sea anemone toxins; ShK: *S. helianthus* K channel toxin, BgK: *B. granulifera* K channel toxin, and AsKS: *A. sulcata* K channel toxin. Sequences were aligned at Cys residues (filled-in boxes). Boxed residues are conserved amino acids. Disulfide bridges of HmK, and the other toxins, are represented schematically. The total number of amino acids (αα) in each toxin is indicated at the end of the respective sequence.

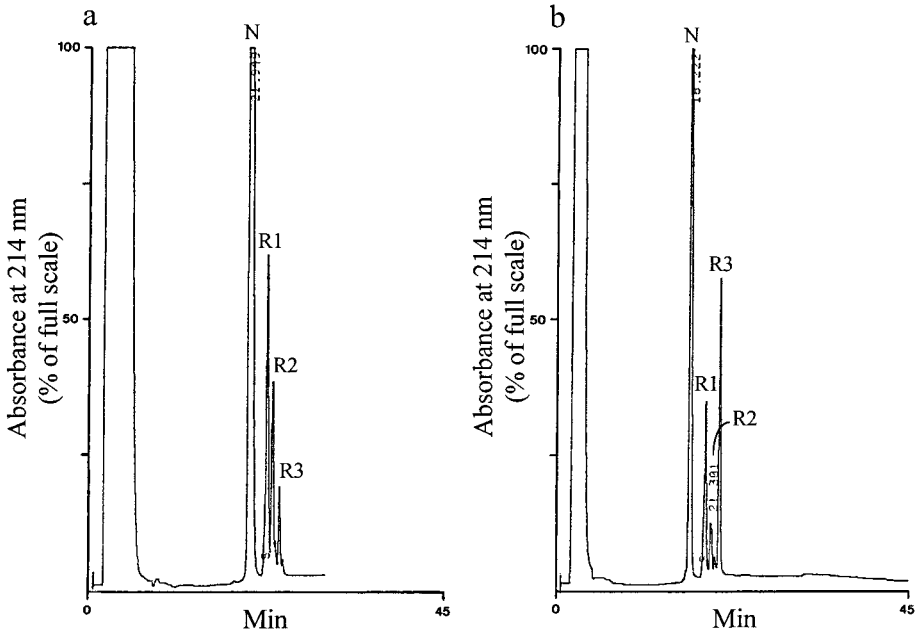

FIGURE 3: Reverse-phase HPLC purification of products from partial reduction of HmK on an Aquapore RP-300 column at 200 μL/min. Solvent A, 0.1% (v/v) TFA; solvent B, 0.085% (v/v) TFA in 70% (v/v) acetonitrile. Elution was performed using gradient of 5−100% from 0−45 min. Full-scale absorbance, 0.5. Peptides eluting at the indicated positions are referred to as N, R1, R2 and R3. (a) Native HmK, N, treated with 10 mM TCEP at pH 3, 20 min, 25 °C. (b) N treated with 10 mM TCEP at pH 3, 10 min, 65 °C.

Cys^{32} bond is only partially exposed. The slower rate of reduction of the Cys^{12}−Cys^{28} bond as compared to the two previous linkages perhaps reflect that this linkage is more deeply buried inside the molecule, thus making it accessible only after the first two bonds have been broken. These results and interpretation are consistent with the NMR-derived structure of ShK toxin (Tudor et al., 1996) and BgK toxin (Dauplais et al., 1997).

