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Effects of potassium channel toxins from *Leiurus quinquestriatus hebraeus* venom on responses to cromakalim in rabbit blood vessels

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1 The effects of fractionated *Leiurus quinquestriatus hebraeus* venom on cromakalim-induced $^{86}Rb^+$ efflux in rabbit aortic smooth muscle were examined.

2 Crude venom (0.1–30 $\mu g\,ml^{-1}$) produced a concentration-dependent decrease of 1 μM cromakalim-induced $^{86}Rb^+$ response. The maximum blocking activity attainable was approximately 60%.

3 Fractionation of crude venom by gel permeation chromatography and subsequent chromatography on a cation ion-exchange column, produced two fractions (X and XI), active in the $^{86}Rb^+$ blocking assay.

4 Fraction XII contained charybdotoxin (~85% pure). After a final high performance liquid chromatography (h.p.l.c.) purification step, the purified toxin failed to inhibit the cromakalim-stimulated $^{86}Rb^+$ efflux although it was a potent inhibitor of A23187-induced K^+ flux in human erythrocytes and the large conductance calcium-activated potassium channel in rabbit portal vein smooth muscle.

5 Subsequent purification of fraction X by h.p.l.c. yielded a minor peak which contained $^{86}Rb^+$ blocking activity. This subfraction was also capable of inhibiting apamin-sensitive, angiotensin II-stimulated K^+ flux in guinea-pig hepatocytes.

6 It is concluded that the potassium channel opened by cromakalim in rabbit aortic smooth muscle is not blocked by charybdotoxin but by another distinct toxin in the venom of *Leiurus quinquestriatus hebraeus*.

Introduction

Potassium channels are involved in an unusually diverse range of biological activities. For example, some channels are sensitive to membrane depolarization while others are activated by neurotransmitters, intracellular messengers and adenosine 5'-triphosphate (ATP), as well as changes in cell volume (reviewed by Cook, 1988). Several different types of potassium channel often occur within the same cell.

The anti-hypertensive drug cromakalim (BRL 34915) produces an increase in potassium permeability in a variety of isolated smooth muscles (Cook 1988). Little is known of the properties of the potassium channel opened by cromakalim. However, it has been shown that the increase in potassium permeability produced by the drug is neither dependent on the entry of calcium through dihydropyridine-sensitive calcium channels, nor is it blocked by apamin (Weir & Weston, 1986; Quast 1987), a toxin which selectively blocks many calcium-activated potassium channels ($K_{(Ca)}$ channels) of low conductance. Preliminary reports have indicated that cromakalim increases the open time of large $K_{(Ca)}$ channels in both cultured aortic smooth muscle cells and in aortic smooth muscle membranes incorporated into planar lipid bilayers (Kusano *et al.*, 1987; Gelband *et al.*, 1988).

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In portal vein, the cromakalim-mediated increase in $^{86}Rb^+$ tracer efflux can be inhibited by the venom of the Israeli scorpion, *Leiurus quinquestriatus hebraeus* (Quast & Cook, 1988). This venom has been shown to contain at least two different toxins which block distinct $K_{(Ca)}$ channels (Castle & Strong, 1986). One is charybdotoxin, a selective inhibitor of high conductance $K_{(Ca)}$ channels in both skeletal and smooth muscle cells (Miller *et al.*, 1985; Beech *et al.*, 1987; Valdivia *et al.*, 1988; Talvenheimo *et al.*, 1988).

This present study demonstrates that the cromakalim-induced $^{86}Rb^+$ efflux in rabbit aorta, presumed to be indicative of the potassium channel 'opened' by cromakalim, is not blocked by charybdotoxin but by another component of the venom. A purification procedure, that allows the isolation and characterization of both of these two toxins is described.

A preliminary account of the findings has been presented to the British Pharmacology Society (Weir & Strong, 1988).

