M-type K^+ current inhibition by a toxin from the scorpion *Buthus eupeus*

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Abstract A number of invertebrate venoms have been tested for effects on M-type K^+ currents ($I_{K(M)}$) in differentiated mouse neuroblastoma × rat glioma NG108-15 cells. Among the venoms tested, *Buthus eupeus* scorpion venom reversibly inhibited $I_{K(M)}$ by ~44% at 50 μg/ml. Inhibition was not due to activation of bradykinin or nucleotide (pyrymidine) receptors. On venom fractionation, a polypeptide of 4 kDa was purified that inhibited $I_{K(M)}$ by ~45% with an IC_{50} of ~33 nM. Neither the crude venom nor the purified polypeptide affected the Ca^{2+} current or the delayed rectifier K^+ current. While the crude venom prolonged the Na^+ current, the polypeptide did not. Thus, the 4 kDa *Buthus eupeus* polypeptide appears to be a selective inhibitor of $I_{K(M)}$ in NG108-15 cells.

Key words: Invertebrate venom; M-type K^+ current; Scorpion; Polypeptide toxin

1. Introduction

Polypeptide toxins have proved useful in the characterization and purification of potassium (K^+) channels (e.g. [1,2]). However, one type of K^+ channel which is insensitive to common K^+ channel-blocking peptides (such as dendrotoxin, apamin or charybdotoxin) is the M-type channel ($I_{K(M)}$), a low-threshold, voltage-gated K^+ channel which is widely distributed in vertebrate neurones [3]. Since this channel is also insensitive to conventional organic blocking agents [3] and has not yet been purified or sequenced, we thought it might be helpful for its further characterization to try to identify a peptide toxin capable of blocking it. To this end, we have screened a number of crude invertebrate venoms from spiders and scorpions on the species of $I_{K(M)}$ present in NG108-15 neuroblastoma × glioma hybrid cells [4]. From this, and subsequent fractionation, we now report the presence of a 4 kDa polypeptide in the venom of the scorpion *Buthus eupeus* which inhibits this species of $I_{K(M)}$ in nanomolar concentrations.

2. Materials and methods

2.1. Electrophysiology

Membrane currents were recorded from differentiated NG108-15 mouse neuroblastoma × rat glioma hybrid cells using the tight-seal, whole-cell patch clamp technique as described previously [4]. Dishes of cells were placed on the stage of an inverted microscope and superfused at 34°C (5–10 ml/min) with a modified Krebs solution of the following composition (mM): NaCl, 120; KCl, 3; glucose, 11.1; $NaHCO_3$, 22.6; $MgCl_2$, 1.2; Hepes, 5; $CaCl_2$, 2.5; tetrodotoxin (TTX), 0.0005; pH was 7.36 when gassed with 95% O_2-5% CO_2. Electrodes (2–4 MΩ) were normally filled with a solution containing (mM): potassium acetate ($KOOCCH_3$), 90; KCl, 20; Hepes, 40; $MgCl_2$, 3; EGTA, 3; $CaCl_2$, 1. The calculated free calcium concentration was 40 nM (program REACT2.01; G.L. Smith, Physiology Department, Glasgow University); measured resting levels of calcium were around 45 nM [5]. M-currents ($I_{K(M)}$) were recorded by voltage-clamping cells at −20 or −30 mV to activate the current and deactivation tails measured during 1s hyperpolarizing steps (see [4]). Current-voltage relationships were obtained using incremental voltage steps of −10 mV between −110 and −20 mV. Currents were measured at the end of hyperpolarizing steps. For dose-response curves, currents were measured at −20 or −30 mV from pseudo steady-state current-voltage relations obtained as indicated or using ramp voltage command of 20 s duration from −20 to −100 mV [4]. The leak component of current was estimated in both cases by extrapolating a line fitted by eye from the region of the current-voltage relationship (negative to −60 mV) where only ohmic currents were observed. Current inhibition was calculated from the fractional reduction of the M-current deactivation tails during hyperpolarizing steps or from the reduction in leak-subtracted steady-state outward current at the holding potential. Delayed rectifier K^+ currents were recorded as described by Robbins and Sim [6]; Na^+ currents were recorded by omitting tetrodotoxin from the bathing solution; and Ca^{2+} currents were recorded using a CsCl-based intracellular solution as described by Caulfield et al. [7]. In a number of experiments, M-currents in dissociated rat superior cervical sympathetic neurones were recorded as described in [8].

