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Polypeptide Toxins from the Venoms of Old World and New World Scorpions Preferentially Block Different Potassium Channels

MORDECAI P. BLAUSTEIN, ROBERT S. ROGOWSKI, MARY J. SCHNEIDER, and BRUCE K. KRUEGER

Departments of Physiology (M.P.B., R.S.R., M.J.S., B.K.K.) and Medicine (M.P.B.), University of Maryland School of Medicine, Baltimore, Maryland 21201

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SUMMARY

Venoms from five Old World and two New World scorpions were tested for their ability to block various K^+ channels in rat brain synaptosomes. A ^{86}Rb efflux kinetic assay was used to identify three types of K^+ channels, Ca^{2+}-independent, voltage-gated, inactivating (A-type) and noninactivating (delayed rectifier) K^+ channels and Ca^{2+}-activated K^+ channels [*J. Physiol.* (*Lond.*) **361**:419–440, 441–457 (1985)]. The venoms from the Old World scorpions all blocked the A-type K^+ channel but not the delayed rectifier K^+ channel; only venom from the Israeli scorpion, *Leiurus quinqestriatus hebraeus* (*Lqh*), blocked the Ca^{2+}-activated K^+ channel. In contrast, venoms from the two New World scorpions selectively blocked the delayed rectifier K^+ channel. Water-soluble components from *Lqh* venom and the venom from the Brazillian scorpion, *Tityus serrulatus* (*Ts*), were separated by ion exchange high performance liquid chromatography (HPLC). Seven components that blocked synaptosome K^+ channels were isolated from *Lqh* venom by ion exchange HPLC. All seven components blocked the A-type K^+ channel; the five most potent toxins had IC_{50} values of 18–40 nM. Two of the components from *Lqh* venom (one identified as charybdotoxin and the other denoted as Lq_{k4}) also blocked a Ca^{2+}-activated K^+ channel (IC_{50} = 15 and 60 nM for charybdotoxin and Lq_{K4}, respectively). Five K^+ channel-blocking components were isolated from the *Ts* venom; all five blocked the delayed rectifier channel selectively, and the two most potent components had IC_{50} values of 8 and 30 nM. Several of the more potent *Lqh* and *Ts* toxins were purified to near-homogeneity by reverse phase HPLC. These toxins should be useful as ligands for K^+ channel purification, for elucidation of K^+ channel structure, and for studies of K^+ channel function.

A number of polypeptides from the venoms of some scorpions and snakes, certain molluscs, and other organisms have neurotoxic activity as a result of their selective, high affinity interactions with ion channels. Several scorpion venoms contain a number of polypeptide toxins that specifically block or alter the gating properties of Na^+ or K^+ channels. These polypeptides appear to be very useful for identifying and characterizing ion channels in excitable cells.

The K^+ channel toxins are particularly interesting because there are a number of different types of K^+ channels, and some scorpion toxins apparently block certain K^+ channels selectively. For example, ChTX, a polypeptide from *Lqh* venom, preferentially blocks a large-conductance ("maxi") Ca^{2+}-activated K^+ channel (1); at slightly higher concentrations, however, it also blocks rapidly inactivating, voltage-gated (A-type) K^+ channels (2, 3). In contrast, certain polypeptides from *Cn* and *Ts* preferentially block noninactivating voltage-gated (delayed rectifier-type) K^+ channels (4, 5), although at 10-fold higher concentrations NTX (from *Cn*) also blocks the maxi Ca^{2+}-activated K^+ channel (6). The unfractionated venom from *Pi* also blocks voltage-gated K^+ channels (7, 8).

Elucidation of the structure-activity relationships of the K^+ channel toxins from scorpion venoms may shed new light on the structures of various K^+ channels. With this as a long range goal, we developed a rapid and convenient assay to identify the selective effects of various scorpion venoms and purified toxins on A-type, delayed rectifier, and Ca^{2+}-activated K^+ channels in rat brain synaptosomes. Preliminary reports of some of these findings have been published in abstract form (9, 10).

This article is dedicated to the memory of Howard A. Schneiderman, whose love of physiology and life inspired one of us (M.P.B.) in his choice of career.

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ABBREVIATIONS: ChTX, charybdotoxin; NTX, noxiustoxin; TFA, trifluoroacetic acid; *Lqh, Leiurus quinquestriatus hebraeus; Lqq, Leiurus quinquestriatus quinquestriatus; Pi, Pandinus imperator; Cn, Centruroides noxius; Ts, Tityus serrulatus; Cs, Centruroides sculpturatus*; NH_4Ac, ammonium acetate; HPLC, high performance liquid chromatography; 4-AP, 4-aminopyridine; *Bj, Buthotus judaicus; Aa, Androctonus australis*; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; α-DaTX, α-dendrotoxin.

Materials and Methods

Synaptosomes. Synaptosomes (pinched-off presynaptic nerve endings) were prepared from homogenized rat forebrains as described (11). In the present study a crude synaptosome preparation, the pellet from the second 10,000 × *g* centrifugation (P2), was used for all tracer flux studies.

Tracer flux (^{86}Rb) assay. The methods for loading synaptosomes with ^{86}Rb and for using an ^{86}Rb efflux assay to identify the components of the efflux associated with A-type K^+ channels, delayed rectifier-type K^+ channels, and Ca^{2+}-activated K^+ channels have been described (9, 12–14). The compositions of the "5K" (standard 5 mM K^+), "100K" (100 mM K^+), and "100K(Ca)" (100 mM K^+ plus 1 mM Ca^{2+}) physiological salt solutions have been published (3). Further details are given in Results.