Methods

Fractionation of L. quinquestriatus hebraeus *venom*

Crude venom (200 mg batches) was placed in a 15 ml centrifuge tube and extracted for 20 min with deionised water (5 ml, 4°C) three times. The extract was centrifuged (45,000*g*, 20 min, 4°C) and the supernatant carefully removed from a viscous opaque gel. The gel was extracted three more times and the supernatants pooled (final volume, 15 ml). The combined supernatants were chromatographed at 4°C on a Sephadex G-50 (superfine) column (90 × 2.6 cm diameter) previously equilibrated with 50 mM ammonium formate buffer, pH 3.5. The column was eluted with equilibration buffer at 40 ml h^{-1} and the eluant was monitored at 278 nm. The peaks containing biological activity in the $^{86}Rb^+$ flux assay from two batches of crude venom were diluted with an equal volume of water and re-chromatographed on an S-Sepharose ion exchange column (10 × 1.6 cm diameter) equilibrated with 50 mM ammonium acetate buffer pH 6.0. After washing the column with equilibration buffer to remove unbound material, the column was eluted with a linear ammonium acetate salt gradient (50–600 mM, pH 6.0, total volume 800 ml) at 90 ml h^{-1}. Fractions were monitored at 278 nm. Fraction X was further chromatographed on a reverse phase column (Pharmacia ProRPC 5/2), equilibrated with 0.01 M sodium acetate/0.04 M acetic acid (buffer A). Bound material was eluted from the column with a linear gradient of 0–100% buffer B (50% aqueous methanol/0.05 M acetic acid, 1:1) at a flow rate of 1 ml min^{-1}. The column was monitored at 280 nm.

$^{86}Rb^+$ *efflux experiments*

Male mongrel rabbits (1.8–3.0 kg, supplied by Sandoz) were killed by a blow to the head. After the thoracic cavity was exposed, the aorta was removed. The aorta was cut into 36 equally sized rings and then each ring was cut along its longitudinal axis. Each ring segment was then mounted on a gassing manifold as described previously by Hamilton *et al.* (1986). The tissues were allowed to equilibrate, firstly in Krebs-Henseleit solution (KHS) for 45 min and then in KHS containing $^{86}Rb^+$ (5 μCi ml^{-1}) for 2 h. During the following efflux experiment, samples were taken at 2 min intervals throughout the entire procedure (80 min). Initially, $^{86}Rb^+$ was allowed to efflux from the tissues into normal KHS for 10 min. Tissues were then exposed to KHS containing PN 200-110 (0.5 μM) (PN/KHS) for 20 min before the transfer to PN/KHS solution supplemented with the toxin under test. After 20 min in toxin solution, the tissues were challenged with cromakalim (1 μM) for a further 20 min. Finally, tissues were washed in PN/KHS for 10 min. Control tissues were treated similarly but exposed to vehicle (0.1% ethanol) alone. Radioactivity in the efflux samples was counted in the Cerenkov mode at 50% efficiency. The amount of radioactivity remaining in the tissue at the end of the experiment was determined by dissolving the tissue in 500 μl Lumasolve (Lumac) at 50°C overnight, 500 μl 1N HCl and 10 ml Optifluor (Packard) was added to the solubilised sample which was then counted in the ^{32}P channel at 100% efficiency. The efflux data were expressed as the first order rate constant (k) of $^{86}Rb^+$ efflux (min^{-1}) (Quast, 1987). Cromakalim-induced $^{86}Rb^+$ efflux was expressed as the area under the curve (AUC) in mg as determined by weighing (see also Quast, 1987).

Single channel recordings

Single smooth muscle cells were isolated from rabbit portal vein by incubating the tissue in a nominally calcium-free physiological medium containing papain, bovine serum albumin and dithiothreitol (Beech & Bolton, 1989) and were used within 12 h. Patch-clamp experiments were performed at room temperature on isolated outside-out patches (Hamill *et al.*, 1981) in quasi-physiological medium of the following composition (mM); bath (external) solution: Na^+ 126, K^+ 6, Ca^{2+} 1.7, Mg^{2+} 1.2, Cl^- 138, glucose 14, HEPES 10.5; pipette (internal) solution: K^+ 134, Mg^{2+} 1.2, Cl^- 136, EGTA (ethylene glycol *bis*-(2-aminoethylether) N,N,N',N'-tetraacetic acid) 0.8, glucose 14, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) 10.5. Both solutions were titrated to pH 7.2 with NaOH. Analogue signals were recorded on FM tape (7.5 in s^{-1}) and

low pass filtered at 2 kHz (8 pole Butterworth filter: Barr and Stroud). The data were then digitized using a CED 401 A/D converter (Cambridge Electronic Design) in conjunction with a BBC microcomputer and displayed on a plotter.

Erythrocyte potassium efflux assay

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, inosine 10, Tris HCl 10, pH 7.4. Net potassium fluxes were measured using a potassium-sensitive electrode placed in the cell suspension (Cook & Haylett 1985; Castle & Strong 1986). The venom fractions to be tested were pre-incubated with 2 ml of erythrocyte suspension for 2 min at 37°C prior to the addition of A23187 (final concentration 5 μM). Potassium loss during the subsequent 2 min was expressed as a percentage of the total cell potassium content. The latter was evaluated after each experiment by adding digitonin (final concentration 200 μM) to the cell suspension. Control experiments were performed concurrently, without venom fractions.

