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A Novel Conotoxin from *Conus betulinus*, κ-BtX, Unique in Cysteine Pattern and in Function as a Specific BK Channel Modulator*

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A novel conotoxin, κ-conotoxin (κ-BtX), has been purified and characterized from the venom of a worm-hunting cone snail, *Conus betulinus*. The toxin, with four disulfide bonds, shares no sequence homology with any other conotoxins. Based on a partial amino acid sequence, its cDNA was cloned and sequenced. The deduced sequence consists of a 26-residue putative signal peptide, a 31-residue mature toxin, and a 13-residue extra peptide at the C terminus. The extra peptide is cleaved off by proteinase post-processing. All three Glu residues are γ-carboxylated, one of the two Pro residues is hydroxylated at position 27, and its C-terminal residue is Pro-amidated. The monoisotopic mass of the toxin is 3569.0 Da. Electrophysiological experiments show that: 1) among voltage-gated channels, κ-BtX is a specific modulator of K^+ channels; 2) among the K channels, κ-BtX specifically up-modulates the Ca^{2+}- and voltage-sensitive BK channels (252 ± 47%); 3) its EC_{50} is 0.7 nM with a single binding site (Hill = 0.88); 4) the time constant of wash-out is 8.3 s; and 5) κ-BtX has no effect on single channel conductance, but increases the open probability of BK channels. It is concluded that κ-BtX is a novel specific biotoxin against BK channels.

Biotoxins have been used widely to identify different subtypes of ion channels in excitable cells. In many cases, determination of a new subtype of ion channel has depended on finding a specific biotoxin (1, 2). In particular, K^+ channels are much more diverse than Na^+ and Ca^{2+} channels. Therefore, new, subtype-specific biotoxins provide valuable tools to identify distinct subtypes of K^+ channels and to study their functions in native systems (3–6).

Cone snails are predatory, venomous mollusks that use a common general strategy to capture their prey. As a genus, cones use a rather diverse spectrum of prey, including at least three different types: fishes, other mollusks, and marine worms. Their venoms contain a large number of small, conformationally constrained peptides that display highly potent and specific biological activity. For example, α-conotoxins are competitive antagonists of the nicotinic acetylcholine receptor; δ- and μ-conotoxins act at sites VI and I of voltage-sensitive sodium channels, respectively; ω-conotoxins selectively inhibit presynaptic calcium channels at neuromuscular junctions; and κ-conotoxin PVIIA inhibits *Shaker*-type potassium channels (7–13). The conotoxins are much smaller than other peptide toxins, with only 10–30 amino acids. The pattern of disulfide bridges of each conotoxin family is relatively conserved (14). A large number of conotoxins have been characterized, most of them from piscivorous (fish-hunting) and molluscivorous (mollusk-hunting) snails, since they have relatively high toxicity to vertebrates. In contrast, the venom of vermivorous (worm-hunting) snails has been seldom studied.

A number of conotoxins were characterized in a recently published work (15). In this study, we investigated the venom of a vermivorous species, *Conus betulinus*, which is found in the South China Sea. Although not highly toxic to vertebrates, the venom of *C. betulinus* causes obvious symptoms such as aggressiveness, stiff tail, paralysis, convulsions, and even death when injected intraventricularly into mice. In the first part of this report, we describe the purification and characterization of a new family of conotoxins, κ-conotoxin BtX (κ-BtX),[1] by using protein sequence determination, gene cloning, and functional assays. This toxin is characterized by the presence of four instead of two or three disulfide bonds, as usually found in other conotoxins, and shares no sequence homology with any others.

In the second part of this report, we describe the electrophysiological findings from whole cell patch clamp recording that κ-BtX is a K^+ channel up-modulator, not a blocker, tar-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF208661.

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[1] The abbreviations used are: κ-BtX, κ conotoxin BtX; ChTX, charybdotoxin; IbTx, iberiotoxin; HPLC, high performance liquid chromatography; RACE, rapid amplification of the cDNA end; TPCK, tosylphenylchloromenthyl ketone; TEA, tetraethylammonium chloride; MS, mass spectrometry; MALDI, matrix-assisted laser desorption ionization; TOF, time of flight; BK, voltage-dependent big conductance K(Ca^{2+}) channel; SK, small conductance K(Ca^{2+}) channel; RACC, rat adrenal chromaffin cell; PTH, phenylthiohydantoin.

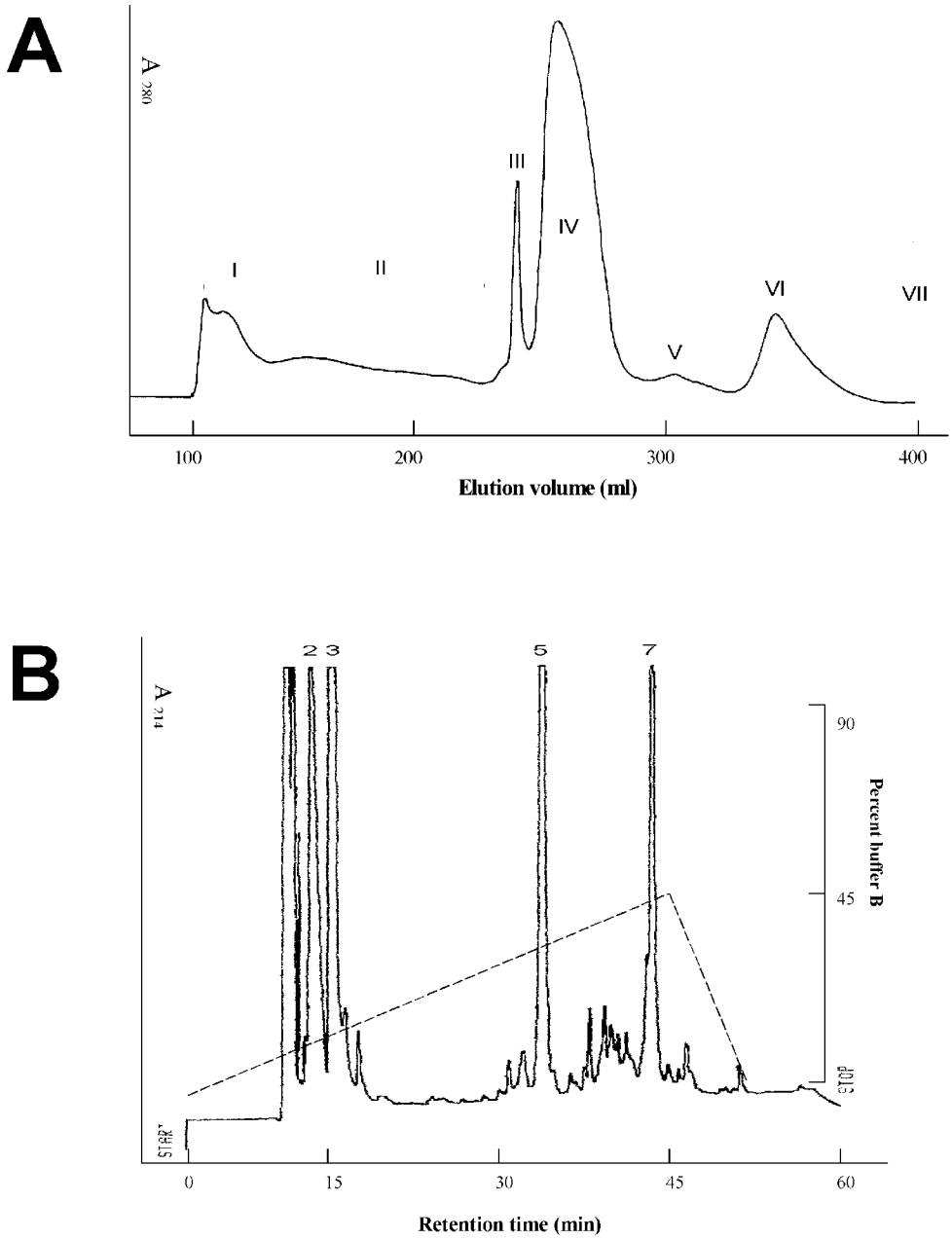

FIG. 1. **Purification of κ-conotoxin BtX.** As shown in *A*, extracted crude venom was applied to a Sephadex G-25SF column (100 × 2.6 cm) eluted with 1.1% acetic acid. The fractions were collected at a flow rate of 0.35 ml/min. As shown in *B*, freeze-dried fraction IV was applied to a Phenomenex C18 semipreparative column (25 × 1 cm) and eluted with a linear gradient from 100% A, 0% B to 55% A, 45% B over 45 min and then with 100% A, 0% B for 5 min at a flow rate of 2 ml/min. Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.1% trifluoroacetic acid in acetonitrile. The κ-BtX fraction was present in peak 5, which was repurified under the same conditions.

geting the Ca^{2+} and voltage dependent K^+ channel in rat adrenal chromaffin cells (RACCs). κ-BTX is therefore a novel and unique pharmacological tool to dissect out the functional properties of the different subtypes of K^+ channels.

