Purification and Characterization of a Unique, Potent Inhibitor of Apamin Binding from Leiurus quinquestriatus hebraeus Venom

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An inhibitor of apamin binding has been purified to homogeneity in three chromatographic steps from the venom of the scorpion, Leiurus quinquestriatus hebraeus. The inhibitor, which we have named leiurotoxin I, represents less than 0.02% of the venom protein. It is a 3.4-kDa peptide with little structural homology to apamin although it has some homology to other scorpion toxins such as charybdotoxin, naxitoxin, and neurotoxin P2. Leiurotoxin I completely inhibits 125I-apamin binding to rat brain synaptosomal membranes (Kᵢ = 75 nM). Thus, it is 10–20-fold less potent than apamin. Leiurotoxin I is not a strictly competitive inhibitor of this binding reaction. Like apamin, leiurotoxin I blocks the epinephrine-induced relaxation of guinea pig teniae coli (ED₅₀ = 6.5 nM), while having no effect on the rate or force of contraction in guinea pig atria or rabbit portal vein preparations. Thus, leiurotoxin I of venom and apamin of honeybee venom demonstrate similar activities in a variety of tissues, yet are structurally unrelated peptides. These two peptides should be useful in elucidating the role of the small conductance, Ca²⁺-activated K⁺ channels in different tissues.

Material and Methods

Apamin was obtained from Sigma. 1,3,4,6-Tetrachloro-3α,6α-diphenyl glycoaluril (IODO-GEN) was obtained from Pierce and Na¹²⁵I (carrier-free) was obtained from Amersham. Leupeptin and chymostatin were purchased from Peninsula Laboratories. Male Sprague-Dawley rats (sesarian-derived, approximately 350 g each) were obtained from Charles River. Lyophilized L. quinquestriatus hebraeus venom was from Latoxin Scorpion Farm (Rosans, France). Crystaline bovine serum albumin used as a protein standard was from Sigma. Bradford protein reagent and electrophoresis reagents were purchased from Bio-Rad. Molecular weight markers (range 2,512–16,949) were obtained from LKB.

Iodination of Apamin—Apamin was purified by high performance liquid chromatography (HPLC) on a C₁₈ reverse-phase column (Zorbax ODS, 4.6 mm × 25 cm, Du Pont-New England Nuclear) using a 5–50% gradient of methanol in 0.05% trifluoroacetic acid. Purified apamin was stored at 1 mM in 0.1 N acetic acid at −20°C. Apamin was iodinated by a modification of the IODO-GEN method (6). Briefly, 10 μg of apamin were dried by vacuum centrifugation in a Reacti-Vial containing 2 μg of dried IODO-GEN. Next, 5 μl of 0.1 M Tris buffer, pH 8.6, and 1 ml of Na¹²⁵I (10 μl) were added. The capped vial was held at 20°C for 15 min, after which the contents of the vial were transferred to a Microfuge tube containing 15 μl of methanol. The tube remained uncapped for 5 min to allow any unincorporated 125I to dissipate. The iodination mixture was subjected to HPLC over a C₁₈ reverse-phase column as described above in order to separate 125I-apamin from unlabelled apamin. 125I-Apamin peak fractions (specific activity, 0.9–1.0 mCi/μg) were pooled and stored at −20°C.

I25I-Apamin Binding to Rat Brain Cortex Membranes—Gradient-purified rat brain cortex synaptosomal membranes were prepared as previously described (9). Membranes (70 μg of protein as determined by the Bradford method) were incubated with 125I-apamin (20 pm) in 0.2 ml of 5 mM Tris, pH 7.5, 5 mM KCl, 50 μg/ml leupeptin, 50 μg/ml chymostatin, and 0.1% crystalline bovine serum albumin in polyethylene tubes for 30 min at 0°C or 20°C as indicated. Samples were filtered over Whatman GF/F filters, presoaked in 0.1% polyethyleneimine, to separate bound from free ligand. The tubes and filters were then washed four times with 2.5 ml of ice-cold wash buffer (incubation buffer without inhibitors). Filters were counted for radioactivity in a γ scintillation counter. Filters bound less than 1% of ligand in the absence of membranes. Rat brain synaptosomal membranes bound 40–50% of the ligand in the absence of unlabeled apamin, while nonspecific binding to the membranes in the presence of excess unlabeled apamin (1 nM, unless stated otherwise) was less than 3% of the ligand added. Assays were conducted in duplicate.