Hepatocyte potassium efflux assay

Hepatocytes were prepared from male Hartley guinea-pigs by collagenase digestion (Burgess *et al.*, 1981). 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^{-1}. Net potassium fluxes were measured in a similar way to those described above for the erythrocyte assay, except that potassium release was stimulated with 100 nM angiotensin II, instead of A23187 (Cook & Haylett, 1985).

Amino acid sequence determination

Purified fraction XII was reduced with dithiothreitol and alkylated by pyridylethylation essentially as described by Tarr *et al.* (1983). The toxin was dissolved in 50 μl Tris buffer (50 mM, pH 8.0) containing 6 M guanidine hydrochloride. Dithiothreitol (200 μg) was added and the mixture incubated for 2 h at room temperature under an atmosphere of argon. Vinylpyridine (0.2 μl) dissolved in 0.8 μl isopropanol was added and the incubation continued for another 2 h. Reagents and toxin were separated by high performance liquid chromatography (h.p.l.c.) on a C_{18} column using a linear 0–100% acetonitrile gradient in aqueous 0.1% heptafluorobutyric acid. For Lys-C digestion, pyridylethylated toxin was dissolved in 50 μl Tris buffer (50 mM, pH 8) containing 4 M urea. Endoproteinase Lys-C (0.4 μg) was added and the mixture incubated for 4 h at 37°C. The digest was separated by h.p.l.c. on a C_{18} column using a linear 0–80% acetonitrile gradient in aqueous 0.1% heptafluorobutyric acid. For V8 protease digestion, pyridylethylated toxin was dissolved in 100 μl sodium phosphate buffer (50 mM, pH 7.8); 1 μg *Staphylococcus aureus* protease V8 was added and the solution incubated for 20 h at 37°C. The digest was separated on a C_4 column using the same conditions described for V8 peptides. N-terminal amino acid sequences were determined by automated Edman degradation in a gas-phase sequencer (Applied Biosystems 470A). After conversion of cleaved amino acids to the corresponding phenylhydantoin derivatives, the latter were subsequently analysed on line (Applied Biosystems 120A h.p.l.c. system).

Materials

Leiurus quinquestriatus hebraeus venom was purchased from Latoxan (Rosans, France). Cromakalim (BRL 34915; (±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol) and PN 200-110 (isradipine; 3,5-pyridinedicarboxylic acid, 4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl, 1-methylethylester) were synthesized at Sandoz, Basel. Stock solutions of cromakalim (1 mM) and PN 200-110 (1 mM) were prepared in 50% ethanol: 50% twice distilled water and further dilutions were made with a modified Krebs-Henseleit solution (KHS; Cook *et al.*, 1988a). Solutions of PN 200-110 were protected from light and all $^{86}Rb^+$ efflux experiments were done under sodium light. Chromatography resins were obtained from Pharmacia; A23187, collagenase (type IV), papain, (±)-dithiothreitol, angiotensin II and digitonin were obtained from Sigma. $^{86}Rb^+$ was purchased from New England Nuclear. All other chemicals were reagent grade and were obtained from Merck.

Results

Cromakalim (0.03–300 μM) produced a concentration-dependent increase in $^{86}Rb^+$ efflux from rabbit aortic tissue (Cook *et al.*, 1988b). In the presence of PN 220-110 (0.5 μM), there was no significant difference in either the shape of the curve or in the maximum response produced by cromakalim. From these results, 1 μM cromakalim was chosen for subsequent flux studies described in this paper, as it represented approximately an EC_{50}. PN200-110 was included in the wash solution as a protective

measure against unknown components in the venom which could themselves stimulate $^{86}Rb^+$ loss from the tissue and thus hamper the interpretation of the data (Quast & Cook, 1988).

Crude *Leiurus quinquestriatus hebraeus* venom (0.1–30 $\mu g\,ml^{-1}$) produced a concentration-dependent decrease of the rubidium response stimulated by 1 μM cromakalim (Figure 1). However the venom failed to inhibit completely the rubidium response induced by the drug. The maximum blocking activity observed was approximately 60% and with higher concentrations of venom a stable plateau was obtained and no further inhibition was observed.