EXPERIMENTAL PROCEDURES

Materials—Sephadex G-25 was purchased from Amersham Biosciences. Trifluoroacetic acid and acetonitrile for HPLC were from Merck. The 3′-RACE and 5′-RACE kits, TRIzol reagent, and T4 DNA ligase were purchased from Invitrogen. Restriction endonucleases and *Taq*DNA polymerase were from MBI Company and Sangon Company, respectively. The DNA sequencing kit was purchased from Promega, and [α-^{32}P]dATP was purchased from Amersham Biosciences. Acrylamide, bisacrylamide, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropyl-thio-β-D-galactoside, and all other reagents were of analytical grade. All chemicals used in the electrophysiological experiments were from Sigma.

Peptide Purification—Specimens of *C. betulinus* were collected from Sanya, Hainan Province in the South China Sea. The venom apparatus was dissected out, and the venom duct was cut in sections and homogenized. The venom was then extracted with 1.1% (v/v) acetic acid containing the protease inhibitor phenylmethylsulfonyl fluoride. The sample was centrifuged at 12,000 rpm at 4 °C for 5 min. The residue was resuspended and soaked in the same buffer for 10 min, and then centrifuged again. Finally, the supernatants were combined, lyophilized, and stored at −20 °C. Lyophilized samples were reextracted with 1.1% acetic acid. After centrifugation, the supernatant was placed on a pre-equilibrated Sephadex G-25SF column (100 × 2.6 cm) and eluted with 1.1% acidic acid at 4 °C. The fractions were collected at flow rate of 0.35 ml/min (Fig. 1*A*). The fractions from each peak were pooled and lyophilized. The freeze-dried fractions IV from gel filtration were dissolved in 0.1% trifluoroacetic acid and applied to HPLC with a Phenomenex C18 semipreparative column (25 × 1 cm, micron). The peptides were eluted with a gradient of 0.1% trifluoroacetic acid in

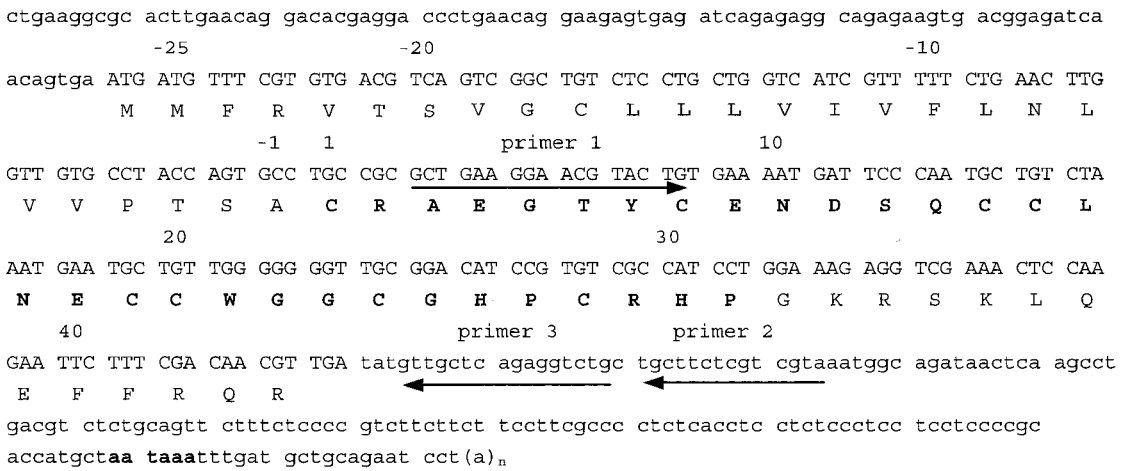

FIG. 2. **cDNA and predicted amino acid sequence of κ-conotoxin BtX.**

acetonitrile. The κ-BtX fraction was located in peak 5 (Fig. 1*B*), which was repurified under the same conditions.

Amino Acid Sequence Determination—About 200 μg of the purified peptide was dissolved in 1 M Tris-HCl, 6 M guanidine hydrochloride, and 1 mM EDTA at pH 8.5 and reduced with 2 mg of dithiothreitol at 37 °C for 5 h. Then it was alkylated with 5 mg of idoacetic acid and kept in the dark for 30 min at room temperature. The modified peptide was purified by HPLC. This carboxymethylated peptide (Rcm-κ-BtX) was directly used for automatic sequencing on a PE ABI model 491 peptide/protein sequencer and PTH analyzer using the program provided by the manufacturer.

Enzyme Hydrolysis—Rcm-κ-BtX- and TPCK-treated trypsin were mixed at a ratio of 50:1 and incubated in 0.5 ml of buffer (20 mM Tris-HCl, 10 mM calcium chloride, pH 7.8) at 37 °C for 10 h. The cleaved fragments were separated and purified on a C-18 reverse-phase HPLC.

Mass Spectrometry—Mass spectral analyses of the native toxin, its modified form Rcm-κ-BtX, and degraded fragments were performed on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA). The apparatus was equipped with an electrospray ionization source with a spray voltage of 4.5 kV. Mass spectra of the native toxin were also obtained using a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source and a matrix assistant laser desorption ionization (MALDI) TOF mass spectrometer (Brucker Biflex III).

3′- and 5′-RACE—3′- and 5′-RACE were used to clone the cDNA of κ-BtX from the fresh venom ducts of *C. betulinus* by using the TRIzol reagent kit. Five μg of total RNA was converted into cDNA using superscript II reverse transcriptase and a universal oligo(dT)-containing adapter primer (5′-GGCCACGCGTCGACTAGTAC($dT)_{17}$-3′). Using the degenerate codons, a gene-specific primer 1 with a *Bam*HI restriction site (as underlined in 5′-CGGGATCC GCC AAC GGT AC(G/A/T/C) TA(T/C) TG-3′) encoding residues 3–8 of κ-BtX was designed and synthesized. Primer 1 was paired with an abridged universal primer with a *Hin*dIII restriction site (as underlined in 5′-GCAAGCTTACGCGTC-GACTAGTAC-3′) similar to the adapter primer but devoid of the polyT for 3′RACE amplification. The amplification products with a size of about 300 bp were purified, cloned into the T-vector, and transformed into DH5α, and the positive clones were sequenced.