2.2. Purification of Buthus toxin

Crude venom was obtained by brief low frequency electric stimulation of *B. eupeus* scorpions collected in Turkmenistan. A protein fraction from the lyophilized powder of scorpion venom was precipitated by the cold acetone method [9] for removing mucoid materials from the mixture. 60 mg of protein fraction obtained was consistently purified by a size-exclusion and reversed-phase chromatography. Portions of crude venom (6 mg) dissolved in 100 ml of running buffer were subjected to gel filtration on a TSK-200 SW HPLC column (7.5 × 600 mm, 10 mm) (Beckman) in a 0.05 M ammonium acetate buffer (pH 5.7), containing 0.15 M NaCl, flow rate 0.5 ml/min. All collected fractions were then tested for their effects on M-currents in NG108-15 cells and active fractions obtained from 10 times gel filtration were further fractionated by reversed-phase HPLC using a preparative column Diax 130 C_4T (15 × 250 mm, 6 mkm) (Chromatoservice, Russia) in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile concentration, flow rate 5 ml/min. These fractions were further tested on M-currents and one eluted in a range 20–30% of acetonitrile concentration was separated on a semipreparative column (Ultrasphere ODS, 10 × 250 mm, 5 mkm (Beckman) in 0.1% trifluoroacetic acid at a flow rate of 3 ml/min with a linear gradient of acetonitrile concentration (see Fig. 2). The final purification was achieved on the same column by a different linear gradient of acetonitrile concentration. A polyacrylamide gel electrophoresis was carried out in a 2050 Midget electrophoresis unit (Pharmacia-LKB, Sweden) using the method described [10]. Gels were stained with Coomassie Blue.

2.3. Drugs and chemicals

The following drugs and chemicals were used (sources are given in parentheses): tetrodotoxin (TTX, Calbiochem, La Jolla, CA, USA), uridine triphosphate (UTP), acetylcholine, bradykinin (Sigma, MO, USA). All the venoms were supplied by Tashkent Zoofarm, Uzbekistan.