Amino Acid Alignment Analysis. Comparison of BgK, ShK and AsKS sequence based on an alignment of Cys residues shows that six other amino acids are identical (D^5, S^{20}, and the dipeptides KY^{22-23} and KT^{30-31}). These amino acids are also conserved in HmK (Figure 2), therefore strongly suggesting common functional roles for these residues. KY^{22-23} (in ShK and the corresponding KY^{25-26} in BgK) were shown to be crucial residues in these toxins for binding to rat brain K channels based on "Ala scan" analyses (Pennington et al., 1996; Dauplais et al., 1997). The same residues are also presumed to be crucial in HmK and AsKS as they are also conserved in these toxins. The highly conserved D^5 and K^{30} (Figure 2) are involved in the formation of a salt bridge between the ionized side chains of the amino acids, as evident from the NMR structure of ShK (Tudor et al., 1996), and has been suggested to be crucial in the proper folding of the toxin (Pennington et al., 1995). K^9 and R^{11} are also important residues in ShK based on electrophysiological assessment of Kv 1.3 using a patch clamp assay on lymphocytes. However, it remains to be established in HmK, BgK, and AsKS, which residues have similar lymphocyte Kv 1.3 channel blocking capability, since these residues are not conserved in these toxins (Figure 2).

Biological Activity. Purified toxin displaced [^{125}I]-α-dendrotoxin from its binding sites on rat brain synaptosomal membranes (Figure 5). Binding of 10 pM [^{125}I]-α-dendrotoxin was inhibited to 50% (IC_{50}) by 13 ng/mL (3.2 nM) toxin. The apparent K_i for HmK (1.1 nM) was calculated from the Cheng-Prusoff formula (Cheng & Prusoff, 1973), assuming K_d for dendrotoxin = 0.7 nM.

Figure 6a shows the effects of exogenously applied HmK (1−25 nM) on the outward K^+ currents recorded from B82 mouse fibroblast cells transfected with Kv 1.2 channels. HmK produced a concentration-dependent reduction in the maximal current of Kv 1.2 channels. Figure 6b shows an original record of K^+ currents blocked by 10 nM HmK.

The time course of HmK block of cloned Kv 1.2 channels was also examined using whole-cell recording B82 cells. Figure 6c shows the current amplitudes recorded in response to pulses to +40 mV, every 10 s, in the absence and presence of 10 nM HmK. The toxin caused a reversible reduction of 60% in current amplitudes, but after washout of the toxin, the current did not recover completely to control levels. The onset of block after the initial application of 10 nM HmK equilibrated with a time constant of 60 s, and recovered during drug washout with a time constant of 100 s. These