Purification of Apamin Binding Inhibitor (Leiurotoxin I) from Scorpion Venom—Lyophilized L. quinquestriatus hebraeus venom (80 mg/10 ml) was extracted in 20 ml of 20 mM sodium borate, pH 9.0, at 4°C and was chromatographed on a Mono S cation exchange column.
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I. Purification of inhibitor of apamin binding from *L. quinquestriatus hebraeus* venom by Mono S cation exchange chromatography. Mono S column chromatography was performed on 0.5-mg of extracted crude scorpion venom as described under "Materials and Methods." Fractions were manually collected and assayed in apamin binding assay. Bottom, absorbance profile (280 nm) of Mono S column eluant. Vertical lines represent fraction collection. Slanted line represents NaCl gradient of elution buffer. Top, inhibitory activity found in Mono S fractions. Inhibitory units are arbitrary designations representing percent inhibition per fraction aliquot times fraction dilution.

![Fig. 1](image-url)

**FIG. 1.** Purification of inhibitor of apamin binding from *L. quinquestriatus hebraeus* venom by Mono S cation exchange chromatography. Mono S column chromatography was performed on 0.5-mg of extracted crude scorpion venom as described under "Materials and Methods." Fractions were manually collected and assayed in apamin binding assay. Bottom, absorbance profile (280 nm) of Mono S column eluant. Vertical lines represent fraction collection. Slanted line represents NaCl gradient of elution buffer. Top, inhibitory activity found in Mono S fractions. Inhibitory units are arbitrary designations representing percent inhibition per fraction aliquot times fraction dilution.

**RESULTS**

125I-Apamin specifically binds to rat brain synaptosomal membranes in a saturable manner. The peptide binds with high affinity ($K_D = 17.2$ pM) to a single class of relatively low density sites (83 fmol/mg of membrane protein) as indicated by a linear Scatchard plot (15) (data not shown). These data are consistent with those previously reported by Hugues et al. (2). Unmodified apamin and nonradioactive iodoapamin inhibit binding with $K_D = 7.4$ and 13.1 pM, respectively (data not shown). Thus, iodination of apamin does not substantially affect the ability of the peptide to bind to rat brain synaptosomal membranes. Similar potency values are obtained at both 0 and 20°C (data not shown).

A potent inhibitor of apamin binding (leuropotoxin 1) is purified from *L. quinquestriatus hebraeus* venom in three chromatographic steps. The dried venom is extracted with 20 mM sodium borate, pH 9.0, and chromatographed on a Mono S cation exchange HPLC column (Fig. 1). Most of the extracted material absorbing at 280 nm is not retained by the column and does not inhibit apamin binding (data not shown). The retained material is eluted with a linear NaCl gradient (0.75 M/h), and inhibitory activity is found in the fractions corresponding to the flat region of the 280 nm absorbance tracing immediately following the large absorbance peak at 17 min (Fig. 1). This step gives a 12-fold purification of binding inhibitory activity with a 28% recovery of activity (Table I).

The Mono S fractions with the highest levels of inhibitory activity, eluting with 250–290 mM NaCl, are fractionated further by C4 reverse-phase chromatography using a linear 15–30% acetonitrile gradient in 0.05% trifluoroacetic acid (Fig. 2, top). A minor peak representing 19% of inhibitory activity recovered co-elutes with the major absorbance peak (220 nm) at 8 min and has not been characterized further. The major peak of inhibitory activity elutes at 21 min (18% acetonitrile) with the trailing shoulder of a UV-active peak. This step gives a 31-fold purification with a 91% recovery of activity (Table I).

The activity is pooled and purified to homogeneity by C4 reverse-phase chromatography using a linear 20–35% acetonitrile gradient in 0.05% heptfluorobutyric acid (Fig. 2, bottom). The major peak of inhibitory activity (>95% of activity recovered) co-elutes with the highest peak of absorbance (220 nm) at 35 min (28% acetonitrile). Less than 5% of the activity which is recovered elutes in a minor peak at 22 min (23% acetonitrile) and has not been characterized further. Recovery of the major peak of activity in this step is 27% with a 36% increase in specific activity (Table I). Thus, the total recovery of activity during the purification is 6.9%. The estimate of recovery does not include activities found in the trailing shoulder of the Mono S HPLC step (Fig. 1) or the minor, active peaks of the two C4 reverse-phase HPLC steps (Fig. 2).