5′-RACE is based on the partial cDNA sequence determined by 3′-RACE. A gene-specific primer 2 with a *Bam*HI restriction site (as underlined in 5′-CGGGATCCTACGACGAGAAGCA-3′) corresponding to the cDNA sequence downstream of the stop codon at bp 21–34 was designed and synthesized. The first strand cDNA was transcripted with primer 2 using 1 μg of total RNA as template. After the cDNA was purified on a Glassmax column, the homopolymeric dC was then tailed to its 3′-end. The dC-tailed cDNA was further amplified using a nested primer 3 with an *Xba*I restriction site 3 bp downstream from the stop codon (as underlined in 5′-CGTCTAGACAGACCTCTGAGCAAC-3′) and an abridged anchor primer (5′-GGCCACGCGTCGACTAGTACGG-GIIGGGIIG-3′) complementary to the dC tail. To obtain a high yield of toxin-specific cDNA, a second PCR was performed using primer 3 and another anchor primer devoid of dG with a *Hin*dIII restriction site (as underlined in 5′-GCAAGCTTACGCGTCGACTAGTAC-3′). The final PCR products were sequenced as described above. The whole cDNA sequence of κ-BtX was generated by combining the two fragments amplified by 3′- and 5′-RACE.

Amplification of the Genomic DNA—The total genomic DNA was isolated from cone venom glands using a $NaClO_4$ extraction procedure followed by RNase treatment (16). Primer 4 with an *Xba*I restriction site (as underlined in 5′-CGTCTAGA TGC CGC GCT GAA GGA-3′), corresponding to amino acid residues 1–5 (Cys-Arg-Ala-Glu-Gly), and primer 2 were used as a pair to amplify the genomic DNA of κ-BtX. The amplified product with a size of around 200 bp was purified and cloned into the T-vector for sequencing.

Cell Culture—RACCs were prepared from adult Wistar rats (250–300 g) as described previously (17, 18). Single cells were obtained after a 40-min digestion in enzyme solution. The cells were cultured with Dulbecco's modified Eagle medium in a CO_2 incubator. Cells were used in experiments after 1–6 days in culture.

Electrophysiology—The standard external solution (bath solution) contained 140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, at pH 7.4. The "high TEA (tetraethylammonium chloride)" solution was the same as the standard solution, except that the 20 mM NaCl was replaced by 20 mM TEA. The "high Cs^+" solution was the same as the standard, but KCl was replaced by CsCl. The standard internal solution in the patch pipette contained 145 mM KCl, 8 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, and 250 μg/ml nystatin, pH 7.2. Samples of κ-BtX were dissolved in the bath solution at the final concentration needed.

The voltage-gated membrane currents were recorded under whole cell voltage clamp using the nystatin perforated patch clamp technique (3, 4). The series resistance (R_s) was typically 10–20 megaohms before R_s compensation. Before cell recording, 75–90% R_s compensation was applied to allow fast voltage clamp of the voltage-dependent big conductance K(Ca^{2+}) channel (BK) currents. An outside-out patch was obtained by excising the patch from a cell in the whole cell configuration. Experiments were done using patch clamp amplifiers Axon 200B (Axon Instruments, Foster City, CA) and PC-2B (INBIO Inc., Wuhan, China). Data were analyzed with Igor software (AveMatrix, Lack Oswego, OR).

The κ-BtX was applied to the cell under investigation through a glass micropipette by applying slight positive pressure (3). Control/wash-out solutions and toxins other than κ-BtX were puffed locally onto the cell via an RCP-2B multichannel microperfusion system (INBIO Inc., Wuhan, China), which allowed fast change of solutions by electronic switching between seven solution channels (18). The puffer pipette (100-μm tip diameter) was located about 120 μm from the cell. We determined, by a conductance test using pure water as perfusion solution, that the recorded cell would only be contacted by puffer solution if the application speed was 100 μl/min or faster. All of the pharmacological experiments in this study met this requirement. All these experiments were done at room temperature (22–25 °C).

Data Analysis—Each data point of a dose-response curve represents the mean ± S.D. of at least five cells. Dose-response curves were fit to Equation 1

$$\gamma = 100/\{1+([\text{toxin}]/EC50)^n\} \quad \text{(Eq. 1)}$$

TABLE I
Measured and calculated molecular masses for κ-BtX

Species	LCQ-MS(ESI)	MALDI-TOF-MS	Q-TOF-MS(ESI)
	Average mass	Average mass	Monoisotopic mass
Natural κ-BtX	3572	3440	3569.0
(Calculated)	(3572)	(3572)	(3569.2)
Rcm-κ-BtX	4044	3912	
(Calculated)	(4044)	(4044)	

where γ is the relative response (%); n is the Hill coefficient; [toxin] is the drug concentration; and EC_{50} is the dissociation constant.

To define the average open probability (P_o) of single channels in a patch containing more than one BK channel, we determined the probability that r channels were open simultaneously (19). The parameter r follows a binomial distribution, as shown in Equation 2

$$P(r) = \sum_{r=0}^{n} C_n^r P_0^r (1 - P_0)^{n-r} = \frac{n!}{r!(n-r)!} P_0^r (1 - P_0)^{n-r} \quad (r = 0,1, \cdot \cdot \cdot \cdot n) \qquad \text{(Eq. 2)}$$

where n is the total number of channels in the patch and P_o is the open probability. Equation (2) was used to fit the mean open time distribution and determine P_o. This formula is ideal for non-inactivating channels. However, since the inactivation rate of BK channels was not altered by κ-BtX (see Figs. 4 and 7), we could still use this formula to calculate *relative changes* of P_o with and without κ-BtX. Total open time of the single channels was defined as the sum of open times from all single channels during a given pulse. If two channels opened simultaneously, the total open time was the sum of the open times of both channels.

RESULTS

Amino Acid- and cDNA-deduced Sequences of Conotoxin κ-BtX—The κ-BtX purified from *C. betulinus* venom was subjected to automatic sequencing directly after reduction and carboxymethylation. The partial N-terminal sequence was determined as follows: CRANGTYCNNDSQCCLN. Since the retention times of PTH-Gla and PTH-Asn were very close, Gla residues 4 and 9 were at first misinterpreted as Asn (underlined). According to the N-terminal partial sequence of the toxin, the gene-specific primer 1 was designed and synthesized for 3′-RACE. Despite the mismatch in the synthesized oligonucleotide resulting from the misinterpreted amino acid in position 4 (corresponding to codon AAC instead of GAA in the primer), the 3′-RACE could still be performed successfully. Based on the partial 3′-cDNA sequence, primer 2 was used for the 5′-RACE. The whole cDNA sequence of the conotoxin κ-BtX was subsequently obtained by combining the two amplified fragments (Fig. 2).

The deduced amino acid sequence consisted of a putative signal peptide of 26 residues and a protoxin of 44 residues. According to the amino acid analysis (data not shown) and the partial N-terminal sequence, the sequence of the mature toxin devoid of an extra C-terminal was as follows: CRAEGTYCENDSQCCLNECCWGGCGHPCRHP* (the asterisk indicates C-terminal amidation). The C terminus of the mature toxin was followed by an extra 13-residue peptide, as a propeptide, which was removed during post-translational processing. The calculated molecular mass of this mature toxin was 148 Da less than that measured. Furthermore, there was a 132 Da difference between the LCQ-MS and the MALDI-TOF-MS analyses (Table I). These differences suggested that the toxin might be post-translationally modified, for example by carboxylation of glutamate, hydroxylation of proline, and amidation of the C terminus, as is often the case for conotoxins. If all three Glu residues at positions 4, 9, and 18 were carboxylated (Gla), one of the two Pro residues at position 27 or at 31 hydroxylated, and the C terminus amidated, then the calculated molecular weight (3569.2) would be quite consistent with the measured value (3569.0). Furthermore, the presence of Gla residues was verified by the mass spectrometric analysis. When the MALDI source was used instead of the electrospray ionization source, the decarboxylation of the peptide resulted in a total loss of 132 Da, corresponding to three decarboxylated groups of Gla residues.