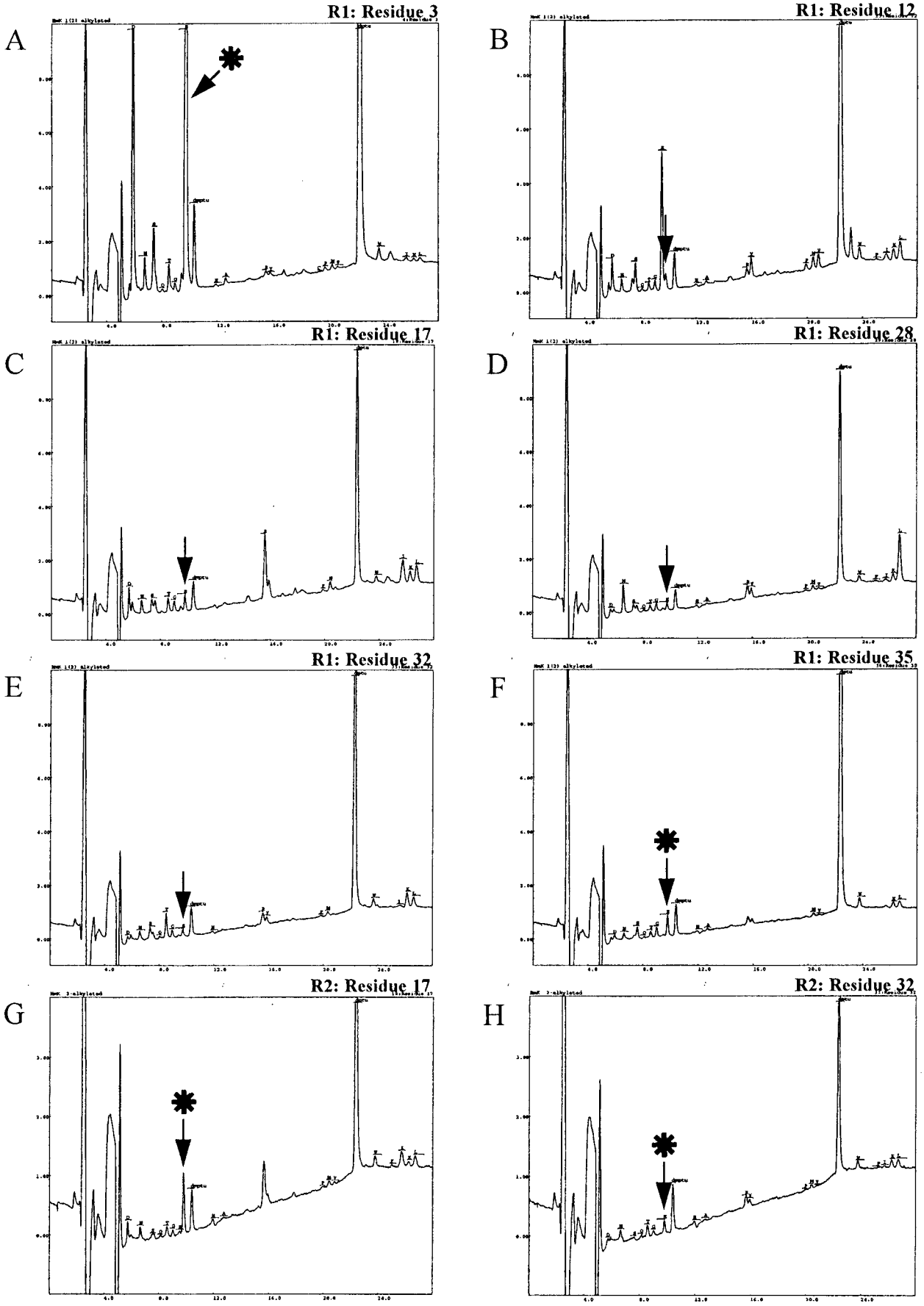

FIGURE 4: Protein sequence analysis of partially reduced and alkylated HmK derivatives. (A−F) HPLC profiles of sequencing product at cycles corresponding to Cys positions in the partially reduced and alkylated peptide R1. (G,H) HPLC profiles of sequencing product at cycles 17 and 32 of the partially reduced and alkylated peptide R2. PTH-Cys(Cam) peaks are indicated by arrows. Significant alkylation of Cys residues are indicative from the peak heights, in relation to non-alkylated derivatives. These are indicated by "*".

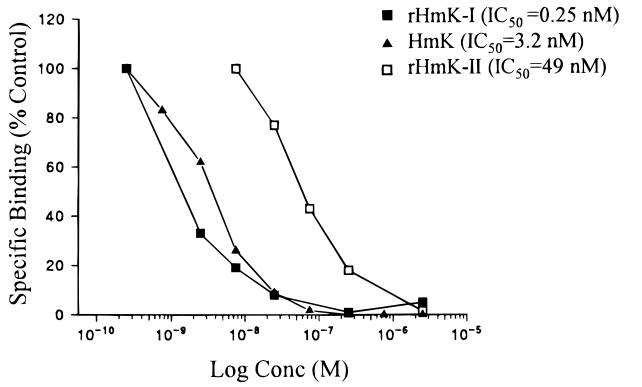

FIGURE 5: Inhibition of [^{125}I]-α-dendrotoxin binding by native and recombinant HmKs. Rat brain synaptosomal membranes were incubated with 10 pM [^{125}I]-α-dendrotoxin in the presence of various concentrations of native HmK and recombinant HmKs, rHmK-I, and rHmK-II .

rates are only approximations of the true rates of channel block and are certainly influenced by the time required to completely exchange the bath solutions.

HmK (≥75 nM) augmented twitch responses of indirectly stimulated chick biventer cervicis preparations without affecting responses to acetylcholine, carbachol, or KCl (Figure 7a) and produced a time-dependent increase in twitch responses to indirect stimulation (Figure 7b). HmK (250 nM) had no effect on twitches in response to direct muscle stimulation (data not shown).