Silver-stained SDS-polyacrylamide gels of the major active species (Fig. 2, bottom) show a single 3.37-kDa polypeptide (Fig. 3), whose amino acid composition appears in Table II. Edman degradation of the same material demonstrates a
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**Table 1**

Purification summary of leiurotoxin I from *L. quinquestriatus hebraeus* venom

<table>
<thead>
<tr>
<th>Purification began with 480 mg of lyophilized crude venom.</th>
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<tr>
<td><strong>Extracted crude venom</strong></td>
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<tr>
<td>Total protein</td>
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<tr>
<td>mg</td>
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<tr>
<td>Extracted crude venom</td>
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<tr>
<td>Mono S HPLC pool</td>
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<td>1st C, HPLC pool (15–30% acetonitrile/0.05% trifluoroacetic acid)</td>
</tr>
<tr>
<td>2nd C, HPLC pool (20–35% acetonitrile/0.05% heptafluorobutyric acid)</td>
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* 50% IU are arbitrary inhibitory activity units obtained by dividing the total volume of the pool at each step by the volume of each pool required to achieve 50% inhibition of ^125^I-apamin binding in the apamin binding assay.

* Fold purification equals specific activity of pool/specific activity of extracted crude venom.

* Protein determined by modified Lowry protein assay (16) using bovine serum albumin as a standard.

* Protein determined by amino acid analysis.

A unique, potent inhibitor of apamin binding has been purified to homogeneity from *L. quinquestriatus hebraeus* venom. The inhibitor, leiurotoxin I, is a minor component of the crude scorpion venom representing less than 0.02% of the total protein. Abia *et al.* (7) previously identified an inhibitor in scorpion venom which was partially purified by Castle and Strong (6) using S-Sepharose ion exchange chromatography. The inhibitor inhibited angiotensin II-stimulated K⁺ efflux from guinea pig hepatocytes and ^125^I-apamin binding to hepatocytes. Leiurotoxin I, purified to homogeneity herein, inhibits epinephrine-induced relaxation of guinea pig teniae coli (ED₅₀ = 6.5 nM) and inhibits ^125^I-apamin binding to rat brain synaptosomal membranes (Kᵯ = 75 pm), being 5–10- and 10–20-fold less potent than apamin, respectively. Thus, the relative biological potency of leiurotoxin I as measured by the teniae coli assay correlates well with its relative potency in binding to the rat brain synaptosomal membranes.

Leiurotoxin I is a mixed-type inhibitor of apamin binding to rat brain synaptosomal membranes since it increases the apparent Kᵯ for ^125^I-apamin binding and reduces the number of binding sites. The similarity in the activities of leiurotoxin I and apamin in the guinea pig teniae coli assay indicates that, like apamin, leiurotoxin I acts as a blocker of a Ca²⁺-activated K⁺ channel.

In a search of the National Biological Research Foundation (NBRF) protein sequence data bank (Release 13, June 1987), leiurotoxin I appears to be unique. Leiurotoxin I demonstrates little sequence homology to apamin (Fig. 7). Both peptides are, however, very basic and both have an amidated histidine at the carboxyl terminus. Castle and Strong (6) reported that crude venom (100 μg/ml) of *L. quinquestriatus* does not cross-react with apamin antibodies, which suggests that there is no immunological cross-reactivity between apamin and leiurotoxin I. This is understandable in light of the lack of homology of the two peptides.