Different batches of crude venom contained varying amounts of mucopolysaccharides which resulted in a viscous, stringy gel upon rehydration of the lyophilised venom at any concentration greater than 1 mg dry weight of venom per ml. After centrifugation at 45,000 *g* for 20 min the supernatant was often still too viscous for chromatography, causing many gel exclusion columns to dehydrate. This problem was overcome by acidification of the venom supernatant with formic acid and chromatography of the supernatant on a Sephadex G50 column equilibrated with 50 mM ammonium formate pH 3.5. Batches of crude venom (200 mg) processed in this manner (20 mg dry weight ml^{-1}) afforded one major peak (G50-C, molecular mass ~7000 kDa) which represented 65% of the A_{280}-absorbing material applied to the column and 94% of the cromakalim-induced $^{86}Rb^+$ flux blocking activity of crude venom (Figure 2). Peaks G50-A and B contained, amongst others, material of a mucopolysaccharide nature and peaks G50-D and E were non-proteinaceous U.V.-absorbing compounds (data not shown).

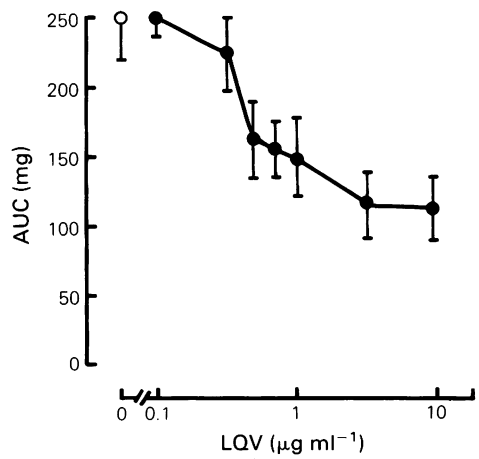


Figure 1 Inhibitory log concentration-effect curve for crude *Leiurus quinquestriatus hebraeus* venom (●) against 1 μM cromakalim in rabbit aorta. (○) Control response to cromakalim in the absence of venom. Ordinate scale: area under the curve (AUC) in mg as a measure of the stimulation of $^{86}Rb^+$ efflux; 100 mg corresponds to an increase in *k* (first order rate constant) by $0.2 \times 10^{-2}\,min^{-1}$ for 20 min. Abscissa scale: venom concentration, log scale. Data represent the means from four experiments (s.e.mean shown by vertical bars).

Fractions constituting peak G50-C were pooled from two batches of venom processed separately, diluted with an equal volume of water, and re-chromatographed on a cation exchange column of S-Sepharose equilibrated with 50 mM ammonium acetate pH 6.0. Of the A_{278}-absorbing material applied to the column, 18% was retained under these initial equilibration conditions. On application of a linear salt gradient, twelve distinct fractions were obtained (Figure 3). Each fraction was assayed for its ability to inhibit cromakalim-stimulated $^{86}Rb^+$ fluxes as before. As shown in Figure 3, most blocking activity was found in fractions X and XI. Fraction XII (containing charybdotoxin, see later), showed an insignificant ability to inhibit cromakalim-induced $^{86}Rb^+$ efflux. In contrast some fractions (most notably fraction IV), caused a *stimulation* of $^{86}Rb^+$ efflux.

Both fractions X and XI were potent inhibitors of the cromakalim-mediated increase in $^{86}Rb^+$ efflux, as indicated by their individual concentration-response curves (Figure 4). Unlike crude venom, both fractions X and XI produced more than 60% inhibition of the cromakalim-induced $^{86}Rb^+$ efflux. We were unable to test higher concentrations due to the small amounts of toxins available. Fraction X was re-chromatographed on a C_1/C_8 reverse phase

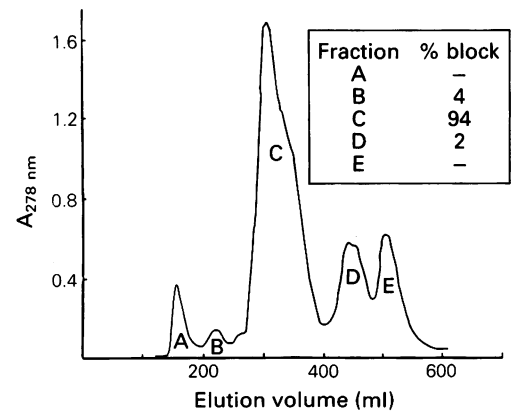


Figure 2 Sephadex G-50 chromatography of crude scorpion venom. Ordinate scale: absorbance at 280 nm. Abscissa scale: column elution volume (ml). Individual fractions making up the five major peaks were pooled separately and assayed for blocking activity in the cromakalim-induced $^{86}Rb^+$ flux assay. Activity was standardised to the total amount of A_{278} absorbance present in each fraction.