Venoms and toxins. Venoms from two New World scorpions, *Cs* (Mexico) and *Ts* (Brazil), and one Old World scorpion, *Lqq* (North Africa), were purchased from the Sigma Chemical Co. (St. Louis, MO). Additional supplies of *Ts* venom were obtained from Dr. C. R. Diniz (Federal University of Minas Gerais, Belo Horizonte, Brazil) and Dr. E. X. Albuquerque (University of Maryland School of Medicine, Baltimore, MD). Venoms from four Old World scorpions, *Lqh* (Israel), *Pi* (Morocco), *Bj* (Israel), and *Aa* (North Africa), were obtained from Latoxan (Rosans, France). Purified NTX from the New World scorpion *Cn* (Hoffman, Mexico) was generously provided by Dr. Jeffrey Smith (Merck, Sharp & Dohme, West Point, PA), and α-DaTX was purified by us (14) from green mamba venom (Sigma).

Purification of toxins from crude scorpion venoms. In early experiments, *Lqh* venom was extracted with acetone (3, 15); after lyophylization, the acetone-soluble material was dissolved in 20 mM NH_4Ac and fractionated by cation exchange HPLC. Subsequently, the extraction procedure was simplified (with similar results); both *Lqh* and *Ts* venoms were extracted with 20 mM NH_4Ac buffer at pH 6.8 (30 mg of venom/300 μl of buffer). The suspensions were centrifuged at 10,000 × *g* for 10 min; the clear supernatants were removed and saved, and the pellets were reextracted twice in 20 mM NH_4Ac. The three supernatants from each venom were pooled and applied to an Aquapore CX-300 cation exchange (Pierce Chemical Co., Rockford, IL) HPLC column (220 × 4.6 mm) equilibrated with 20 mM NH_4Ac (pH 6.8). The polypeptide components were eluted with a linear NH_4Ac gradient (0.02–0.75 M) at a flow rate of 1 ml/min. Fig. 1A shows a representative elution profile for the polypeptides from *Lqh* venom, and Fig. 1B shows a similar profile for the components from *Ts* venom. The fractions containing the various peptides were collected and lyophilized three times in order to remove all traces of NH_4Ac; they were dissolved in appropriate physiological solutions before use (see Results).

Several of the more potent and prevalent toxins were further purified by reverse phase HPLC. These toxins were dissolved in 0.075% TFA and applied to an Aquapore RP-300 reverse phase (Pierce) HPLC column (220 × 4.6 mm); the toxins were eluted with a linear gradient from 0.075% TFA in water to 0.075% TFA in acetonitrile over a 30- or 60-min period. The fractions were dried in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY); they were dissolved in appropriate physiological solutions before use (see Results).

Protein measurements. The protein concentrations of some of the fractions from the HPLC columns (e.g., Lq_{K2}, Lq_{K4}, Lq_{K5}, Ts_{K2}, and Ts_{K4}) were determined directly with a Pierce Micro bicinchoninic acid assay. The protein concentrations of the other peptides (which were present in very small amounts; see Fig. 1) were determined from the relative sizes of the integrated areas under the A_{280} peaks, using the directly measured protein concentrations as references; the peak areas were determined with Beckman System Gold HPLC software. Several of the most potent toxins were purified to near-homogeneity by reverse phase HPLC; the molecular weights were then determined by SDS-PAGE (16), and the toxin concentrations were based on these molecular weights. The concentrations of the other K^+ channel toxins were approximated on the assumption that their molecular weights were