Charybdoxin from *L. quinquestriatus* venom is an inhibitor of high conductance, apamin-insensitive Ca²⁺-activated K⁺ channels. Recently, it has been purified, characterized as a basic 4.353-kDa protein, and its tertiary structure has been...
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FIG. 2. Purification of inhibitor of apamin binding from *L. quinquestriatus hebraeus* venom by C$_4$ reverse-phase chromatography. *Top,* active fractions from Mono S chromatography (Fig. 1, 1.55 mg of protein) were chromatographed with 15–30% acetonitrile/0.05% trifluoroacetic acid as described under “Materials and Methods.” Fractions (1 min, 0.5 ml) were collected in polyethylene tubes and were assayed in the apamin binding assay. Hatched bars represent the inhibitory activity found in each fraction. Inhibitory units are arbitrary designations for percent inhibition per fraction aliquot times fraction dilution. *Bottom,* active fractions of major peak from above (55 pg of protein) were resolubilized in 20% acetonitrile in 0.05% heptafluorobutyric acid and were chromatographed with 20–35% acetonitrile/0.05% heptafluorobutyric acid as described under “Materials and Methods.” Fractions (1 min, 0.5 ml) were collected in polyethylene tubes and were assayed in apamin binding assay. Data are expressed as above.

Toxins which have been shown to be homologous to charybdotoxin (11). The best alignment found between leiurotoxin I and noxiustoxin or neurotoxin P2 is 4.7 or 2.8 S. D. from random, respectively. The probabilities are less than 1 X 10$^{-4}$ and 2.6 X 10$^{-3}$ that the values 4.7 and 2.8 reflect zero correlation of leiurotoxin I with the noxiustoxin and neurotoxin P2 sequences, respectively. It should be noted that the highest degree of homology among these proteins is in the carboxyl terminal region of the molecule. Perhaps this suggests that this may be the specific region of interaction of all these toxins with homologous regions of different channels which evolved from a common ancestral gene coding for a singular ion channel.

The similarity in the pattern of side-chain solvent exposure of charybdotoxin and leiurotoxin I can be evaluated by the method of Sweet and Eisenberg (20) using the normalized solvent exposure indexes (11). The correlation coefficient ($r_H$) obtained is 0.64, a value which is 4.6 S. D. from random for proteins of this size. The three-dimensional structural homology is also supported by analysis of the minimum mutation distance between these two sequences (21). The minimum mutation distance is 0.97 mutation per amino acid,
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Fig. 4. Sequence analysis of purified carboxymethylated leiurotoxin I. Purified carboxymethylated leiurotoxin I (3.2 nmol) was sequenced on an Applied Biosystems sequenator with an on-line phenylthiohydantoin analyzer as described under "Materials and Methods." The natural logarithms of the amounts injected into the detector (40% of the collected volume) are plotted. The repetitive yield was 91% per cycle.

Fig. 5. Scatchard analysis of [125I]-apamin binding to rat brain synaptosomal membranes in the absence or presence of leiurotoxin I. Rat brain synaptosomal membranes (70 μg) were incubated with increasing concentrations of [125I]-apamin (0.367 pM, 0.3 mCi/μg) in the absence (closed circles) or presence (open circles) of leiurotoxin I (430 pM) as described under "Materials and Methods" with the exception that nonspecific binding was measured using 1 μM apamin. Scatchard data were calculated using linear regression analysis. Correlation coefficients for Scatchard data in the absence and presence of leiurotoxin I are −0.964 and −0.974, respectively. B/F, bound/free.

approximately 6.13 S. D. from random. Preliminary results suggest that the leiurotoxin I sequence is compatible with the three-dimensional model proposed for charybdotoxin.2

2 M. A. Navia, personal communication.

Charybdotoxin is a major component of the trailing shoulder of inhibitory activity eluting from the Mono S HPLC column with approximately 325 mM NaCl (Fig. 1). When purified, it inhibits [125I]-apamin binding to rat brain synaptosomal membranes (IC50 = 0.8 μM), but is 2000-fold less potent than leiurotoxin I (data not shown). In contrast, charybdotoxin inhibits [125I]-apamin binding to the same membrane preparation with IC50 = 29 pM, whereas apamin (1 μM) and leiurotoxin I (0.6 μM) demonstrate approximately 20% inhibition.3 These binding data further suggest that leiurotoxin I and charybdotoxin may share some secondary and tertiary structural characteristics.

More extensive studies will be necessary to delineate the

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structural features responsible for the divergent functions of these various toxin molecules. Leiurotoxin I, a novel toxin purified from scorpion venom, should prove to be a useful tool in identifying and classifying various types of apamin-sensitive K⁺ channels.

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REFERENCES