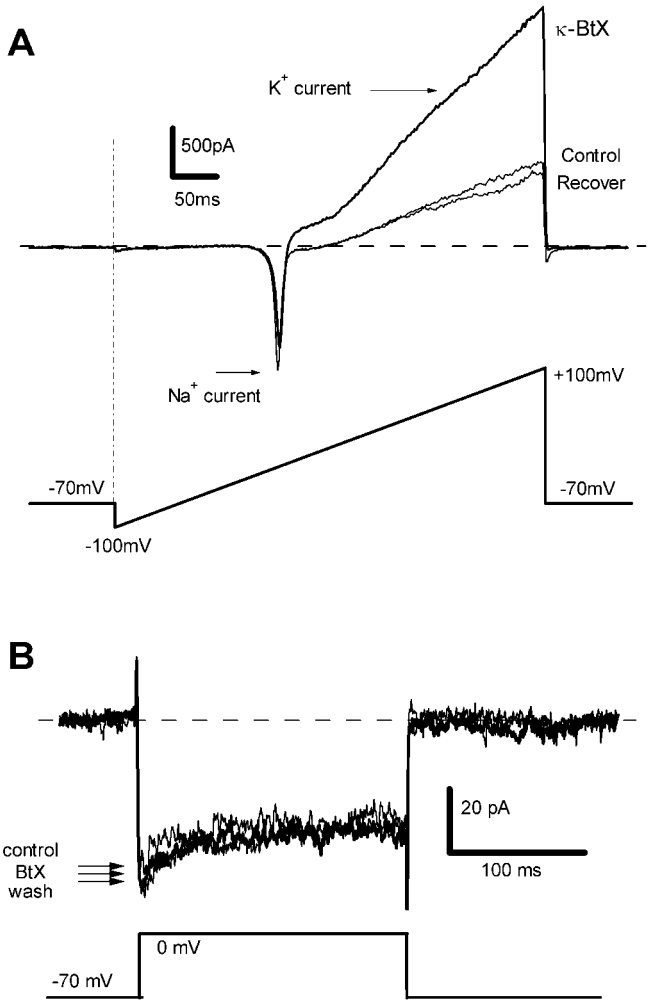

FIG. 3. **Effects of κ-BtX on Na^+, K^+, and Ca^{2+} currents in RACCs.** As shown in *A*, whole cell currents were induced by 100-ms voltage ramps (−100 to 100 mV). κ-BtX had negligible effects on the downward (inward) Na^+ current. However, it increased the outward K^+ current around 2-fold. The average up-modulation was 164 ± 10% ($p < 0.01$, $n = 11$). *B*, effect of κ-BtX on Ca^{2+} currents. Ca^{2+} currents were induced by depolarizing pulses in high TEA (20 mM) in the bath and high CsCl (145 mM) internal solution. All records were from the same cell with 90-s intervals. The Ca^{2+} current induced during κ-BtX application was nearly the same as in control. The slight decline of Ca^{2+} current during the recovery was due to the rundown of Ca^{2+} channels.

To determine in which position the hydroxyproline was located, the reduced and carboxymethylated conotoxin was further digested with TPCK-trypsin. As there are only two cleavage sites for TPCK-trypsin in the toxin (Arg at positions 2 and

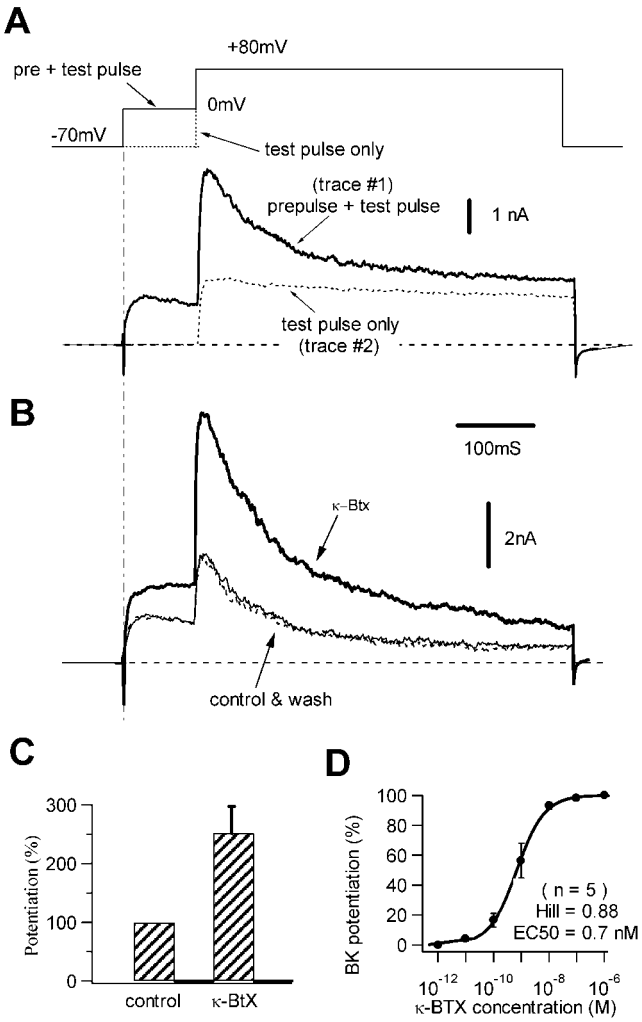

FIG. 4. **κ-BtX increases Ca^{2+}- and voltage-dependent BK channel currents.** *A*, the protocol to record BK currents in RACC. Two current traces from the same patch were recorded and superimposed according to the stimulation time (*upper*). The first voltage protocol (*solid line*) consisted of two pulses. The second voltage protocol (*dashed line*) was similar to the first protocol, except there was no prepulse before the 80 mV pulse. The current induced by the second (no prepulse) protocol (*trace 2*) was mainly due to Kv channels (see "Results"). The difference between *traces 1* and *2* gave the pure BK current ($n > 100$). As shown in *B*, κ-BtX increased the pure BK current by 229% in this cell. The traces show the pure BK currents obtained with the protocol described in *panel A*. The three current traces, before, during, and after application of 10 nM κ-BtX, were superimposed according to stimulation time. *C*, statistics of κ-BtX up-modulation of BK currents shown in *panel B*. On average, κ-BtX increased BK currents by 2.52 ± 0.47-fold ($p < 0.01$, $n = 65$). *D*, dose-response curve of κ-BtX up-modulation of the pure BK currents. The pure BK current was elicited by the protocol shown in *panel A*. The curve was fitted by the Hill function (see "Experimental Procedures"). The EC_{50} was 0.7 nM, and the Hill coefficient was 0.88 ($n = 5$).

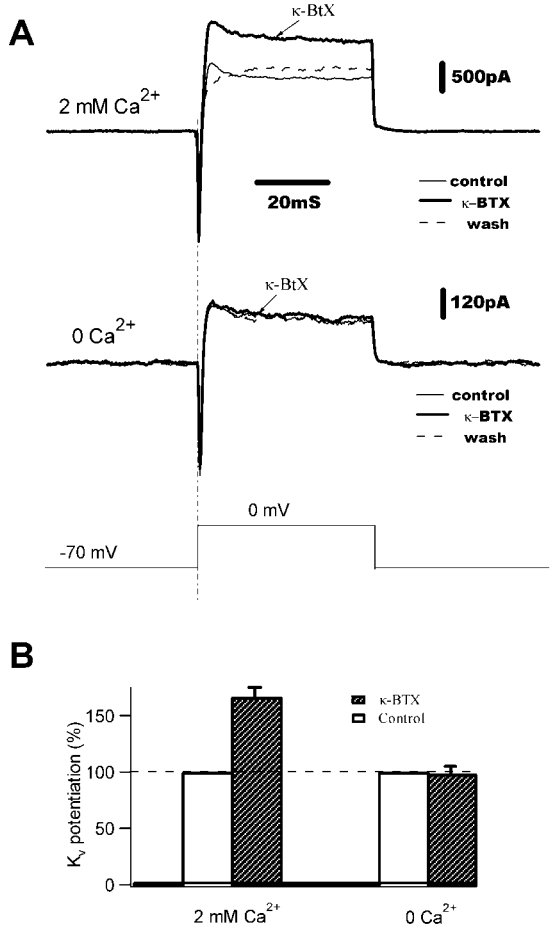

FIG. 5. **κ-BtX has no effect on Ca^{2+}-independent but voltage-dependent K^+ currents.** As shown in *A*, κ-BtX has no effect on K^+ currents in Ca^{2+}-free bath. The outward current of the depolarization-induced currents was K^+ current. κ-BtX increased the outward K^+ current to 160% of the control level in 2 mM Ca^{2+} bath (*top panel*). However, κ-BtX had no effect on the outward K^+ current in 0 Ca^{2+} bath (*middle panel*), indicating that all Ca^{2+}-independent K^+ currents were insensitive to κ-BtX. All traces were from the same cell. As shown in *B*, on average, κ-BtX increased the BK current to 167 ± 7% of control. In the same cells, removing Ca^{2+} from the bath abolished the κ-BtX effect completely (98 ± 4% of control). This suggests that κ-sensitive K^+ currents are exclusively Ca^{2+}-dependent ($n = 5$).