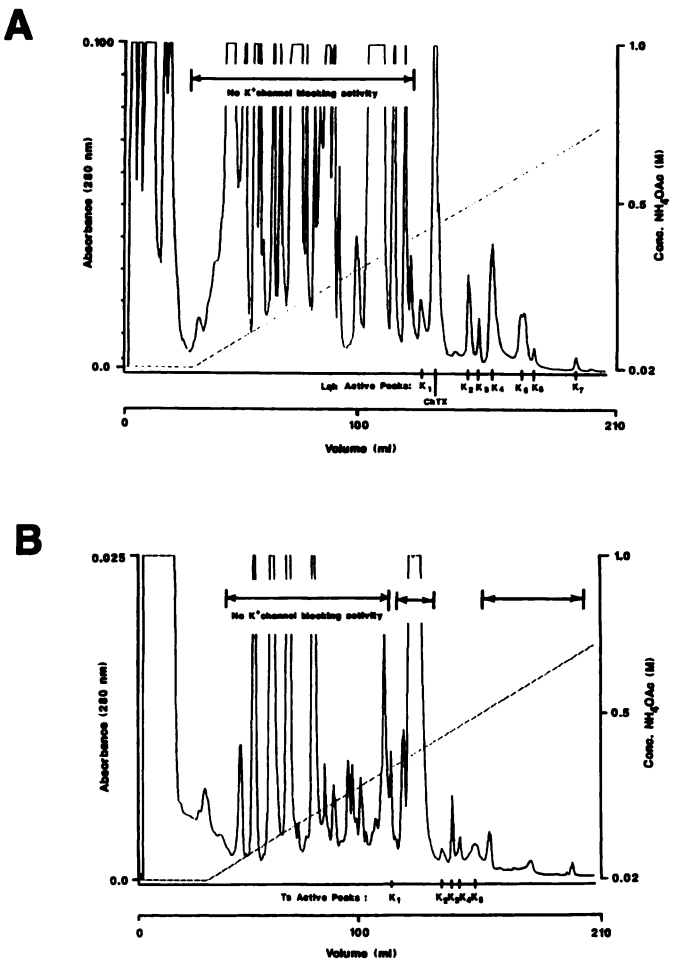

Fig. 1. HPLC elution patterns of acetone-extracted *Lqh* venom (A) and NH_4Ac-extracted *Ts* venom (B). Adsorbed proteins were eluted with a linear NH_4Ac gradient (0.02–0.75 M), at a flow rate of 1 ml/min. Fractions that were observed to inhibit ^{86}Rb efflux from synaptosomes are designated as fractions K_1 through K_5 (*Ts*) or K_1 through K_7, plus ChTX (*Lqh*), in the order of their elution from the column.

6000 (the mean value for the purified K^+ channel toxins from *Lqh* and *Ts* venoms; see Table 1).

Statistics. Values shown in the figures are presented with standard error bars when the standard errors extend beyond the symbols. For time course experiments, the regression lines were calculated by the method of least squares (SigmaPlot; Jandel, Corte Madera, CA). Dose-response curves were fitted to the Hill equation using SigmaPlot software.

Results

Assay of Unfractionated (Crude) Venoms for K^+ Channel-Blocking Activity

Assay for voltage-gated K^+ channels. Fig. 2 shows the effects of the five Old World (Fig. 2A) and two New World (Fig. 2B) scorpion venoms on the time course of ^{86}Rb efflux from synaptosomes (in the absence of Ca^{2+}, to prevent the opening of Ca^{2+}-activated K^+ channels; see below). The long-lived ^{86}Rb isotope was used here, rather than ^{42}K, because Rb passes through most K^+ channels nearly as well as K^+ itself (12).

The data in Fig. 2 show that, when the synaptosomes were depolarized by raising the external K^+ concentration from the

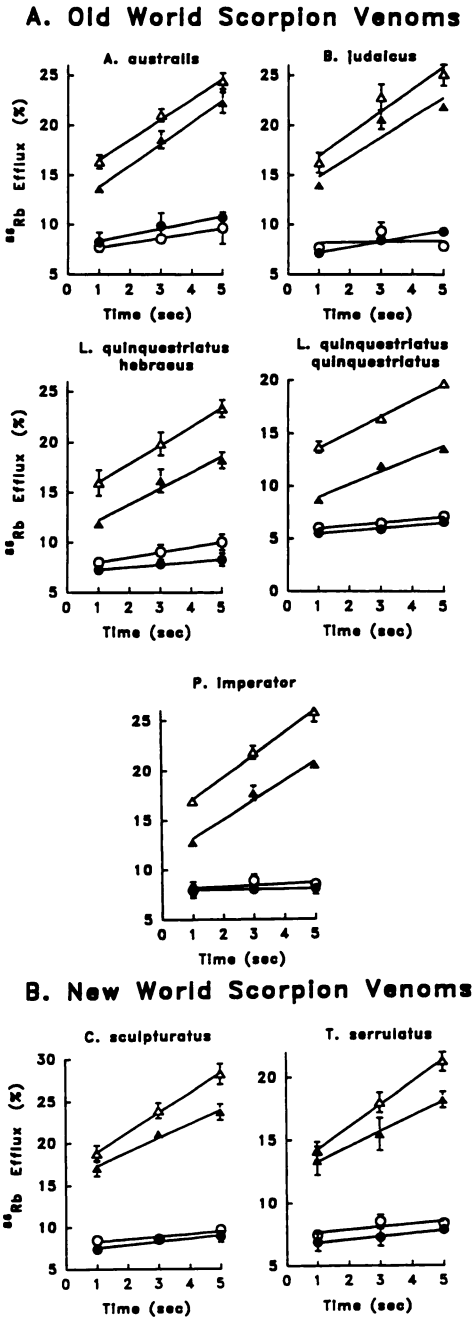

Fig. 2. Effects of five Old World scorpion venoms (A) and two New World scorpion venoms (B) on the time course of ^{86}Rb efflux from rat brain synaptosomes in the absence of extracellular Ca^{2+}. Efflux into 5K (*circles*) and into 100K (*triangles*) was measured in the absence (*open symbols*) or presence (*solid symbols*) of the various venoms. When present, the venom concentrations were 5 μg/ml, except in the case of *Pi* venom (10 μg/ml). Tetrodotoxin (100 nM) was added to all the preincubation and incubation solutions, to prevent interference by the Na^+ channel toxins that are present in some or all of these venoms. Each *panel* shows data from a different synaptosome experiment; the data were averaged from five or six measurements each; *standard error bars* are shown where they extend beyond the *symbols*. The linear regression lines were determined by the method of least squares. Similar results for each venom were obtained in at least two other experiments.

normal 5 mM to 100 mM (at 0 time), there was a large increase in ^{86}Rb efflux (the K^+-stimulated ^{86}Rb efflux) (12). In the absence of venom, depolarization increased the rate of efflux (slope) as well as the ordinate intercept. The increment in the