The IC_{50} value of HmK in binding experiments was comparable to those of BgK (IC_{50} = 1.4 nM; Aneiros et al., 1993) and ShK (IC_{50} = 0.7 nM; Castaneda et al., 1995). The four peptide toxins for voltage sensitive K channels recently purified from the sea anemone *Anemonia sulcata* (AsKS, AsKC1−3) had higher IC_{50} values (27−500 nM) (Schweitz et al., 1995). The longer AsKC1−3 (58 or 59 amino acid residues) were unique, in exhibiting Kunitz-like trypsin inhibitors properties, in addition to blocking voltage sensitive K channels.

Cloning and Sequencing of HmK cDNA. A combination of RT-PCR and RACE techniques enabled us to clone and characterize the full-length HmK cDNA. A 105 bp product was amplified from total RNA (5 μg) using M-MuLV reverse transcriptase and antisense primer (P2). Degenerate oligonucleotide primers P1 and P2 used (see Experimental Procedures) were designed based on earlier amino acid sequence information. Inosine (I) was used as the third base of a codon in instances of high degree of degeneracy. The 105 bp product RT-PCR was purified and sequenced. It encoded an amino acid sequence with complete homology to HmK. Flanking sequence information was obtained using RACE technique. The coding region of HmK together with its 5′-flanking sequence was amplified using an antisense RACE adaptor primer (AP1; 5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′) and a sense gene-specific primer (GSP1; 5′-TTT CAT TGA CGT TCT ACA TCG GAT ATC-3′). The 3′-flanking sequence, including the poly-A tail, was amplified using GSP2 sense primer (5′-CCA GTC AGT GAA TGT ACT GAT ATC CGA TGC-3′) and the same AP1. The full-length cDNA of HmK, together with its 5′- and 3′-flanking regions, is shown in Figure 8.

Analysis of cDNA Sequence. The full-length cDNA of HmK was 563 bp. This includes the adaptor sequences found at both ends of the cDNA (underlined in Figure 8),