29), the enzymatic cleavage removed two peptides, both with two residues, from the N and C termini. The remaining fragment (from residues 3 to 29) was then purified on HPLC and subjected to mass spectrometric analysis. A mass of 3492 Da was measured, matching quite well with the calculated value (3492.5 Da). Thus, the hydroxyproline was located at position 27, and the full sequence of κ-BtX is as follows: CRAγ GTYCγNDSQCCLNγCCWGGCGHOCRHP* (γ is γ-carboxyglutamate; O is hydroxyproline; the asterisk indicates C-terminal amidation).

Cloning and Sequencing Genomic DNA of κ-BtX—Based on the elucidated cDNA sequence of κ-BtX, primers 2 and 4 were used to amplify the genomic DNA corresponding to the κ-BtX gene. The analysis showed that the genomic sequence is identical to the cDNA sequence. This finding implies that there is no intron in the genomic DNA structure of κ-BtX.

Effects of κ-BtX on Voltage-gated Channels in Chromaffin Cells—Chromaffin cells are endocrine cells that differentiate from the neural crest during embryonic development. They are excitable cells with typical neuronal voltage-gated Na^+, K^+, and Ca^{2+} channels and are widely used as a neuronal model (3, 17, 22–24). To investigate whether κ-BtX affects voltage-gated ion channels, we studied the effect of κ-BtX on these channels in RACCs (Fig. 3). In Fig. 3*A*, the *top panel* shows current traces induced by a voltage ramp (*bottom panel*). Three ramp-induced currents were superimposed. The cell was stimulated by the first ramp in the absence of toxin (*Control*). The inward transient current was activated at about −30 mV and corresponded to a typical, voltage-gated Na^+ current. The outward current (positive current component) was activated from −30 mV and increased in amplitude toward positive voltages, as expected for a typical, voltage-gated outward K^+ current (17, 24). The cell was subsequently stimulated by a second ramp in the presence of 1 μM of κ-BtX, which resulted in a 2-fold in-

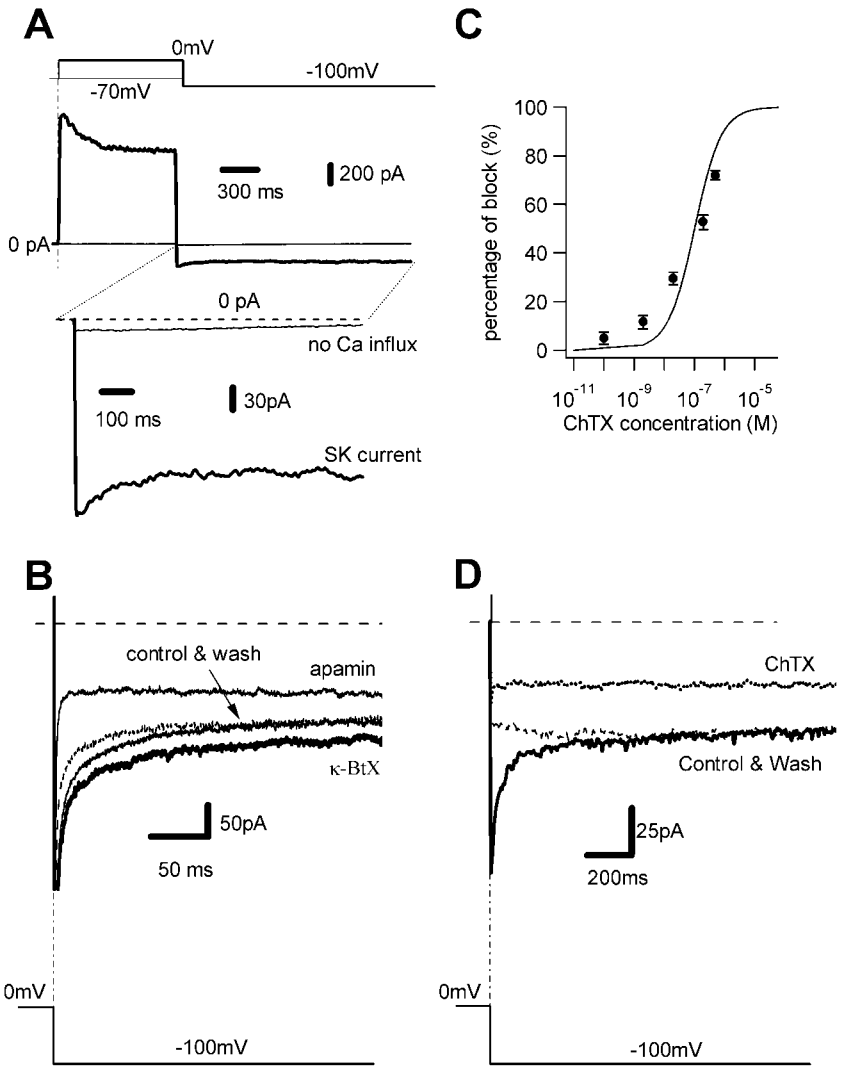

FIG. 6. **κ-BtX has little effect on SK currents.** *A*, protocol to detect pure SK in RACCs. The slow tail current was induced by the double-pulse protocol and recorded during the second pulse at −100 mV in 2 mM Ca^{2+} bath solution. The SK current was activated by Ca^{2+} influx during the 1-s prepulse in the 2 mM Ca^{2+} bath (*thicker trace*). In the same cell, SK currents were absent when no prepulse was given to induce Ca^{2+} influx (*thinner trace*, $n = 4$). As shown in *B*, SK currents were induced using the protocol described in *panel A* before, during, and after application of κ-BtX. In this cell, κ-BtX (1000 nM) increased the SK current by only 11%. The average increase of SK by κ-BtX was 9 ± 4% ($n = 13$). Apamin (100 nM) blocked 50% of the current, indicating that the Ca^{2+}-sensitive inward current at −100 mV was indeed SK current. *C* and *D*, effects of ChTX on SK channels. SK was blocked by 200 nM ChTX (*D*). The dose-response curve of ChTX shows that 200 nM blocked 52 ± 3%. The Hill coefficient was 0.95, indicating a single binding site between ChTX and SK ($n = 8$).

crease of the peak outward current. The up-modulating effect on the outward current was statistically significant. The K^+ current in response to the third ramp, after removal of κ-BtX, recovered to the control level. Along with Na^+ and K^+ currents, RACCs have voltage-gated Ca^{2+} channels of the L-, N-, P/Q-, and R-types (23). Fig. 3*B* shows the effect of κ-BtX on Ca^{2+} currents, which were sensitive to 200 μM Cd^{2+} (data not shown). To isolate the Ca^{2+} currents, the Na^+ current was blocked by removing Na^+ from the external solution. All voltage-gated K^+ currents were blocked by high extracellular TEA and high internal Cs^+. The three Ca^{2+} current traces were recorded as control, test (1 μM κ-BtX), and recovery, sequentially at 120-s intervals. All three Ca^{2+} currents were nearly identical, indicating that κ-BtX had little or no effect on the voltage-gated Ca^{2+} channels ($n = 7$).