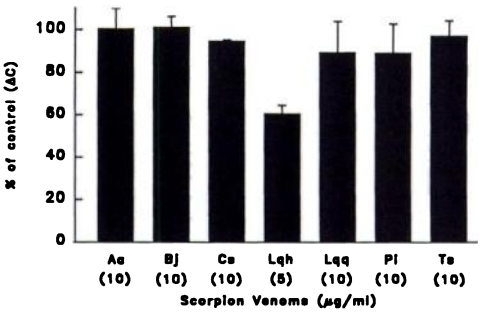

Fig. 3. Effects of several Old World and New World scorpion venoms on the Ca^{2+}-dependent component of the 5-sec 100 mM K^+-stimulated ^{86}Rb efflux (ΔC) from rat brain synaptosomes. All solutions contained 100 nM tetrodotoxin to block Na^+ channels and 10 mM 4-AP to block the voltage-gated inactivating and noninactivating K^+ channels. ΔC was calculated as the Rb efflux in 100K(Ca) minus the efflux in Ca^{2+}-free 100K, either without or with venom present. The venom concentrations were 10 μg/ml except for *Lqh* venom (5 μg/ml). Each *bar* indicates the difference between the means of the differences for five or six measurements in both 100K and 100K(Ca) for two (*Aa, Bj, Cs*, and *Lqq*) or three (*Lqh, Pi*, and *Ts*) experiments. The data are normalized to the control ΔC, 4.7 ± 0.2% (five experiments). Standard errors for the ΔC values were calculated as described (34); only the ΔC for *Lqh* venom was significantly smaller ($p < 0.01$) than control ΔC (ΔC in the absence of venom).

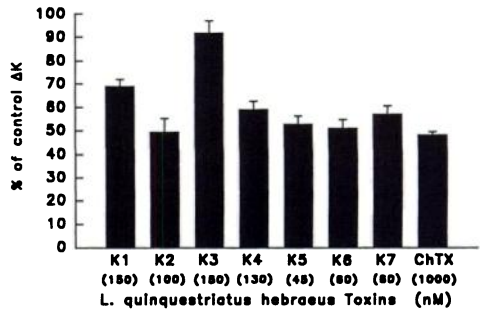

Fig. 4. Effects of various fractions of *Lqh* venom from the ion exchange HPLC column (see Fig. 1A) on the 5-sec Ca^{2+}-independent, K-stimulated ^{86}Rb efflux (ΔK) in synaptosomes. Six replicate determinations were made for each condition. The *bars* indicate the percentage of control ΔK observed in the presence of each of the toxins at the concentrations indicated in *parentheses* (concentrations were calculated as described in Materials and Methods). Control ΔK (i.e., in the absence of toxins) was 13.7 ± 0.4%. Standard errors for the ΔK values were calculated as described (34); all of the fractions except K3 significantly reduced ΔK ($p < 0.01$). Complete block of both the A-type and delayed rectifier K^+ channels would be expected to reduce ΔK to about 30% of control.

slope of the line represents an efflux component that corresponds, in part, to an increase in a voltage-gated noninactivating Rb efflux or delayed rectifier-type K^+ conductance and, in part, to Rb efflux mediated by the voltage-insensitive ("resting") K^+ conductance (17). The increment in ordinate intercept reflects an efflux component that corresponds to a voltage-gated inactivating (A-type) K^+ conductance (5, 13). As will be further illustrated below, ΔK (i.e., the efflux into 100K medium minus the efflux into 5K in the absence of Ca^{2+}) at 5 sec consists of these three components (the two slope components and the increase in ordinate intercept), which are nearly equal in magnitude (17). The data in Fig. 2 were obtained in the absence of external Ca^{2+} and thus do not reflect ^{86}Rb efflux through Ca^{2+}-activated K^+ channels (13).

The venoms from *Aa, Bj, Lqh, Lqq*, and *Pi* all reduced the ordinate intercept of the Rb efflux into 100K but had little effect on the steady state rate of the efflux (slope) in K^+-rich

