Identification of two toxins from scorpion (*Leiurus quinquestriatus*) venom which block distinct classes of calcium-activated potassium channel

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Two polypeptide toxins from scorpion (*Leiurus quinquestriatus*) venom which block distinct classes of calcium-activated potassium channels have been identified and partially purified. One toxin, at 50–100 ng/ml, blocks apamin-sensitive potassium fluxes in hepatocytes and inhibits [^{125}I]monoiodoapamin binding. The other, more basic, toxin blocks apamin-insensitive potassium fluxes in erythrocytes at 200 ng/ml and, to our knowledge, is the first toxin shown to block the erythrocyte calcium-activated potassium channel with high affinity. The possible co-identity of this latter toxin with charybdotoxin is discussed.

(Leiurus quinquestriatus) *Apamin* *Charybdotoxin* *Ca^{2+} activation* *K^+ channel*

1. INTRODUCTION

Of the many types of transmembrane K^+ channels, those activated by a rise in cytosolic Ca^{2+} are of great current interest. They are a heterogeneous group which can be distinguished both on the basis of their unitary conductance and on their sensitivity to blockade by specific peptide toxins. Apamin, a peptide present in bee venom, has been shown to block, with high affinity, the Ca^{2+}-activated K^+ channels ($J_{(Ca)}$ channels) found in guinea-pig hepatocytes, intestinal smooth muscle, embryonic skeletal muscle as well as in certain neuronally derived tissues [1–5]. More recently another peptide toxin, charybdotoxin, which is a minor component of *Leiurus quinquestriatus* (LQ) scorpion venom, has been reported to block the large conductance, apamin-insensitive $K_{(Ca)}$ channel ('BK' channel) in skeletal muscle T-tubules and cultured mammalian kidney cells [6,7]. Crude LQ venom has also recently been shown to block apamin-sensitive K^+ movements in guinea-pig hepatocytes and the apamin-insensitive $K_{(Ca)}$ channel of intermediate conductance found in erythrocytes [8].

This report describes the identification of two toxins present in LQ venom which specifically block either the apamin-sensitive $K_{(Ca)}$ channel in guinea-pig hepatocytes or the apamin-insensitive $K_{(Ca)}$ channel in human erythrocytes. The latter toxin is the most effective blocker of the erythrocyte $K_{(Ca)}$ channel yet identified.

MATERIALS AND METHODS

2.1. Materials

L. quinquestriatus hebraeus venom was obtained from Latoxan, Rosans, France. Apamin was purified from *Apis mellifera* venom [9] and [^{125}I]monoiodoapamin was prepared and purified as described [10]. Chromatography media were purchased from LKB and Pharmacia, and A23187 and angiotensin II were obtained from Sigma.

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2.2. *Fractionation of* L. quinquestriatus *venom*

Crude venom (140 mg) was extracted with distilled water and centrifuged as in [6]. The supernatant was chromatographed on an S-Sepharose ion-exchange column (7×0.9 cm) equilibrated with 0.01 M NH_4OAc, pH 7.0. The column was washed with equilibration buffer until the UV absorbance returned to baseline and was then eluted with a linear salt gradient of NH_4OAc (0.01–0.8 M, 200 ml total volume). 1 ml fractions were collected at a flow rate of 12 ml/h.

Peak X (see later) was diluted 4× with distilled water and re-applied (final volume, 55 ml) to a CM-Trisacryl ion-exchange column (4×0.6 cm), equilibrated with 0.05 M sodium phosphate buffer, pH 7.4. After washing off unbound material, the column was eluted with a linear salt gradient of NaCl (0–0.8 M, 400 ml total volume) in equilibration buffer. 1 ml fractions were collected at a flow rate of 12 ml/h. Protein concentrations were determined according to Lowry et al. [11].

Crude venom and semi-purified fractions were analysed by SDS-PAGE using a Laemmli buffer system as modified by Fling and Gregerson [12] for analysis of low-M_r polypeptides.

2.3. *Preparation of hepatocytes and erythrocytes*

Hepatocytes were prepared from male Hartley guinea-pigs by collagenase digestion [1]. Cells were incubated and experiments carried out at 37°C in Eagles MEM (Wellcome) supplemented with 2% bovine serum albumin and 10% new-born calf serum at a density of approx. 1×10^7 cells/ml.

Erythrocytes from freshly drawn human blood were separated from plasma, platelets and leucocytes by sedimentation in Dextran 70 (6%, w/v, in saline) and resuspended to a haematocrit of 7% in a medium containing (mM): NaCl, 145; KCl, 0.1; $MgCl_2$, 1; $CaCl_2$, 1; Tris-HCl, 10 (pH 7.4 at 37°C) and inosine, 10 [13].