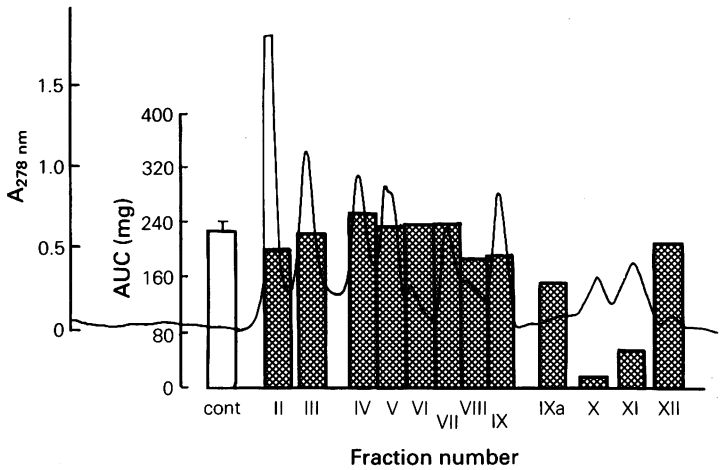


Figure 3 S-Sepharose ion exchange chromatography of fraction G-50-C (see Figure 2). Ordinate scale: absorbance at 280 nm. Abscissa scale: column elution volume. Individual fractions making up identified peaks II–XII were pooled separately and assayed for blocking activity in the cromakalim-stimulated $^{86}Rb^+$ efflux assay. Activity was standardized to the total amount of A_{278} absorbance present in each fraction.

column (Figure 5). Material bound under the starting conditions was eluted from the column with a linear gradient of acetic acid/methanol. Biological activity was located in one of the minor peaks, representing less than 2% of the total amount of A_{280} absorbing material applied to the column and less than 0.02% of total A_{280} absorbing material in the crude venom used at the start of the purification procedure. Fractions eluting from this reverse phase column were also assayed for their ability to block apamin-sensitive low conductance $K_{(Ca)}$ channels in hepatocytes, using an angiotensin II-stimulated potassium efflux assay. The only fraction that showed activity in this assay was the dominant fraction that inhibited chromakalim-induced $^{86}Rb^+$ fluxes (Figure 5).

Rechromatography of fraction XII on the same C_1/C_8 reverse-phase column, using an acetic acid/methanol gradient resulted in a single major peak (Figure 6, lower). This was identified as charybdotoxin by its elution characteristics both in this system (Smith *et al.*, 1986) and on a trifluoroacetic acid/acetonitrile solvent gradient, run on a C_{18} reverse phase column (Gimenez-Gallego *et al.*, 1988). Reduction and alkylation of purified peak XII, followed by enzymatic digestion with either Lys-C or V8 protease afforded the partial sequence shown (see below), which was identical to the C-terminal region of charybdotoxin (Gimenez-Gallego *et al.*, 1988).

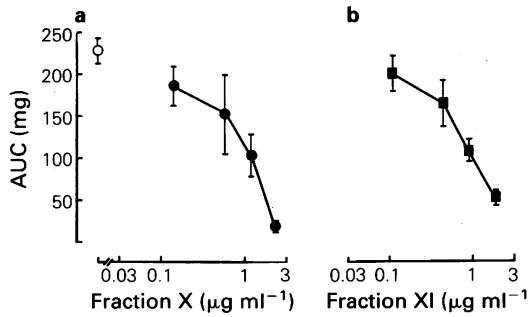


Figure 4 Inhibitory log-concentration-effect curve for fractions X (a, ●) and XI (b, ■) against cromakalim-stimulated $^{86}Rb^+$ efflux in rabbit aorta: (○) represents the control response to 1 μM cromakalim in the absence of any inhibitory fraction. Ordinate scale: area under the curve (AUC) in mg as a measure of the stimulation of $^{86}Rb^+$ efflux; 100 mg corresponds to an increase in k by 0.2×10^{-2} min^{-1} for 20 min. Abscissa scale: fraction concentration (μg ml^{-1}). Data represent the means from three experiments (s.e.mean shown by vertical bars).

Purified fraction XII proved to be a potent inhibitor of A23187-stimulated potassium efflux from human erythrocytes (Figure 6, trace A). In contrast, both fractions X (Figure 6, trace B) and XI were completely devoid of activity in the erythrocyte assay. Fractions X and XI also did not show any

(a) Z F T N V S C T T S K E C W S V C Q R L H N T S R G K C M N K K C R C Y S

(b) E C W S V C Q R L H N T S R G K C M N K K C R C Y S

(a) Charybdotoxin sequence, Gimenez-Gallego *et al.*, 1988; (b) Purified fraction XII, partial sequence.