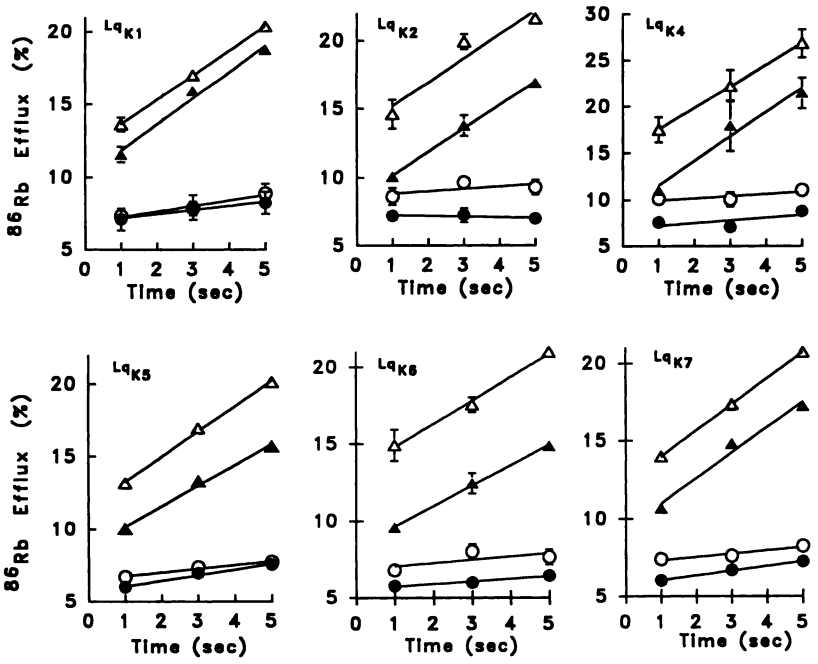

Fig. 5. Effects of six toxins purified by ion exchange HPLC from *Lqh* venom (Lq_{K1}, Lq_{K2}, and Lq_{K4} through Lq_{K7}) on the time course of ^{86}Rb efflux from rat brain synaptosomes in the absence of extracellular Ca^{2+}. *Open symbols*, data obtained in the absence of toxin; *circles*, efflux in 5K; *triangles*, efflux in 100K. The estimated toxin concentrations in the preincubation media (*solid symbols*) were Lq_{K1}, 150 nM; Lq_{K2}, 100 nM; Lq_{K4}, 130 nM; Lq_{K5}, 90 nM; Lq_{K6}, 120 nM; and Lq_{K7}, 45 nM. Each *symbol* indicates the mean of five or six replicate determinations; *standard error bars* are shown where they extend beyond the *symbols*. The *lines* were fitted by the method of least squares.

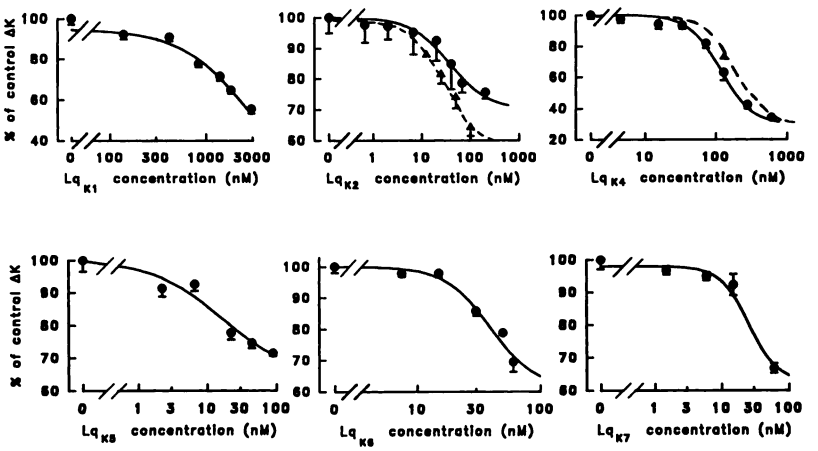

Fig. 6. Dose-response curves for block of the Ca^{2+}-independent, 100 mM K^+-stimulated ^{86}Rb efflux (ΔK) from rat brain synaptosomes by six toxins purified by ion exchange HPLC (*circles* and *solid lines*) from *Lqh* venom (Lq_{K1}, Lq_{K2}, and Lq_{K4} through Lq_{K7}). The *triangles* and *dashed lines* in the Lq_{K2} and Lq_{K4} panels indicate the data obtained with the material purified by reverse phase HPLC. ΔK was calculated as the Rb efflux into Ca^{2+}-free 100K minus the efflux into 5K, either in the absence (*0*) or in the presence of toxin. Each *panel* was obtained with a different synaptosome preparation. The *symbols* correspond to the differences between the means of five or six determinations in 5K and in 100K. Standard errors for the ΔK values were calculated as described (34); *standard error bars* are shown where they extend beyond the symbols. The toxin concentrations were estimated as described in Materials and Methods. The calculated Hill coefficients for the fitted solid curves were Lq_{K1}, 2.0; Lq_{K2}, 1.0 (1.7 after reverse phase HPLC); Lq_{K4}, 2.0; Lq_{K5}, 0.9; Lq_{K6}, 2.4; and Lq_{K7}, 2.0.

media or on the unstimulated efflux in 5K (Fig. 2A). This indicates that each of these venoms contains one or more components that selectively block the A-type K channel.

In contrast, venoms from both New World scorpions, *Cs* and *Ts*, reduced the steady state rate of the Rb efflux in 100K but had negligible effects on the ordinate intercept or on the efflux in 5K (Fig. 2B). Thus, these two venoms contain components that selectively block the delayed rectifier-type K channel. These results demonstrate that the synaptosome Rb efflux assay can be used to test the effects of an agent on both inactivating and noninactivating voltage-gated K^+ channels in a single experiment.

Assay for Ca^{2+}-activated K^+ channels. The addition of Ca^{2+} to the medium when synaptosomes are depolarized by 100K promotes a Ca^{2+}-dependent ^{86}Rb efflux (ΔC) through Ca^{2+}-activated K^+ channels (13). These channels are insensitive to 4-AP, an agent that blocks both the A-type and delayed rectifier K^+ channels (12). The 4-AP-insensitive ΔC component is blocked by tetraethylammonium ions (13) and by ChTX (3) but not by apamin[1]; thus, it has characteristics that are typical of a large conductance (maxi) Ca^{2+}-activated K^+ channel (1, 18).