2.4. *Potassium efflux experiments*

Net K^+ fluxes from hepatocytes or erythrocytes were measured using a K^+-sensitive electrode placed in the cell suspension [1]. Crude LQ venom components to be tested for inhibition of agonist-induced K^+ loss were incubated with 2 ml of cell suspension for 2 min at 37°C before addition of agonist. The stimuli for K^+ release from hepatocytes and erythrocytes were angiotensin II (100 nM) and the calcium ionophore, A23187 (5 μM) respectively. K^+ loss in the first 30 s for hepatocytes (3 min for erythrocytes) after agonist application was expressed as a percentage of total cell content, evaluated for each aliquot of cells by the subsequent addition 100 μM digitonin [13].

2.5. *Competition binding experiments with [^{125}I]monoiodoapamin*

Hepatocytes (0.3 ml) were incubated with 0.2 ml of incubation medium containing [^{125}I]monoiodoapamin (final concentration 100 pM) and varying concentrations of LQ-VIII for 2 min at 37°C. Cell-associated [^{125}I]monoiodoapamin was separated from free labelled apamin by rapid centrifugation of the cells through di(*n*-butyl)phthalate [13,14].

2.6. *Analysis of data*

IC_{50} values were obtained from dose-inhibition curves fitted with the Hill equation using a least-squares computer fit [15]. The fitted Hill coefficients (n_H) were found to be significantly less than 1 ($n_H = 0.59$–0.70) except for the displacement of [^{125}I]monoiodoapamin ($n_H = 0.99$) and the inhibition of K^+ loss, by LQ-VIII ($n_H = 0.94$).

3. RESULTS

Crude LQ venom inhibited both angiotensin II stimulated K^+ efflux from guinea-pig hepatocytes and A23187-induced K^+ loss from erythrocytes with a similar potency, the IC_{50} values being 7.2 and 8.7 μg/ml, respectively. This confirms the work of Abia et al. [8].

Ion-exchange chromatography of the crude venom on S-Sepharose produced ten distinct components (LQ-I to LQ-X, fig.2A) in addition to the material which did not bind to the column under the initial elution conditions (this represented approx. 75% of the absorption at $A_{278\ nm}$ in the venom and is not shown in fig.2A). The peak fraction of each component (diluted 100-fold) was assayed for its ability to block K^+ fluxes from both cell types and the results expressed in terms of the amount of $A_{278\ nm}$ absorption of each fraction. Both the hepatocyte and erythrocyte K^+ blocking activities were clearly resolved by this chromatographic procedure (fig. 2B). Nearly all hepatocyte K^+ flux blocking activity was recovered in LQ-VIII, while the majority of the erythrocyte

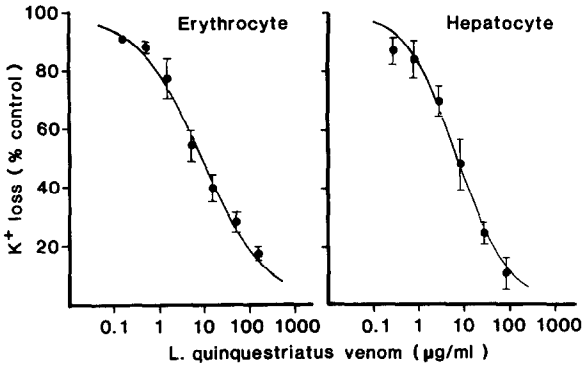

Fig.1. Inhibition of A23187 (5 μM)-stimulated net K^+ loss from human erythrocytes and angiotensin II (100 nM)-induced net K^+ efflux from guinea-pig hepatocytes by crude *L. quinquestriatus* venom. K^+ loss is expressed as the % of cell K^+ lost during the first 30 s (for hepatocytes) or 3 min (for erythrocytes) after exposure to the respective agonists. Points are means ± SE from 3 experiments.

blocking activity was found in LQ-X, with smaller amounts occurring in peaks I–IV.

Individual fractions of LQ-X were pooled diluted with distilled water to reduce the ionic strength and re-chromatographed on a CM-Trisacryl ion-exchange column (fig.3A). Approximately half of the UV-absorbing material (LQ-X/1) did not bind to the column and was devoid of biological activity. A single peak eluted (at ~0.1 M NaCl) after application of a salt gradient. LQ-X/2 retained all the original channel blocking activity of LQ-X and inhibited A23187-stimulated K^+ loss from erythrocytes with an IC_{50} of 198 ng/ml which represented a 44-fold increase in activity compared with the crude venom.