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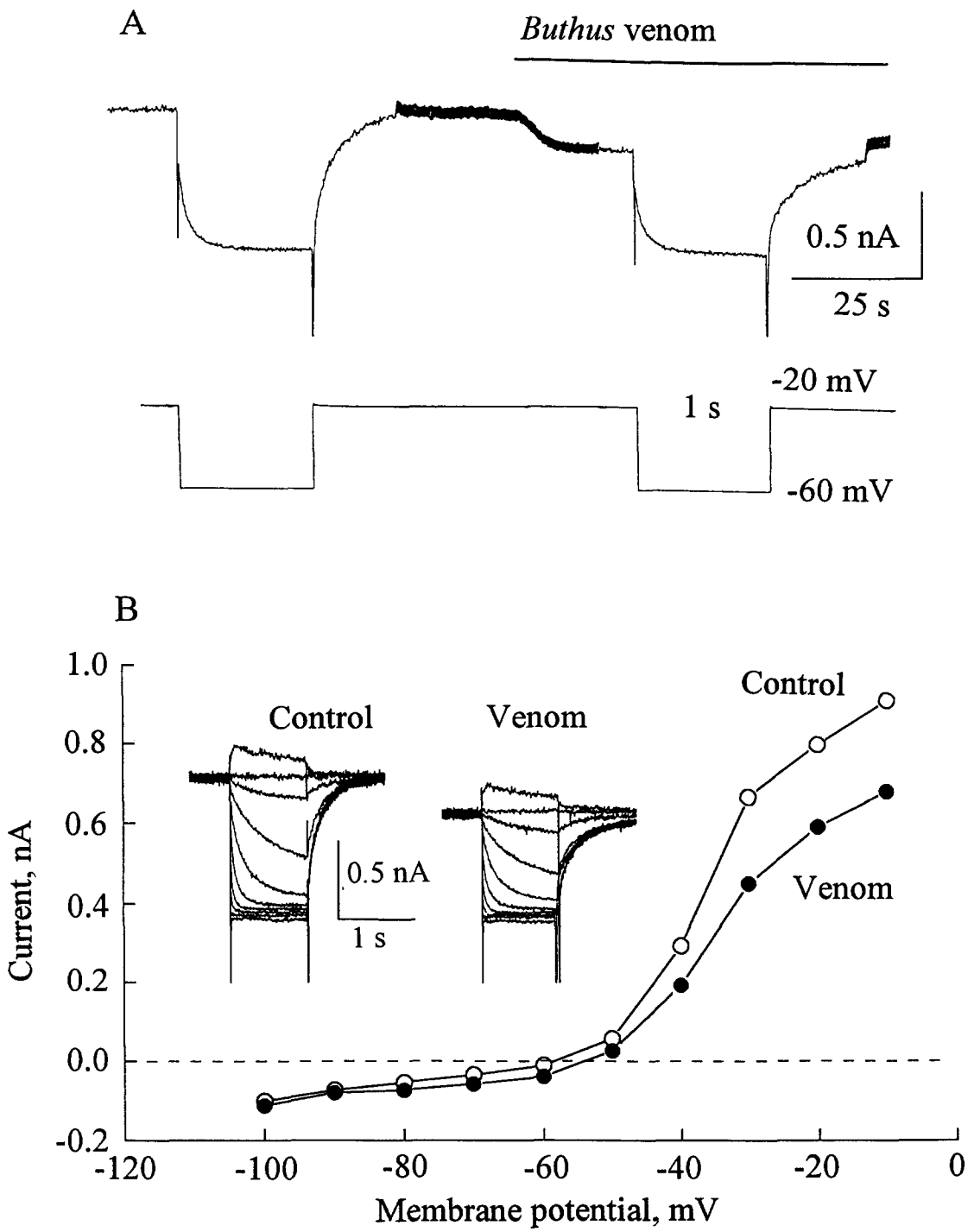

Fig. 1. Effect of crude *Buthus eupeus* venom on M-current ($I_{K(M)}$) in NG108-15 cells. (A) The cell was clamped at −20 mV and stepped to −60 mV for 1 s before and during superfusion with 50 μg/ml crude venom. During these hyperpolarizing steps (lower trace) the recorded current shows a slow inward relaxation, due to deactivation of M-current. (The 25 s time scale refers to the slow speed recording before and during venom application; individual responses to 1 s commands are shown at a ×25 faster speed.) The slowly developing inward current appearing at −20 mV holding potential after venom application results from inhibition of M-current previously activated at −20 mV. (B) Records inset show current responses of the cell to a series of voltage steps from −20 mV holding potential to command potentials between −10 and −100 mV in 10 mV increments, recorded before and 1 min after beginning the superfusion with 50 μg/ml crude venom. The graphs show the absolute current level attained at the end of each voltage step plotted against the command potential before and during superfusion with crude venom. Dotted line indicates zero current level.

3. Results

3.1. Crude venoms

The following crude venoms were tested at up to 100 μg/ml for their effect on M-currents in differentiated NG108-15 cells: from the spiders *Linothele* sp., *Scolopendra singulata*, *Lycosa singoriensis*, *Teganaria domestica*, *Argiopa lobata*, *Segestria senoculata*, *Steatoda paykulliana*, *Zorpsis* sp., *Eresus*