Fig. 3 illustrates the effects of the seven scorpion venoms on this Ca^{2+}-dependent component of the Rb efflux in the presence of 10 mM 4-AP. Only the *Lqh* venom blocked a large fraction of ΔC. Thus, among the several venoms tested, only *Lqh* venom contains substantial concentrations of one or more components that inhibit the maxi Ca^{2+}-activated K^+ channel. As illustrated here, this flux assay can be used to test several samples for maxi Ca^{2+}-activated K^+ channel-blocking activity in a single experiment.

Effects of Purified Toxins from *Lqh* Venom on the Components of Rb Efflux in Synaptosomes

We previously showed that pure ChTX (see Fig. 1A) blocks both maxi Ca^{2+}-activated K^+ channels and Ca^{2+}-independent, voltage-gated, inactivating (A-type) K^+ channels in synaptosomes (3); the respective IC_{50} values were 15 and 40 nM. During purification of ChTX, we identified six other fractions from *Lqh* venom (Fig. 1A, peaks K_1, K_2, and K_4–K_7) that also exhibited K^+ channel-blocking activity; as illustrated in Fig. 4, these fractions all inhibited the 5-sec Ca^{2+}-independent, 100K-

[1]Bartschat and Blaustein, unpublished data.

TABLE 1

Scorpion K^+ channel toxin IC_{50} values

Toxin	Molecular weight[a]	Apparent IC_{50} for block of K^+ channels[b]		
		Ca^{2+}-activated	A-type	Delayed rectifier
			nM	
From *Lqh*				
ChTX	4300	15[c]	40[c]	≫1000[d]
$LqTX_{K1}$	(6000)	>440[d]	760	≫1850[d]
$LqTX_{K2}$	4300	>180	43 (30)[e]	≫300[d]
$LqTX_{K4}$	5100	60 (90)[e]	150 (160)[e]	≫400[d]
$LqTX_{K5}$	8900	>70[d]	18	≫90[d]
$LqTX_{K6}$	(6000)	>150[d]	40	≫150[d]
$LqTX_{K7}$	(6000)	>120[d]	40	≫120[d]
From *Ts*				
$TsTX_{K1}$	6900	ND[f]	≫1700[d]	600
$TsTX_{K2}$	8160	ND	≫200[d]	30 (120)[e]
$TsTX_{K3}$	(6000)	ND	≫1500[d]	1250
$TsTX_{K4}$	3900	ND	≫30[d]	8 (7)[e]
$TsTX_{K5}$	(6000)	ND	≫1500[d]	300
From *Cn*				
NTX	4200	>300	≫300	15

[a] Molecular weights in parentheses were assumed (see Materials and Methods).
[b] Data obtained from fractions separated by ion exchange HPLC separation, except for the values in parentheses (see footnote *e*) and those for ChTX and NTX, which were pure.
[c] Data from Ref. 3.
[d] No detectable block at the highest concentration tested.
[e] Data obtained from reverse phase purified materials.
[f] ND, not determined.

stimulated Rb efflux, ΔK. These K^+ channel blockers were all eluted from the ion exchange HPLC column at an NH_4Ac concentration greater than 0.35 M; no K^+ channel blockers were eluted at lower NH_4Ac concentrations, at which Na^+ channel toxins from *Lqh* venom elute.[2] In the experiment of Fig. 4, and all subsequent experiments illustrated with bar graphs or dose-response curves, toxins were added to appropriate 5K as well as 100K solutions. Thus, the ΔK (or ΔC) values in the presence of toxins are corrected for small toxin-mediated reductions of the Rb efflux in 5K media (e.g., see Fig. 4).

Fig. 5 shows the effects of the six fractions from *Lqh* venom (other than ChTX) (see Ref. 3) with K^+ channel-blocking activity on the time course of ^{86}Rb efflux from synaptosomes in the absence of external Ca^{2+}; each fraction was tested on a different synaptosome preparation. Fractions Lq_{K1}, Lq_{K2}, and Lq_{K4} through Lq_{K7} all selectively reduced the ordinate intercept of the Rb efflux line in 100K; they had no significant effect on the slope of the efflux line. This indicates that they all blocked the inactivating, but not the noninactivating, voltage-gated K^+ channel.

Fig. 6 (*circles* and *solid lines*) shows the dose-response curves for block of this channel by the six toxins. The most potent was Lq_{K5}, with an IC_{50} of about 18 nM; Lq_{K2}, Lq_{K6}, and Lq_{K7} were also quite potent, with IC_{50} values of about 40 nM. The IC_{50} data are summarized in Table 1. The curves in Fig. 6 were all fitted to the Hill equation by computer; as indicated in the figure legend, the Hill coefficients varied between 0.9 (Lq_{K5}) and 2.4 (Lq_{K6}).