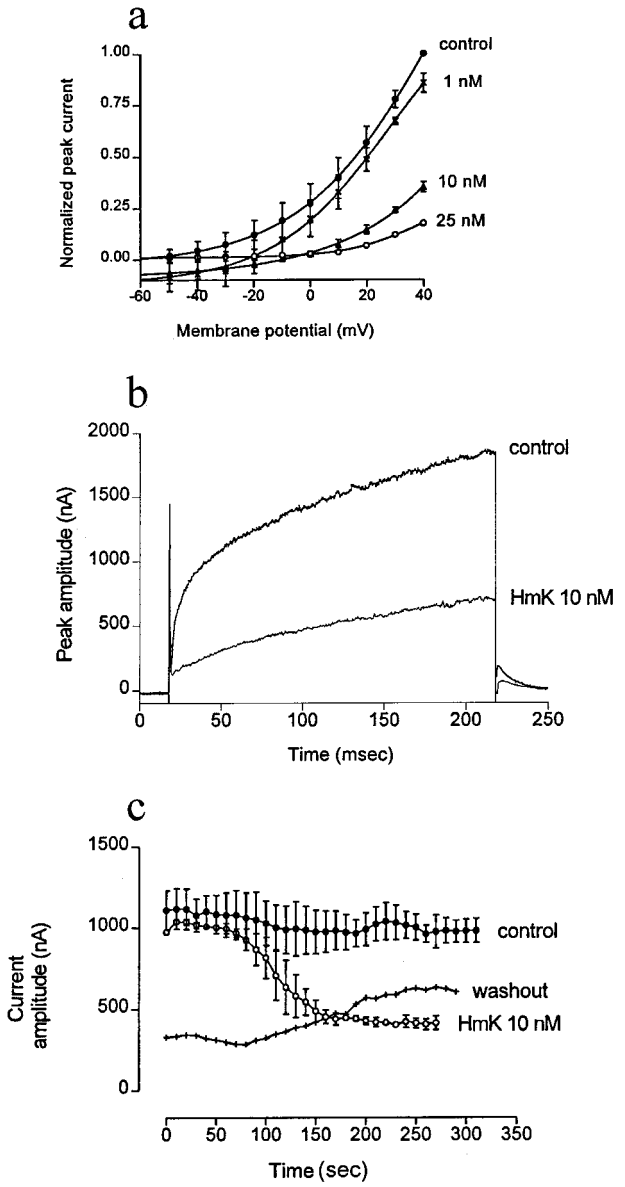

FIGURE 6: (a) Effects of HmK on K^+ currents from cloned Kv 1.2 channels expressed in B82 cells. Control (●; n = 5), and the presence of 1 nM (×; n = 3), 10 nM (▲; n = 2), and 25 nM (○; n = 1) HmK. Currents were evoked from cells held at −60 mV and stepping to the indicated membrane potentials for 250 ms every 10 s. (b) Currents were recorded before and after the addition of 10 nM HmK by a voltage step of +100 mV from a holding potential of −60 mV. (c) Time course of 10 nM HmK block of cloned Kv 1.2 channels expressed in B82 cells. Data shown are control (●), toxin (○), and after toxin washout (+). Mean current amplitudes were measured during 200 ms pulses to +40 mV every 10 s applied from a holding potential of −60 mV.

incorporated as a result of the 5′- and 3′-RACE. The 5′-UTR of HmK cDNA contains the first initiation codon at position 100, following which 222 nucleotides correspond to an open reading frame (ORF), before the first termination codon (TAA) was encountered. The 3′-UTR is therefore 242 bp long. A putative polyadenylation signal (AATAAA) was present 14 nucleotides upstream of the poly-A tail accordingly (Proudfoot & Brownlee, 1976). The ORF predicts a 74 amino acid polypeptide precursor for HmK, which is larger than the toxin that we purified and characterized. The sequence, therefore, infers a signal peptide of 39 amino acids in length, followed by a peptide containing 35 amino acids with a sequence identical to that obtained from

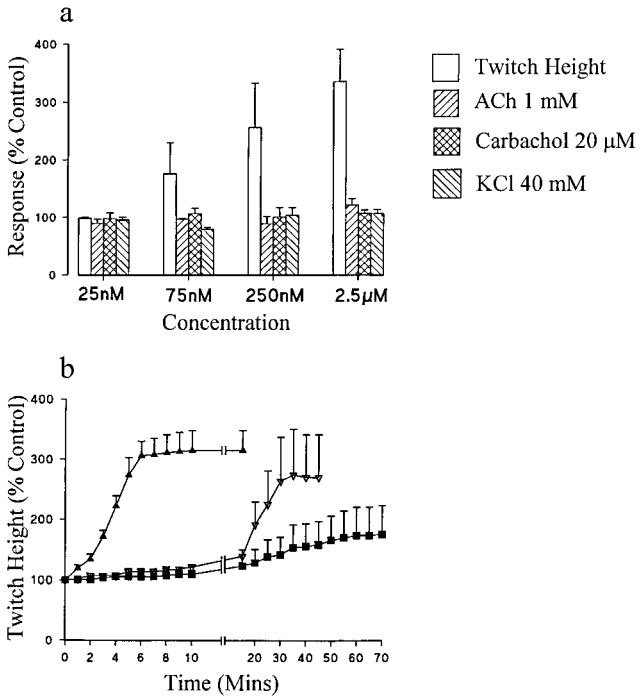

FIGURE 7: (a) Effect of HmK on chick biventer cervicis preparations. Preparations were exposed to HmK until the response to indirect stimulation (electric stimulation of the motor nerve) at 0.1 Hz reached maximum twitch height (see twitch column). Acetylcholine (ACh, 1 mM), carbachol (20 μM), or KCl (40 mM) was then added, and responses without any nerve stimulation were observed. Columns represent the responses after exposure to HmK as a percentage of the pre-toxin control responses. Mean ± SEMS, $n = 4$ (except 2.5 μM, $n = 3$). (b) Time course of the effect of HmK on chick biventer cervicis preparation on indirect stimulation. HmK was used at concentrations 75 nM (■), 250 nM (▽), and 2.5 μM (△). Means ± SEMS, $n = 4$.