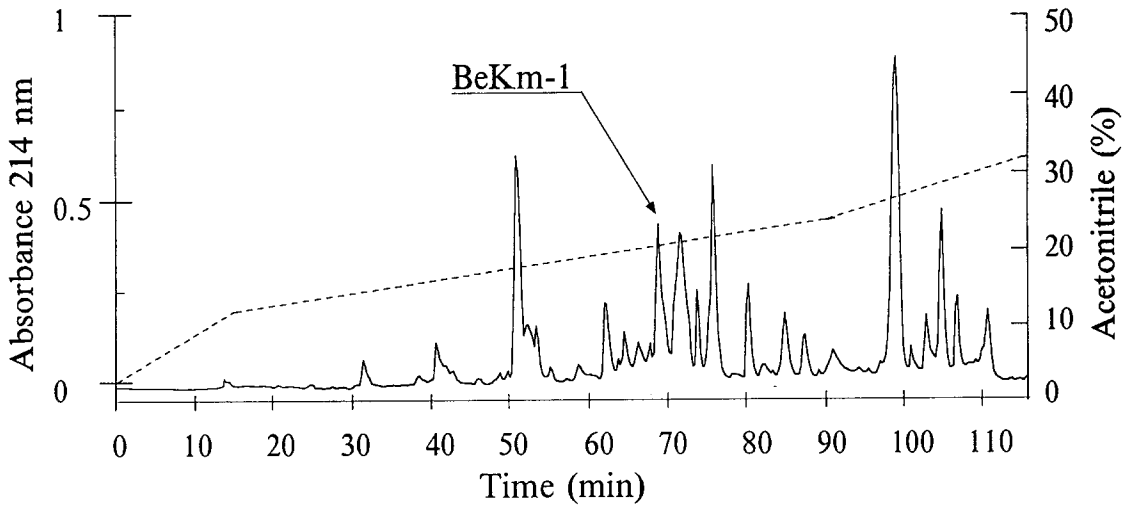

Fig. 2. Separation of a fraction from *Buthus eupeus* venom which inhibits M-current. Graph shows reverse-phase HPLC chromatography of the active fraction obtained by the size-exclusion and reverse-phase separation of crude venom on Ultrasphere ODS column (10×250 mm, 5 mkm) in 0.1% trifluoroacetic acid with a linear gradient of acetonitrile concentration (shown as dashed line). Flow rate 3 ml/min (for details see section 2). Peak BeKm-1 indicates the active fraction which is a pure polypeptide.

niger and *Agelena* sp.; and from the scorpions *B. eupeus, B. crassicauda* and *Orthochirus scrobiculosus*. All venoms except those from *Buthus* sp. were inactive. The venom of *B. eupeus* was used for further studies.

3.2. Crude B. eupeus venom

In NG108-15 cells, the whole venom (50 μg/ml) from *B. eupeus* scorpion reversibly inhibited the M-type potassium current by 44 ± 3% (mean ± S.E.M., n = 9) when measured at −20 mV (Fig. 1; see section 2). The current was inhibited at all potentials within its activation range (Fig. 1B). Agonists which activate phospholipase C-linked receptors (such as bradykinin [11] and UTP [12]) also inhibit $I_{K(M)}$ in NG108-15 cells, but this is normally preceded by an outward current due to release of intracellular Ca^{2+} and opening of Ca^{2+}-activated K^+ channels. *Buthus* venom did not induce an initial outward current (Fig. 1A), so probably did not act through such receptors. In accord with this, the effect of *Buthus* venom persisted in the presence of maximally effective concentrations of bradykinin (20 μM) or UTP (100 μM).

Buthus venom (100 μg/ml) had no effect on the delayed rectifier K^+ current [6] (n = 6) and produced <15% inhibition of the peak Ca^{2+} current measured at 0 mV [7] (n = 3). At >1 μg/ml, crude *Buthus* venom prolonged the Na^+ current, as reported previously [13]. This effect was prevented by adding 0.5 μM TTX to the bathing solution. M-current inhibition persisted in TTX solution.

The venom also inhibited $I_{K(M)}$ in dissociated rat sympathetic neurones, by 14 ± 0.6% (n = 3) at 50 μg/ml and 21 ± 3% (n = 2) at 100 μg/ml.

3.3. Effect of the venom fractions

Initial gel filtration yielded 6 fractions (see section 2). $I_{K(M)}$-inhibiting activity on NG108-15 cells was restricted to fraction 4, containing polypeptides with molecular masses 4–14 kDa. The active fraction was then subfractionated by RP-HPLC on Diax C_4T column to get 4 more fractions. One of these fractions eluted from 20 to 30% of acetonitrile inhibited $I_{K(M)}$. This fraction was further fractionated to obtain 21 fractions using Ultrasphere ODS column (Fig. 2), of which only one inhibited $I_{K(M)}$. Further rechromatography and SDS-polyacrylamide gel electrophoresis showed that this fraction was a pure polypeptide (provisionally named BeKm-1) of about 4 kDa. The content of BeKm-1 in the whole venom protein mass was not more than 1%.