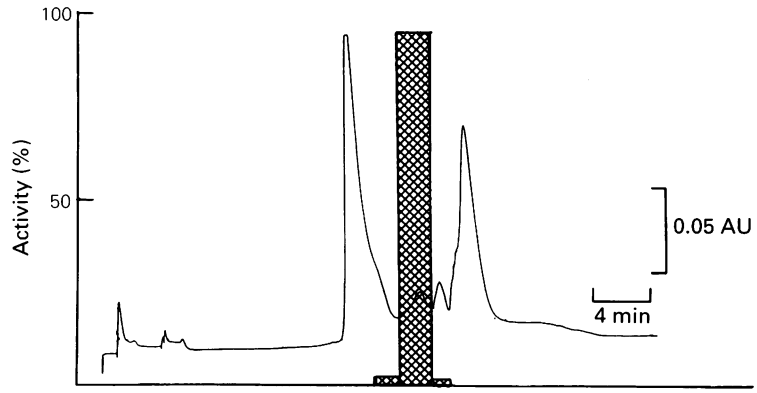


Figure 5 High performance liquid chromatography of fraction X (see Figure 3) on a Pharmacia C_1/C_8 reverse phase column. Ordinate scale: absorption at 280 nm. Abscissa scale: elution time (min). The column was equilibrated with solvent A and eluted with a linear gradient 0–100% solvent B at 1 ml min^{-1} (solvent A: 0.05 M sodium acetate buffer, pH 4.6; solvent B: methanol/100 mM acetic acid, (1/1)). Arrow indicates the start of the linear gradient. Biological activity in the $^{86}Rb^+$ flux assay is indicated by a hatched bar and expressed as a percentage of the total activity found in fraction X.

blocking activity against high conductance $K_{(Ca)}$ channels (see below). Interestingly, cromakalim failed to stimulate potassium efflux from the red blood cells (data not shown).

The activity of purified fraction XII at the single channel level was studied in a series of patch-clamp experiments using outside-out patches from rabbit portal vein smooth muscle cells. Care was taken to

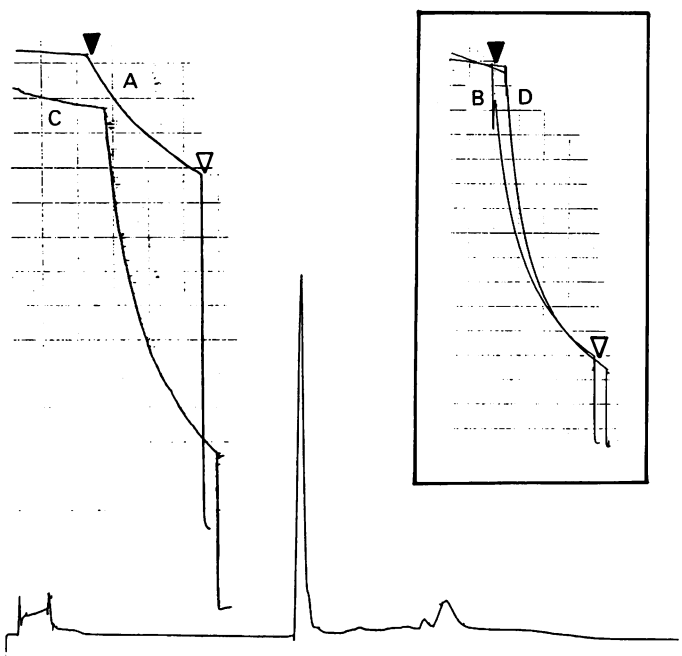
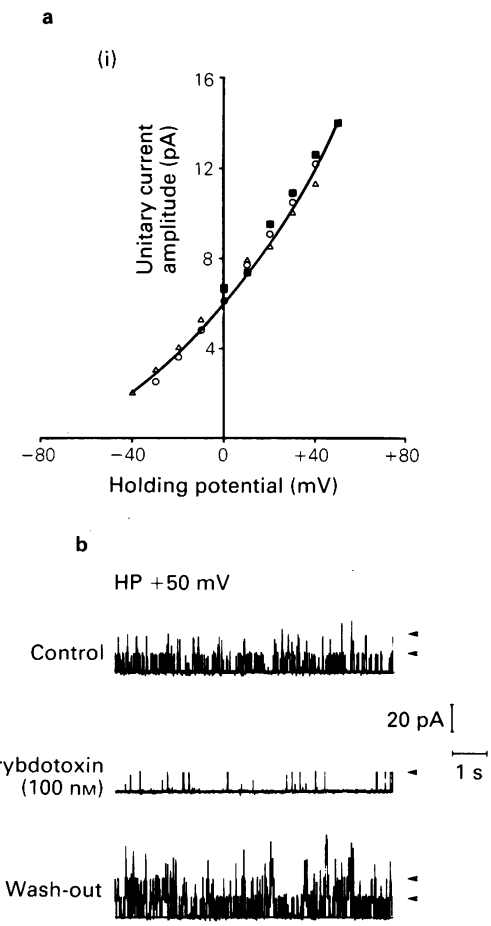