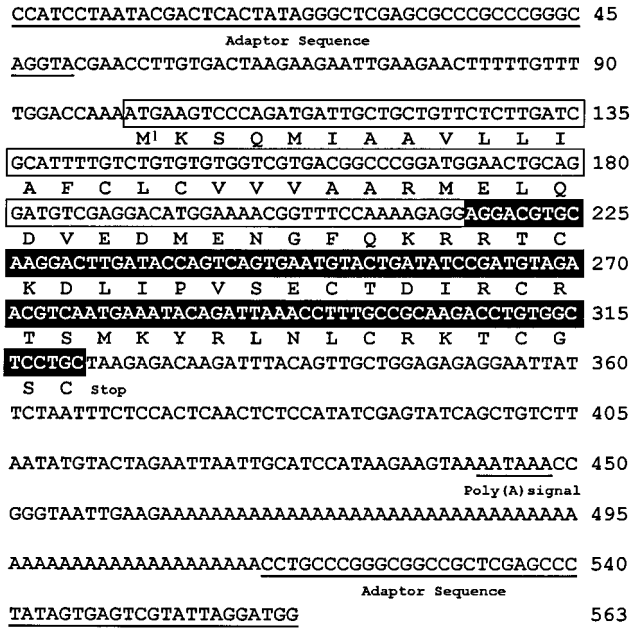

FIGURE 8: Nucleotide sequence of HmK cDNA. ORF sequence is boxed; open box represent the leader sequence, followed by filled-in box carrying the nucleotide sequence of the matured HmK toxin. The deduced amino acid sequence of the ORF is indicated by the single-letter code beneath the second nucleotide of the appropriate codon. The proposed initiating methionine is designated "1". Adaptor sequences and the polyadenylation signal are underlined and indicated in the figure.

direct amino acid sequence analysis of the native HmK. The putative leader sequence following the putative initiating methionine (Figure 8) has structural features distinctive of secreted proteins: a basic N-terminal region, a central hydrophobic region, and a more polar C-terminal region (von Heijne, 1986; Watson, 1984). The nucleotide sequence preceding this Met codon also shows partial homology to the consensus sequence for initiation of translation in vertebrates (Kozak, 1987), having a purine in position −3 (three nucleotides upstream from the ATG codon). There is a purine-rich region (Figure 8 nt 50−100) which is complementary to the conserved 3′-terminal region of many eukaryotic 18S RNA (Hagenbuchle et al., 1987). This purine-rich segment probably represent a putative ribosome binding site during translational initiation.

A Long Signal Sequence Is Not a Prerequisite in the Proper Folding of Short Peptide Toxins with High Density of Disulfide Bonds. Secreted proteins are generally synthesized with amino-terminal extensions, typically 15−60 residues in length, which aid in directing proteins to their final cellular or extracellular destinations (Smith et al., 1990). An earlier review (Smith, 1990) also showed that animal toxin genes previously cloned and characterized from venom glands all encode precursor proteins having signal peptides usually between 16 and 27 residues in length. Our toxin has a leader sequence of 39 amino acids, which is longer than the structural peptide. A long N-terminal region appears to be a characteristic of short peptide toxins with a high density of disulfide bonds as is seen for all Conus toxins. It has been suggested that these long N-terminal regions are probably essential for folding the propeptides into a single biologically active disulfide bonded configuration (Woodward et al., 1990). This notion, however, can be challenged by the successful functional expression of charybdotoxin in *E. coli* (Park et al., 1991) and the recent syntheses of ShK toxin (Pennington et al., 1995) and BgK toxin (Dauplais et al., 1997) which were successfully folded into the biologically active forms containing three intramolecular disulfide bonds, and our successful expression of functionally active HmK in *E. coli* (see later). In these instances, a leader sequence was absent. We suggest, therefore, that the information for proper folding lies solely within the matured peptide, conferring the most thermodynamically stable disulfide configuration. A longer leader sequence, as in our toxin, may then play a more specialized role of targeting and packaging of the inactive precursor molecules in the cnidocytes (the cells specialized for toxin production) until a specific signal induces maturation events that lead to the release of the biologically active peptides (Spagnuolo et al., 1994).