Up-modulation of BK Currents by κ-BtX—The target of κ-BtX was found to be the BK channel (Fig. 4). In a standard double-pulse protocol to test the BK current (25), the first step of the double pulse (from −70 to 0 mV for 100 ms) induced Ca^{2+} influx through the voltage-gated Ca^{2+} channels, which activated the typical large and fast-inactivating BK currents during the strong depolarization of the second step (from 0 to 80 mV for 500ms, Fig. 4*A*, *trace 1*). Most BK currents were absent when the prepulse for loading Ca^{2+} was omitted (Fig. 4*A*, *trace 2*). The difference between *traces 1* and *2* yielded the pure BK currents, and this difference was used to determine the effects of κ-BtX (Fig. 4*B*). During application of 10 nM κ-BtX, the BK currents induced by the double-pulse protocol were 2.3 times greater than the control (or the current after wash-out). On average, κ-BtX (10 nM) increased (or up-modulated) BK currents by 2.51 ± 0.47 times control ($n = 65$, Fig. 4*C*). Since BK current was the dominant component in the double-pulse protocol, the "pure BK currents" in Fig. 4*B* were very similar to *trace 1* in *panel A*. The dose-response curve of the up-modulation effect is illustrated in Fig. 4*D*. κ-BtX had an EC_{50} of 0.7 nM for BK channels. The Hill coefficient was 0.88, indicating that there was a single binding site between κ-BtX and the BK channel.

Selectivity of κ-BtX among K^+ Channels—We found that the BK current was the exclusive subtype of voltage-gated K^+ channel sensitive to κ-BtX in RACCs (Fig. 5). Pure Ca^{2+}-independent but voltage-dependent K^+ current, or K_z current, can be recorded in Ca^{2+}-free bathing solution. The "K_z channel" is a delayed rectifier channel with fast activation and slow inactivation kinetics (6, 24). In the presence of 2 mM Ca^{2+}, application of 10 nM κ-BtX increased K^+ currents induced by the step depolarization pulses 2-fold, having evoked both Ca^{2+} influx and BK currents. When the bath was changed to a Ca^{2+}-free solution, κ-BtX failed to increase the depolarization-induced K^+ current. Statistically, κ-BtX increased total depolarization (single pulse)-induced currents by 67 ± 7% with 2 mM Ca^{2+} and −2 ± 5% in the absence of Ca^{2+} (Fig. 5*B*, $n = 5$).

There is controversy in the literature concerning the effects of charybdotoxin (ChTX) on SK channel, which is another type

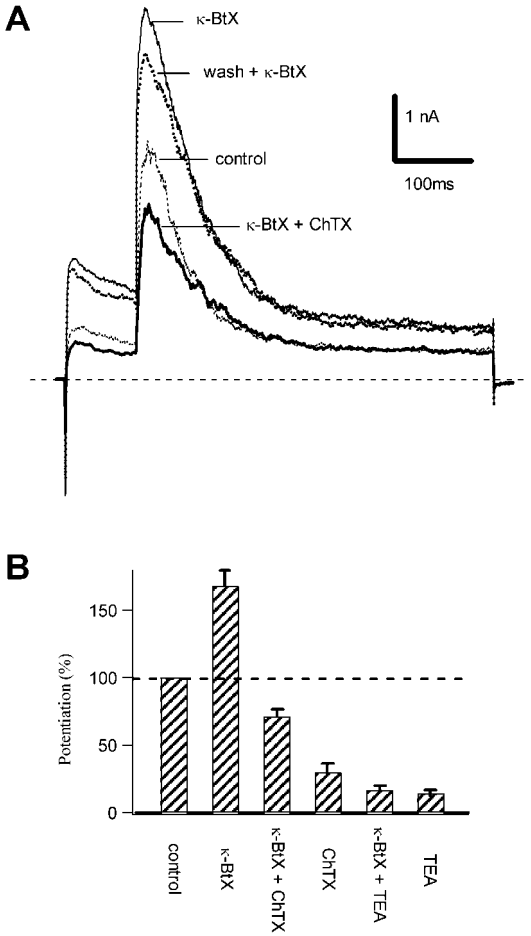

FIG. 7. **ChTX blocks κ-BtX induced BK up-modulation.** As shown in *A*, ChTX (100 nM) blocked the κ-BtX (10 nM)-induced BK up-modulation. BK currents induced by the double-pulse protocol shown in Fig. 1 were superimposed according to stimulus time. All traces were from the same whole cell patch ($n = 7$). As shown in *B*, data were measured using the protocol shown in *panel A*. The up-modulation was normalized to BK current under control conditions. κ-BtX increased BK current to 168 ± 11% of control level. ChTX reduced the total K^+ current to 30 ± 6% (without κ-BtX, $n = 6$) and 71 ± 4% (with κ-BtX, $n = 7$) of its control level ($p < 0.01$). TEA (1 mM) reduced the total K^+ current to 15 ± 2% (without κ-BtX, $n = 8$) and 17 ± 3% (with κ-BtX, $n = 3$) of its control level.

of Ca^{2+} dependent K channel in RACCs (5). To compare the effects of κ-BtX and ChTX, we reexamined the effect of ChTX on SK channels. Pure SK currents were examined by another type of double-pulse protocol (Fig. 6*A*) (17, 26, 27). Again the prepulse (from −70 mV to 0 mV for 1 s) induced Ca^{2+} influx, which activated a Ca^{2+}-dependent and voltage-independent inward current (*thick trace*) during the hyperpolarization of the second pulse (from 0 mV to −100 mV). The hyperpolarization deactivated all voltage-dependent currents, including the BK current. Most of the inward current at −100 mV was absent when the Ca^{2+}-loading prepulse was omitted (Fig. 6*A*, *thin trace*). This Ca^{2+}-sensitive inward current at −100 mV was assumed to be an SK current because the specific SK antagonist apamin (100 nM) blocked it by 50% (Fig. 6*B*). In contrast to the BK current, the extracellular application of a very high concentration of κ-BtX (1000 nM) induced only a minor increase (~10%) in the SK current (Fig. 6*B*). In contrast, the BK channel antagonist ChTX (200 nM) blocked 53 ± 3% of SK currents, so it is as potent as the specific SK blocker apamin (Fig. 6, *C* and *D*).

That the BK channels were the targets of κ-BtX was further supported by experiments using the BK blocker ChTX. As shown in Fig. 7*A*, up-modulation of the BK current by 10 nM

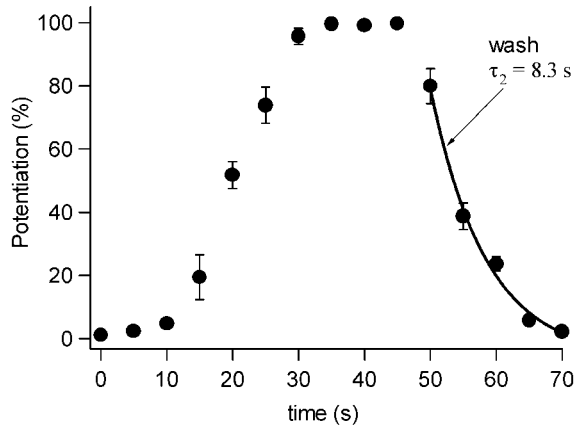

FIG. 8. **Time course of κ-BtX wash-out.** The time constant of κ-BtX (10 nM) wash-out was 8 s, showing that the κ-BtX effect on BK channels was fast and completely reversible ($n = 6$). The up-modulation curve increased to a maximum within about 10 s, which was the typical time required for the puffer system to reach a steady-state concentration of κ-BtX.