Figure 6 (Upper) Erythrocyte potassium flux assay. Experimental traces of (A) 100 nM (0.4 μg ml^{-1}) purified fraction XII (charybdotoxin); (B) 20 μg ml^{-1} fraction X (inset); (C,D) controls. Potassium flux was initiated by application of 5 μM A23187 (in the presence/absence of toxins) at the symbol (▼). Total cell potassium was determined at the end of each assay by adding 200 μM digitonin at the symbol (▽). (Lower) h.p.l.c. purification of fraction XII (charybdotoxin) on a C_1/C_8 reverse phase column, same conditions as in Figure 5.



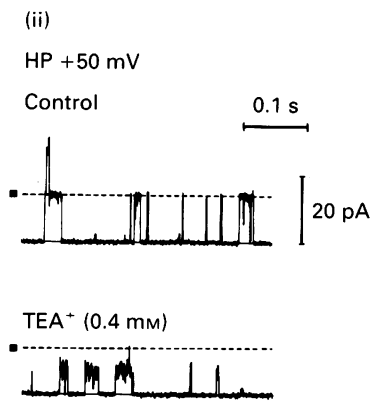


Figure 7 Effect of fraction XII (charybdotoxin) on high conductance $K_{(Ca)}$ channels in an outside-out patch from a rabbit portal vein smooth muscle cell. (a) Identification of the channel: (i) voltage-current relationship (■) for the unitary current amplitude of the channel. Other symbols (○, △) are for comparison with similar channels recorded from different patches under similar conditions. The line, which was drawn by eye, crosses the voltage axis close to the calculated potassium equilibrium potential (−78 mV). (ii) bath-applied tetraethylammonium (TEA^+) (0.4 mM) induced about a 50% decrease in unitary current – characteristic of open channel block. The signal was digitized at 4 kHz. (b) The effect of bath-applied purified fraction XII when the patch was held at +50 mV. The arrows mark the unitary current levels for the fully open state(s) of the $K_{(Ca)}$ channels and the continuous line marks the current when no $K_{(Ca)}$ channels were open. The block was reversible upon wash-out. The signal was digitized at 0.4 Hz.

characterize the channels appearing in these patches, before application of any toxic sample to the patch to ensure that they were indeed high conductance $K_{(Ca)}$ channels. The channels had a unitary conductance of ~90 pS (0 mV) with a physiological potassium gradient and were blocked by 0.4 mM TEA^+ (Figure 7a, right). The opening probability of the channels increased as the negativity at the intracellular side of the membrane was decreased (Figure 7b, left) and qualitative experiments with pipette solutions containing different amounts of free ionized calcium indicated that the opening probability of the channels also increased as the internal calcium concentration increased (data not shown). These experiments therefore provided strong evidence that the channels appearing in outside-out patches were high conductance $K_{(Ca)}$ channels as described by Inoue *et al.* (1985) for these cells.

In the presence of external, purified fraction XII (100 nM, assuming identity with charybdotoxin), the frequency of channel openings was markedly reduced as compared with the control (80–90%; Figure 7b). There was no appreciable effect on the unitary current of the high conductance $K_{(Ca)}$ channels or on the small channel in this patch (of unknown identity), which was observed to be open most of the time during the recording. The block was fully reversible on washout, when the opening probability of the channels appeared to be greater than in the controls. In support of the conclusion that charybdotoxin blocks single $K_{(Ca)}$ channels, the toxin also blocked spontaneous transient outward currents (STOCS), which represent $K_{(Ca)}$ currents in isolated smooth muscle cells (Benham & Bolton, 1986), at the same concentration (Beech & Bolton, 1989). Charybdotoxin affects neither the delayed rectifier potassium current (Beech & Bolton, 1989), the resting membrane resistance nor the cromakalim-induced potassium current of these smooth muscle cells (Beech and Bolton, unpublished).