LQ-VIII, which appeared as a shoulder on LQ-VII, possessed more than 95% of the hepatocyte K^+ flux blocking activity present in the crude venom and exhibited an IC_{50} of 132 ng/ml (fig.4). To establish whether the inhibitory action of LQ-VIII was similar in nature to that reported for apamin, the effect of the toxin on [^{125}I]monoiodoapamin binding was also examined. LQ-VIII clearly inhibited [^{125}I]monoiodoapamin binding (fig.4); the IC_{50} of 54 ng/ml being in good agreement with the data from the K^+ flux assay. This suggested that the action of the toxin related to the blockade of a $K_{(Ca)}$ channel and not, for example, to the antagonism of angiotensin II receptors.

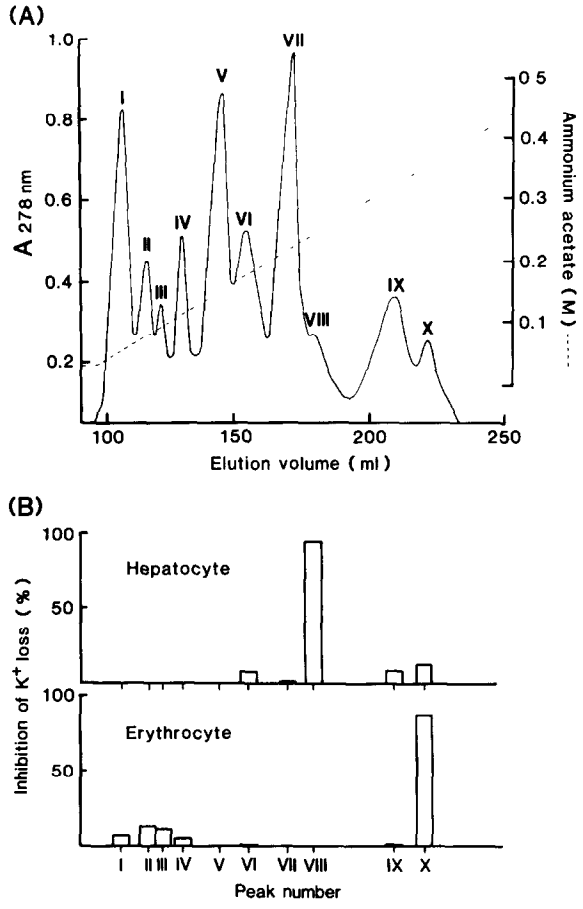

Fig.2. (A) S-Sepharose ion-exchange chromatography of *L. quinquestriatus* venom. (B) Effect of individual peaks (100-fold dilution) on angiotensin II (100 nM)-induced K^+ loss from guinea-pig hepatocytes and A23187 (5 μM)-induced K^+ efflux from human erythrocytes. % inhibition is expressed in terms of the amount of absorption at $A_{27a\ nm}$ of each fraction.

Molecular mass analysis, using SDS-PAGE (Fig.5), showed that, although the crude venom consisted mainly of peptides of 6–8 kDa, the minor venom components, LQ-VIII and LQ-X12, appeared to be considerably smaller (4–5 kDa). The higher molecular mass band present in LQ-VIII possibly reflected contamination with LQ-VII.

It is interesting to note that crude LQ venom (100 μg/ml) did not compete with [^{125}I]monoiodoapamin for apamin antibodies in a competitive radioimmunoassay (not shown) indicating that there was probably no immunological cross-reac-

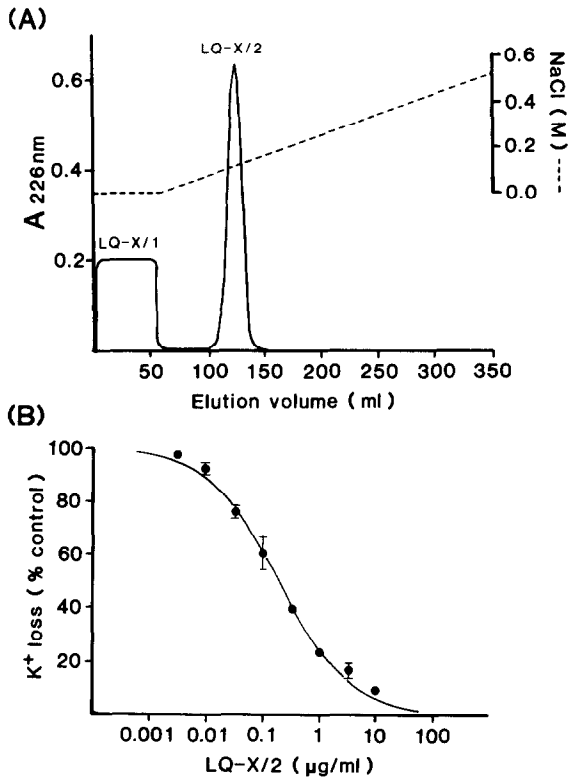

Fig.3. (A) CM-Trisacryl ion-exchange chromatography of LQ-X. (B) Inhibition of A23187 (5 μM)-stimulated K^+ loss from human erythrocytes by LQ-X/2. Points are means ± SE from 3 experiments.