Polypeptide BeKm-1 inhibited $I_{K(M)}$ in NG108-15 cells in a similar manner to the crude venom, with an extrapolated maximal inhibition of 45.3 ± 5.2%, a calculated IC_{50} of 33.1 ± 1.6 nM and a Hill coefficient of 1.0 ± 0.5 (Fig. 3). Un-

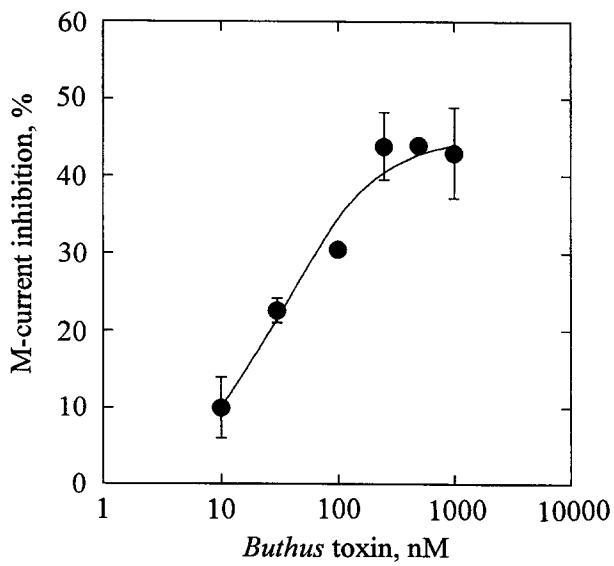

Fig. 3. Dose-response relationship for inhibition of $I_{K(M)}$ in differentiated NG108-15 cells by the polypeptide toxin BeKm-1 purified from *Buthus eupeus* venom. Points are means of 2–5 experiments and show % inhibition of the current recorded between −30 and −20 mV (calculated from *I/V* curves, see section 2) at different polypeptide concentrations. Bars show S.E.M. The curve is a least-squares fit for pooled data to the logistic expression $y = A \cdot X^n / (X^n + IC_{50}^n)$ where X = polypeptide concentration, A = maximum inhibition and n = Hill coefficient.

like the crude venom, however, BeKm-1 (100–250 nM) did not prolong the Na^+ current in either NG108-15 cells ($n = 3$) or sympathetic neurones ($n = 3$). It also had no effect on transient or delayed rectifier K^+ currents in either cell type at 100 nM.

4. Discussion

We have isolated a 4 kDa peptide from *B. eupeus* which we provisionally term BeKm-1, and which inhibits the sustained, voltage-gated M-type K^+ current in NG108-15 cells with an IC_{50} of ~33nM. This is clearly a different peptide from the 8 kDa M7 toxin previously isolated from *B. eupeus* [13] and responsible for inhibiting Na^+ current inactivation. In accordance with this, whereas the crude venom enhanced the Na^+ current in NG108-15 cells, BeKm-1 did not.

BeKm-1 appeared to be selective for $I_{K(M)}$ in NG108-15 cells to the extent that it did not inhibit either 'leak' or delayed rectifier K^+ currents. Available evidence suggests that BeKm-1 acted directly on the K_M channels, rather than through activation of known endogenous phospholipase C-linked receptors, though this needs confirmation by direct M-channel recording [14].

The reason for the apparently incomplete (~50%) inhibition of steady-state $I_{K(M)}$ by BeKm-1 is not yet clear: it might result from an effect on channel gating, or it might imply that more than one pharmacologically different channel type might contribute to the macroscopic current [14]. This again will require further studies on single channel activity.

The limited tests so far suggest that both crude *Buthus* venom and the BeKm-1 peptide may be rather less effective in inhibiting $I_{K(M)}$ in sympathetic neurones than in NG108-15 cells. This would accord with previous inferences that the channels responsible for generating the macoscopic M-current in these cell types, though kinetically similar [14], may exhibit pharmacological differences [4].

Notwithstanding, BeKm-1 is the only peptide toxin so far identified with submicromolar activity on K_M currents and hence warrants further investigation. For this reason, attempts are now in progress to sequence the peptide and to obtain sufficient quantities to extend these studies.

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