Several of these toxins were further purified by reverse phase HPLC. As illustrated in Fig. 7, Lq_{K2} and Lq_{K4} were purified to near-homogeneity; this was confirmed by the SDS-PAGE analysis of the polypeptides, which shows that these toxins run as single bands (Fig. 8A). The block of the noninactivating voltage-gated K^+ channel by the reverse phase-purified toxins

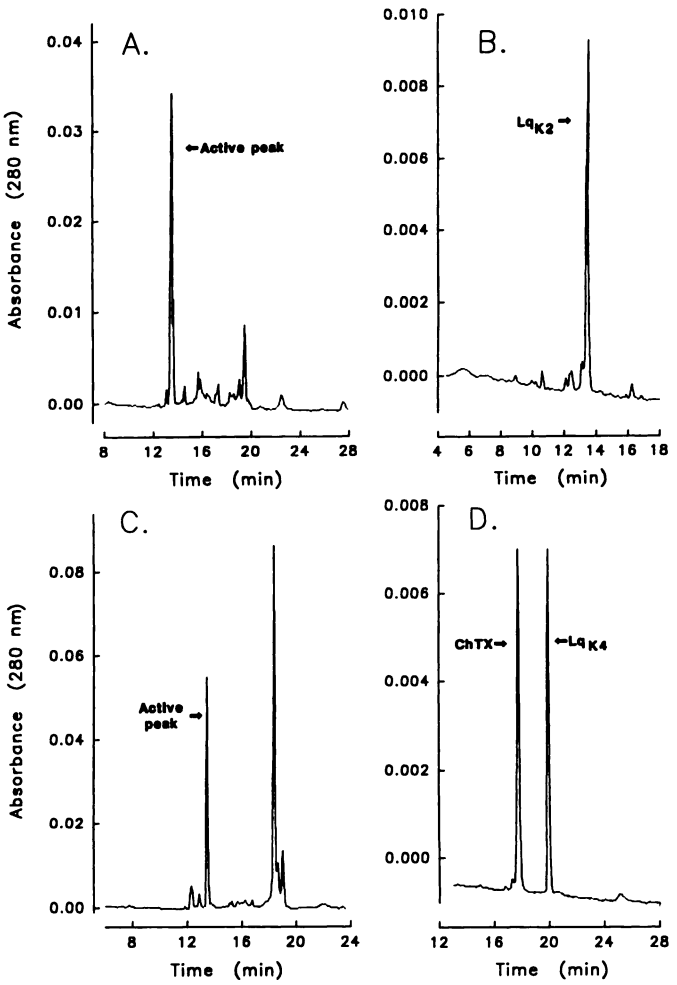

Fig. 7. Purification of Lq_{K2} and Lq_{K4} by reverse phase HPLC. A, Reverse phase HPLC separation of cation exchange fraction Lq_{K2}. The only peak with K^+ channel-inhibitory activity is marked with an *arrow*. B, Rechromatograph of the active peak from A by reverse phase HPLC. C, Reverse phase HPLC separation of cation exchange fraction Lq_{K4}. The only peak with K^+ channel-inhibitory activity is marked with an *arrow*. D, Rechromatograph of the active peak from C, along with ChTX, by reverse phase HPLC. Chromatographs A, B, and C were obtained with 30-min gradients; D was obtained with a 60-min gradient (see Materials and Methods). The purified materials (B and D) were used for SDS-PAGE analysis and tracer flux assays.

is indicated by the *triangles* and *dashed lines* in the respective curves in Fig. 6. Purified Lq_{K2} exhibited an apparent increase in potency for block of this K^+ channel, as anticipated if some of the inactive impurities were removed (Fig. 7A). However, the potency of Lq_{K4} block of this channel appeared to decrease slightly after purification by reverse phase HPLC, despite removal of a substantial amount of impurity (Fig. 7C). This may be explained by the fact that both the reverse phase packing materials and the acetonitrile-TFA eluants may unfold (denature) the proteins and cause protein loss during reverse phase HPLC (19, 20). Indeed, when Lq_{K5} was subjected to reverse phase HPLC, its potency declined precipitously and very little protein was recovered. The dominant polypeptide in the Lq_{K5} fraction from the ion exchange column had a molecular weight of about 8900, and no smaller polypeptides were observed on SDS-PAGE. Only minute amounts of Lq_{K6} and Lq_{K7} were obtained from the ion exchange column (Fig. 1A). Therefore, Lq_{K5}, Lq_{K6}, and Lq_{K7} were not studied further.

[2] B. K. Krueger, unpublished data.