Discussion

Leiurus quinquestriatus venom has been shown previously to contain toxins that block a remarkable number of different types of potassium channels. For example, large conductance $K_{(Ca)}$ channels in smooth muscle cells (Beech *et al.*, 1987; Kusano *et al.*, 1987) and skeletal muscle T-tubular vesicles (Miller *et al.*, 1985); apamin-sensitive low conductance channels in hepatocytes (Abia *et al.*, 1986; Castle & Strong, 1986); apamin-insensitive channels in thymocytes and Erlich cells (Abia *et al.*, 1986); intermediate conductance $K_{(Ca)}$ channels in erythrocytes (Abia *et al.*, 1986); Castle & Strong, 1986; Beech *et al.*, 1987), as well as the cromakalim-stimulated increase in potassium permeability in portal vein (Quast & Cook, 1988). The biological effects of crude venom on large conductance $K_{(Ca)}$ channels can be attributed to charybdotoxin, a peptide toxin first isolated by Miller *et al.* (1985) and recently sequenced (Gimenez-Gallego *et al.*, 1988). Although charybdotoxin has been shown subsequently to block a variety of large conductance $K_{(Ca)}$ channels (Figure 7; Guggino *et al.*, 1986; Beech *et al.*, 1987; Hoshi & Aldrich, 1987), it is becoming increasingly clear that the toxin is not completely selective. Charybdotoxin also blocks a 50 pS $K_{(Ca)}$ channel in *Aplysia* neurones (Hermann & Erxleben, 1987) as well as a voltage-sensitive potassium channel in lymphocytes (Lewis & Cahalan, 1988). In this present study we have confirmed that the toxin which we had previously tentatively identified as charybdotoxin (Castle & Strong, 1986), also blocks the human erythrocyte $K_{(Ca)}$ channel, which has been shown to have a single channel conductance value of ~20 pS (Hamill, 1981; Grygorczyk *et al.*, 1984). Since cromakalim failed to stimulate potassium efflux from erythrocytes, it is extremely unlikely that red cells possess cromakalim-sensitive potassium channels.

The results presented in this study unequivocally show that charybdotoxin does not affect the potassium channel opened by cromakalim (see also data by Talvenheimo *et al.* (1988), which implies a similar conclusion) and that the inhibitory effects of crude *L. quinquestriatus* venom on tracer efflux from aorta is due to another, distinct toxin species. This second toxin seems rather elusive. It appears to be present in the venom in extremely small amounts, in much smaller quantity than charybdotoxin. However, unlike charybdotoxin, we found that the biological activity of this second toxin is not very stable and becomes increasingly so during the final stages of purification; hence quantitation of the amount present may well be an underestimate. Many venoms contain toxic components that are stabilized in the crude venom through secondary non-covalent associations and we cannot exclude the possibility that the inhibitory effects of crude *L. quinquestriatus hebraeus* venom on cromakalim-stimulated tracer efflux might well be due to such a synergism. Purification schemes for fractionating the venom (with the aim of purifying charybdotoxin) often involve an ion exchange step at pH 9 or greater, sometimes approaching pH 11; these extremely basic conditions invariably reduce or destroy the biological activity of the second toxin. The present purification scheme has been designed to minimize this problem. The inability of crude venom to inhibit completely the cromakalim-stimulated $^{86}Rb^+$ efflux is intriguing. This could be explained either by the presence of stimulatory factors in the crude venom (Figure 3) or else by the fact that cromakalim is stimulating, in addition, another venom-insensitive potassium channel. This second possibility is unlikely in view of the fact that semi-purified fractions X and XII, in the concentrations used, can almost completely block efflux.

The most highly purified fraction containing the cromakalim-associated inhibitory activity also inhibited the apamin-sensitive potassium efflux from hepatocytes and we have previously shown that this component also inhibits [^{125}I]-monoiodoapamin binding to hepatocytes (Castle & Strong, 1986). As this paper was in its final stages of preparation, the complete purification and sequence of this apamin-binding toxin was published (Chicchi *et al.*, 1988). At present we suggest that this second toxin has a broad specificity for both apamin-sensitive, $K_{(Ca)}$ channels and those potassium channels that are opened by cromakalim, although we cannot rule out the possibility that these two activities will be shown subsequently to reside on distinct molecules.

The isolation of a voltage-dependent potassium channel gene from the *Shaker* mutant of *Drosophila* (Papazian *et al.*, 1987; Pongs *et al.*, 1988) and the subsequent use of *Drosophila* cDNA libraries to identify a potassium channel gene in mammalian brain (Tempel *et al.*, 1988; Baumann *et al.*, 1988) suggests many structural similarities between different types of potassium channel and therefore perhaps it is not surprising to find toxins that have high affinities for more than one potassium channel subtype.

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