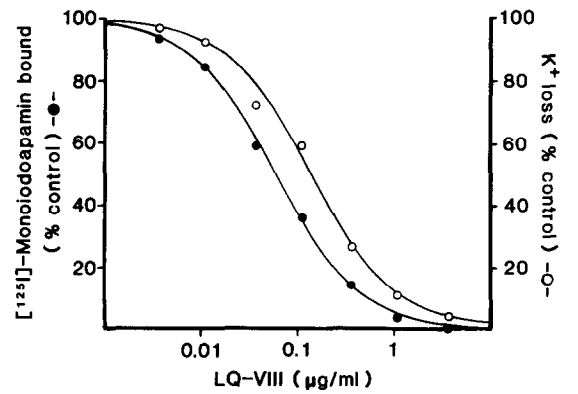

Fig.4. Comparison of the ability of LQ-VIII to inhibit [^{125}I]monoiodoapamin binding to, and angiotensin II-stimulated net K^+ loss from, guinea-pig hepatocytes. Points are the means of 2–3 observations.

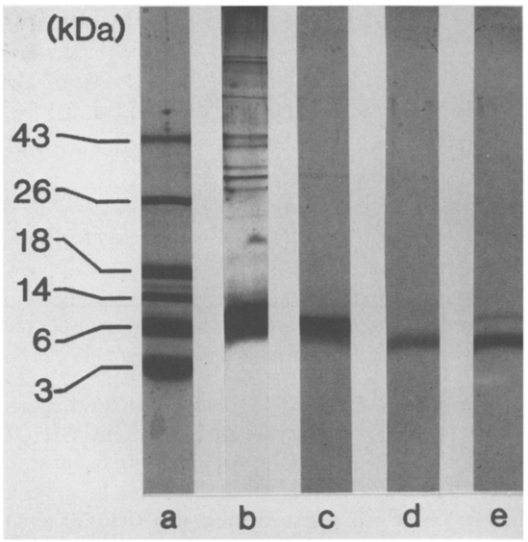

Fig.5. SDS-PAGE of crude *L. quinquestriatus* venom and partially purified $K_{(Ca)}$ channel blocking toxins. Lanes: (a) molecular mass markers, (b,c) crude venom, (d) LQ-X/2, (e) LQ-VIII. The sample in lane b was visualised by silver staining [16] and all other tracks were stained with Coomassie blue.

tivity between the bee venom toxin and the apamin-like component of the scorpion venom.

DISCUSSION

This study has shown that LQ venom contains two distinct toxins which block different classes of $K_{(Ca)}$ channel. Assuming a molecular mass of 5 kDa and 100% purity (clearly an overestimate), IC_{50} values of ~20 and ~40 nM can be obtained for the apamin-like hepatocyte $K_{(Ca)}$ channel blocking toxin (LQ-VIII) and for the erythrocyte $K_{(Ca)}$ channel blocking toxin (LQ-X/2), respectively. These values are upper estimates and are likely to be lower when the toxins have been purified to homogeneity. They are, however, in the same range as the K_d (10 nM) reported for charybdotoxin in mammalian T-tubules [6]. At present it is unclear if either of the two toxins identified in this study corresponds to charybdotoxin. The erythrocyte $K_{(Ca)}$ channel blocking toxin is probably the more likely candidate since like charybdotoxin, it elutes as the most basic polypeptide in its fractionation procedure. Furthermore, a recent report has shown that *Aplysia* $K_{(Ca)}$ channels, which have

an intermediate unitary conductance (20–40 pS) similar to that of erythrocytes, are also blocked by charybdotoxin [17]. LQ-X/2, whether or not identical to charybdotoxin, still represents the first toxin shown to block the erythrocyte $K_{(Ca)}$ channel with high affinity.

Apamin has been frequently used to define one class of $K_{(Ca)}$ channel. It will be extremely interesting to see whether the new apamin-like scorpion venom toxin that blocks hepatocyte $K_{(Ca)}$ channels also blocks all other apamin-sensitive channels; since the two toxins appear to be structurally unrelated (by both size and immunological criteria) they may allow subtle differences in these channels to be detected.

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