Functional Expression of the HmK Toxin as a Fusion Protein in E. coli. We expressed the HmK toxin in *E. coli* as fusion protein with glutathione *S*-transferase (GST). Nondegenerate sense and antisense primers, both with specific restriction enzyme sites were used to amplify and add restriction sites to the matured HmK toxin DNA sequence for cloning into pGEX-KG expression vector. The amplified HmK sequence, flanked by restriction sites, was restricted and fused onto the C-terminal portion of the GST sequence at the *Bam*HI site, and *Sal*I site further downstream. Usage of specific restriction sites for fusion ensures that the HmK sequence was in the proper orientation to produce the GST-HmK fusion. The hexapeptide sequence LVPRGS, which is recognized by the restriction protease thrombin, is

present immediately upstream from the HmK sequence. A translation termination codon was inserted at the end of the HmK coding sequence. The final construct (termed pHMK1) was transformed into *E. coli* JM109. Clones were first screened for the presence of insert in pHMK1 by PCR. Positive clones were initially grown as 10 mL cultures and later induced in the late logarithmic phase by the addition of IPTG, as described under Experimental Procedures. Aliquots of cell extract were analyzed by SDS−PAGE . Extracts from induced cultures showed the increased expression of a band with M_r ~31.5 kDa, when compared with that of extracts from the uninduced cultures (data not shown). The size of the protein fragment observed from SDS−PAGE is in accordance with that expected from a fusion protein of GST and HmK. Consequently, a preparative scale culture (2 L) was grown for subsequent characterization and activity studies. The fusion protein was purified from the cell extract using a glutathione-agarose column and digested with thrombin. The recombinant peptide eluted as a single peak with the same retention time as the native HmK, when the thrombin digest was fractionated by a Mono-S column (data not shown). Reverse-phase HPLC employing a C_8 column (Aquapore RP-300) was used to confirm the purity of the recombinant HmK. Interestingly, the single peak on Mono-S was further resolved into two approximately equal components (Figure 1d). The first peak (termed rHmK-I) had the same retention time with native HmK, while the second peak (termed rHmK-II) had a slightly delayed retention time. The total final yield of recombinant HmK was approximately 100 μg per liter of culture.

The N-terminal sequences of the first 10 amino acids of rHmK-I and rHmK-II were the same (GSRTCKDLIP), and identical to that of the naturally occurring HmK except for the first two amino acids which are the expected residual G and S, left over from the thrombin cleavage site. Both rHmK-I and rHmK-II also displaced [^{125}I]-α-dendrotoxin from rat brain synaptosomal membranes (Figure 5). Whilst the ability of rHmK-I to displaced [^{125}I]-α-dendrotoxin was the same as native HmK (Figure 5), rHmK-II did so at a lower efficacy with an IC_{50} value of 50 nM. The 15-fold decrease in the ability of rHmK-II to displace [^{125}I]-α-dendroroxin suggests that rHmK-II may represent a misfolded form of recombinant HmK. The recombinant toxin rHmK-I therefore is more representative of the native toxin based on its chromatographic behavior on a reverse-phase column and by competitive binding assays with [^{125}I]-α-dendrotoxin.

We are currently establishing the disulfide linkage patterns of rHmK-I, to confirm the authenticity of this recombinant toxin relative to the native HmK, and that of rHmK-II, to shed some light on the nature of its folding, which will prove useful in the renaturation of this isoform.

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