κ-BtX was largely removed by 100 nM ChTX. The κ-BtX induced up-modulation was readily reversible; after a 5-min wash-out, the up-modulation recovered by 90% (Fig. 7*A*). Since the K_d of κ-BtX was 0.7 nM (Fig. 4*C*), 10 nM was a saturating concentration and produced the maximum effect. Note that the peak K^+ current in the presence of ChTX + κ-BtX was even smaller than that without them. This was probably because without κ-BtX, the basal BK current (Fig. 4*A*) was blocked even more by ChTX (Fig. 7*B*). Before adding ChTX, κ-BtX produced a 68 ± 10% increase of the BK current (Fig. 7*B*, $n = 7$). BK current can be blocked more effectively by 1 mM TEA (24). Indeed, 1 mM TEA blocked 85% of the total K current. After 1 mM TEA blockade of BK, the effect of κ-BtX on K current was abolished. Taken together, these experiments provide further independent evidence that κ-BtX is a specific up-modulator of the BK channel.

The time course of onset and recovery of the up-modulation effect was relatively fast in comparison with other biotoxins. The time constant of the κ-BtX wash-out curve was 8.3 s (Fig. 8, $n = 7$). The delay in the puffer device was less than 0.1 s (not shown). The wash-out time of κ-BtX was much faster than that of ChTX (>3 min)[2] and iberiotoxin (IbTX) (5). The association rate was too fast to be determined by our application system. (We did not determine the onset of the κ-BtX action because the drug dialysis at the tip of the puffer pipette prevented testing rapid onsets.)

Mechanisms of κ-BtX Action on BK—Next, we studied the mechanism of κ-BtX action on the BK channels via single channel recordings in outside-out patches. As shown in Fig. 9, κ-BtX had no effect on single BK channel current (Fig. 9*A*) or single channel conductance (Fig. 9*B*), suggesting that the up-modulation induced by κ-BtX was not due to changes in single channel conductance. Note that during κ-BtX application, two or three single BK channels opened simultaneously. In contrast, only a single channel current was visible in the same patch without κ-BtX.

10 nM κ-BtX increased the open probability and total open-time of single BK channels in an outside-out patch (Fig. 10). Single channel BK currents were induced by depolarization from −80 mV to 80 mV with 2 μM Ca^{2+} in the patch pipette in the presence or absence of κ-BtX (Fig. 10*A*). Statistical analysis revealed that κ-BtX increased the open probability by 2.45-fold and total channel open time (see "Experimental Procedures" for

[2] L.-L. He and Z. Zhou, unpublished data.

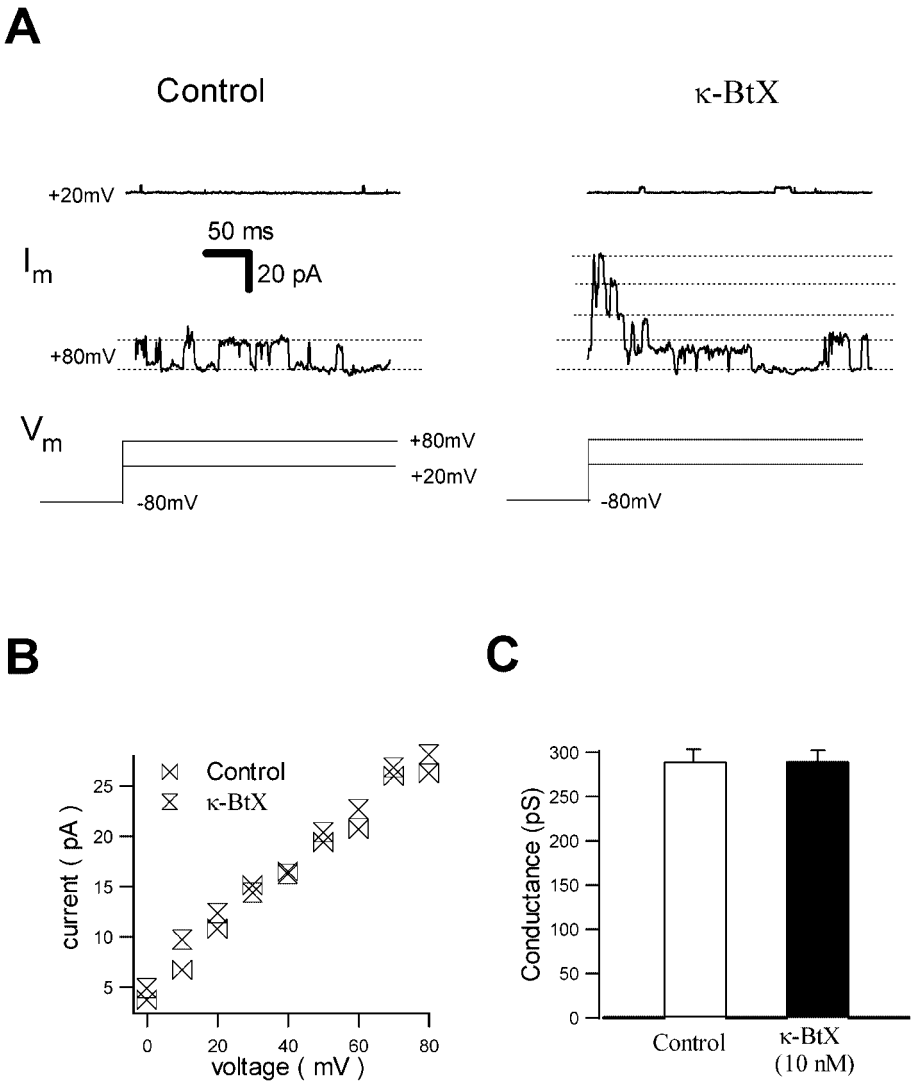

FIG. 9. **κ-BtX has no effect on single channel conductance of BK channels.** *A*, typical sweeps from an excised outside-out patch before and after 10 nM κ-BtX applications at different depolarization potentials. The patch was exposed to 2 μM Ca^{2+} in the pipette and stepped every 3 s from −80 mV to 20 mV (*top panel*) or to 80 mV (*middle panel*) for 500 ms. κ-BtX had no effect on the amplitude of single channel currents. The solutions for the outside-out patch were asymmetric K^+ solutions (see "Experimental Procedures"). As shown in *B*, κ-BtX had no effect on the I-V curve of the single channel current. The single channel conductance was 286 and 284 picosiemens for control and in the presence of 10 nM κ-BtX, respectively. *C*, statistics of the effect of κ-BtX on the single channel conductance. The average conductance was 290 ± 13 picosiemens for control and 289 ± 12 picosiemens for κ-BtX (n = 6).

definition) by 1.93-fold in single BK channel recordings ($p < 0.01$, Fig. 10, *B* and *C*). These values were similar to those of the whole cell BK currents (Figs. 4, 5 and 7). Taken together, the mechanism of κ-BtX induced up-modulation of BK currents is that κ-BtX increases the open probability and thus total open time of single BK channels during a given sweep but does not affect the single channel conductance.

DISCUSSION

This report describes the purification, characterization, and mode of action of a novel *Conus* peptide, κ-conotoxin BtX. The peptide acts as a specific up-modulator of the Ca^{2+}- and voltage-dependent BK channel and does not act as a channel blocker as found for all other conotoxins. Its primary amino acid sequence, including post-translational modifications, was established by partial Edman degradation, 3′- and 5′-RACE amplification, mass spectrometric analysis, and digestion of the carboxymethylated toxin with TPCK-trypsin. Our electrophysiological experiments showed that: 1) κ-BtX had little effect on voltage-gated Na^+ and Ca^{2+} channels, but it increased voltage-gated K^+ currents; 2) the BK channel was sensitive to κ-BtX, with an average increase of 252 ± 47%; 3) other K^+ channels, including the Ca^{2+}-insensitive but voltage-sensitive K_z channel and the Ca^{2+}-sensitive but voltage-insensitive SK channel, were not sensitive to κ-BtX; and 4) κ-BtX affected the open probability but not the conductance of single BK channels.

Structure of κ-BtX—From the viewpoint of the topological structure, the peptide is quite different from other known *Conus* peptides. One unique feature is that κ-BtX contains 8 cysteine residues to form four intramolecular disulfide bonds in a framework of $CX_6CX_5CCX_3CCX_3CX_3CX_3$. Thus, κ-BtX represents a new cysteine framework lacking sequence homology with any other conotoxins. Furthermore, the post-translational modifications of κ-BtX are more complex than those of other conotoxins, with all three Glu residues being γ-carboxylated, one Pro at position 27 hydroxylated, and another C-terminal Pro amidated. In accordance with the proposed rules for conotoxin nomenclature (13), the Roman numeral in a toxin designation should represent its cysteine framework. As the Roman numerals I to IX have already been assigned, the numeral X is therefore designated for the proposed new cysteine framework of κ-BtX (14).

Among the known precursor sequences of the *Conus* peptides, the mature peptides are usually present in the C-terminal part in a form of pre-propeptide. However, the pre-propeptide sequence of κ-BtX is remarkably different. The deduced amino acid sequence consists of a putative signal peptide of 26 residues, a mature toxin of 31 residues and an extra tail of 13 residues at the C terminus. The extra peptide might act as a propeptide and might be removed during post-translational processing. It is well known that the residue Gly is inevitably required for the C-terminal amidation of any protein by peptidylglycine α-amidating monooxygenase. The presence of Gly was also confirmed by

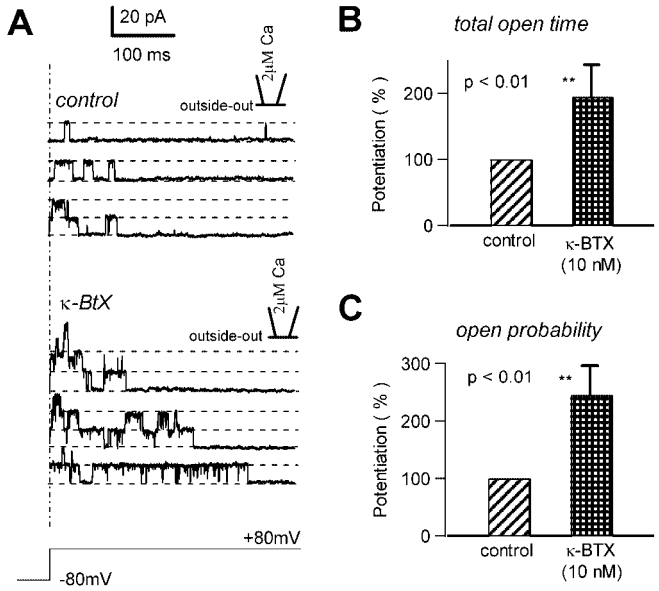

FIG. 10. **κ-BtX increases open probability of single BK channels.** *A*, examples of sweeps from an excised outside-out patch before and after 10 nM κ-BtX applications. The patch was exposed to 2 μM Ca^{2+} in the pipette and stepped every 3 s from −80 mV to 80 mV for 500 ms. *B*, the effect of 10 nM κ-BtX on the total open time of single BK channels. After application of 10 nM κ-BtX, the total open time increased to 1.94 ± 0.48-fold over the control. The effect of 10 nM κ-BtX on the total open time was statistically significant ($p < 0.01$, $n = 6$). *C*, the effect of 10 nM κ-BtX on the open probability of single BK channels. After application of 10 nM κ-BtX, the open probability increased to 2.45 ± 0.5-fold over the control. The effect of 10 nM κ-BtX on the open probability was statistic significant ($p < 0.01$, $n = 6$).

the cDNA-deduced sequence of κ-BtX. Furthermore, this Gly residue is followed by two basic residues, Lys and Arg, that might function as a recognition site for the amidation enzyme.

Specificity of κ-BtX—One important advantage of biotoxins has been their specificity against a particular ion channel, such as tetrodotoxin against the Na^+ channel. However, it was difficult to find biotoxins with high specificity for subtypes of K^+ channels, such as BK channels, other than the scorpion toxins ChTX and IbTX.

The scorpion toxin ChTX is a well known BK blocker and has been widely used in BK studies (2). ChTX is considered to be selective only when one compares K^+ with Na^+ and Ca^{2+} channels. ChTX is not selective between the subtypes of K^+ channels because it blocks not only BK but also SK channels (Fig. 6, *C* and *D*) and delayed rectifier K^+ channels (2). The other scorpion toxin, IbTX, is a specific blocker for BK channels *versus* other delayed rectifier K^+ channels (5). In this work, κ-BtX was found to specifically up-modulate the BK current but not voltage-gated Na^+ and Ca^{2+} channels in RACCs. κ-BtX has little effect on other subtypes of K^+ channels including K_z and SK channels. To our knowledge, κ-BtX is the first K^+ channel up-modulator from a C*onus* venom.

Up-modulator of BK Channels—So far, five compound types have been reported to activate the BK channel: a medical herb, anti-inflammatory aromatic compounds, benzimidazolones, phloretin, and ethanol (28). However, none of them are selective, and they are less potent than κ-BtX, except for DHS-I, an organic compound from a medical herb (29). However, DHS-I increases the BK current only when it is applied intracellularly. Since DHS-I is membrane impermeable, it can be used only when intracellular dialysis with a glass-pipette-electrode is available, which limits its wide application. Thus, κ-BtX is superior because it can affect BK channels extracellularly.

As an up-modulator of BK channels, the biological role of κ-BtX is opposite to that of BK blockers. It is known that the BK antagonists ChTX and IbTX reduce the frequency of action potentials and broaden the spike duration in neurons and adrenal chromaffin cells (8, 9),[3] because BK channels are required for fast repolarization between action potentials. In these cells, κ-BtX should increase action potential frequency and reduce the spike duration. In other cells, the function of BK channels is to keep cells at the resting potential. In these cells, BK up-modulators would act similarly to blockers of voltage-gated Na^+ channels (tetrodotoxin) or Ca^{2+} channels (nifedipine, ω-conotoxin). The worm-hunting snail might use this latter function as its weapon to catch prey, just like the ω-conotoxin case (30).

Mechanisms of κ-BtX Effects—Single channel recording of BK channels revealed that κ-BtX had no effect on single channel conductance (Fig. 9). Instead, κ-BtX increased the open probability of BK channels 2.45-fold. This is close to the 2.52-fold increase of the BK whole cell current (Fig. 4). Thus, the mechanism of action of κ-BtX is that the BK channel opens more frequently after binding with it. There are two possibilities. First, κ-BtX may increase the total open time by increasing the Ca^{2+} sensitivity of the channel. Alternatively, κ-BtX may act directly on the gating site of the channel. Since the Ca^{2+} sensor is intracellular, it is more likely that κ-BtX directly affects the channel gating when it is opened by the combined action of Ca^{2+} and depolarization.

The binding site between ChTX and the BK channel is located in the pore-forming region (31). At present, it is not clear where the exact location of the binding site between κ-BtX and BK channel is. However, the present data provide some useful cues. Firstly, the binding site might be different from that of ChTX because κ-BtX did not remove the ChTX block (Fig. 7). Secondly, BK channel inactivation is through an intracellular "peptide ball" at the N-terminal of the β-subunits (20, 32), and the binding site of κ-BtX is not likely to be associated with β-subunits because the inactivation kinetics remain intact in its presence (Fig. 3*A*). Finally, the single binding site is probably located at the extracellular side of the channel because the κ-BtX effect can be washed out within seconds (Fig. 8).

Although more than 80 conotoxins have been characterized, only 2 of them, κ-conotoxin PVIIA and κA-conotoxin SIVA, target potassium channels (17, 21). Both toxins inhibit voltage-gated potassium channels. On the contrary, the present study has identified κ-BtX as an up-modulator of BK channels in chromaffin cells. Thus, κ-BtX provides a useful new pharmacological tool for the characterization of K^+ channels and possibly even for application in the treatment of disorders caused by membrane hyper-excitability (21).

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