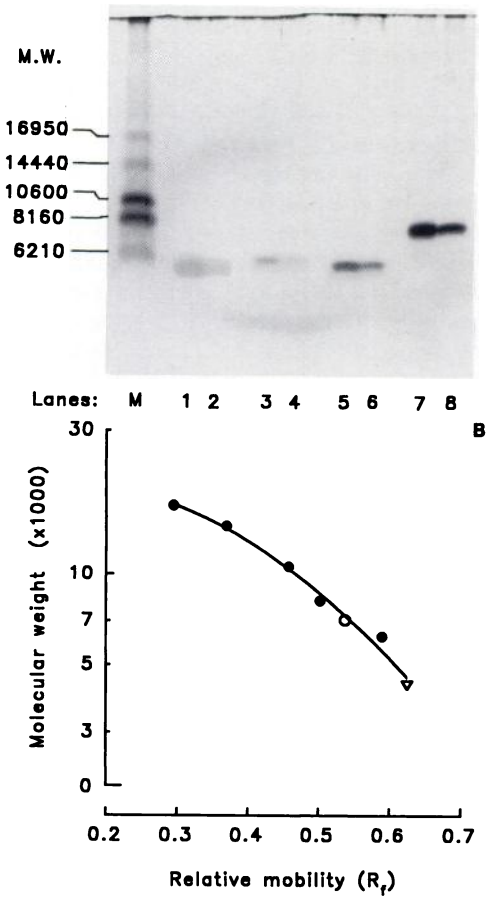

Fig. 8. A, SDS-PAGE separation of purified *Lqh* toxins. The polypeptides were separated using the discontinuous procedure of Schägger and von Jagow (16) and visualized with a silver stain. *Lanes 1* and *2*, 25 and 10 ng, respectively, of Lq_{K2} (purified by reverse phase HPLC), M_r 4300. *Lanes 3* and *4*, 25 and 10 ng, respectively, of Lq_{K4} (purified by reverse phase HPLC), M_r 5100. *Lanes 5* and *6*, 50 and 25 ng, respectively, of ChTX. *Lanes 7* and *8*, 50 and 25 ng, respectively, of α-DaTX. B, Molecular weight calibration curve. Standards and toxins were separated by SDS-PAGE with a tricine-SDS buffer (see Results). The mobilites of the polypeptides were plotted versus the logarithms of the molecular weights. The *solid line* was fitted to a third-order equation by the method of least squares (SigmaPlot). ○ and ▽, positions of α-DaTX and ChTX, respectively, on this curve.

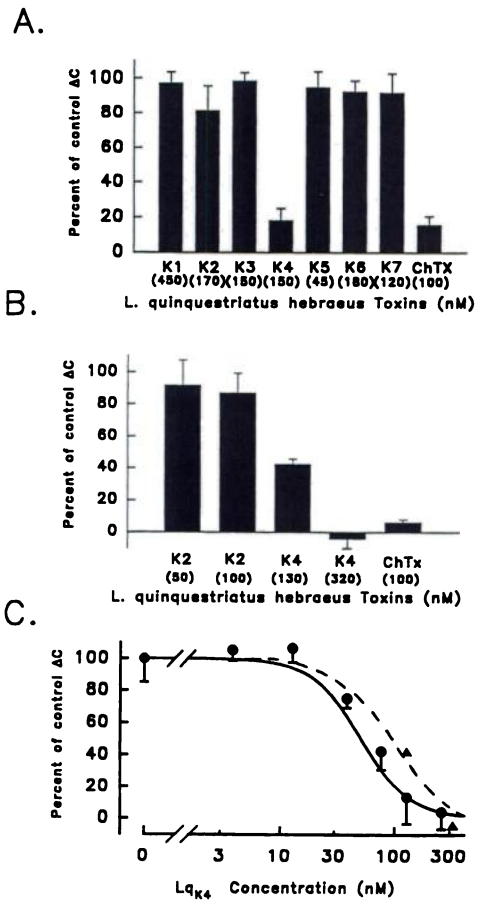

Fig. 9. Effects of seven K^+ channel toxins from *Lqh* venom (Lq_{K1} through Lq_{K7}) on the Ca^{2+}-dependent, 100 mM K^+-stimulated component of ^{86}Rb efflux (ΔC) from rat brain synaptosomes. In these experiments, all of the solutions contained 10 mM 4-AP to block the voltage-gated K^+ channels. Six replicate determinations were made for each condition. A, Screening of fractions from the ion exchange HPLC separation. Data are plotted as percentage of control ΔC; control ΔC was 4.3 ± 0.3%. Toxin concentrations (in nM) are indicated in *parentheses* below each *bar*. Only ChTX and Lq_{K4} significantly reduced ΔC ($p < 0.01$ in both cases). B, Screening of Lq_{K2} and Lq_{K4} purified by reverse phase HPLC. Data are plotted as in A. C, Dose-response curve illustrating the effect of Lq_{K4} on ΔC. The control ΔC was 3.6 ± 0.5%. The calculated Hill coefficient was 2.5. *Circles* and *solid line*, data obtained with material from the ion exchange HPLC separation; *Triangles* and *broken line*, obtained with Lq_{K4} purified by reverse phase HPLC (see B).

The *Lqh* K^+ channel toxins were also tested for their effects on the Ca^{2+}-dependent component of the synaptosome Rb efflux. Fig. 9, A and B, show that Lq_{K4}, like ChTX (3), also inhibited ΔC. In contrast, Lq_{K1}, Lq_{K2}, and Lq_{K5}–Lq_{K7} all had no effect on ΔC. The IC_{50} for block of ΔC by Lq_{K4} (after ion exchange separation) was about 60 nM (Fig. 9C), substantially lower than the IC_{50} for block of ΔK by this toxin (150 nM) (Fig. 6). The IC_{50} values after reverse phase HPLC purification were, respectively, about 115 and 190 nM; i.e., the potency of this toxin for block of both channels was decreased. The Hill coefficient for Lq_{K4} block of ΔC was 2.5. It is noteworthy that toxins Lq_{K2} and Lq_{K5} had little effect on ΔC (Fig. 9A), even though they were potent inhibitors of the noninactivating voltage-gated K^+ channel (Figs. 5 and 6; Table 1).

Effects of Purified Toxins from *Ts* Venom on the Components of Rb Efflux in Synaptosomes

In order to compare the actions of K^+ channel toxins from the venom of a New World scorpion with those from the venom of *Lqh*, the toxins from *Ts* venom were separated by ion exchange HPLC (Fig. 1B). Each of the fractions was screened for voltage-gated K^+ channel-blocking activity (Fig. 10). The fractions labeled Ts_{K1}, Ts_{K2}, Ts_{K3}, Ts_{K4}, and Ts_{K5} in Fig. 1B were the only ones that inhibited the 100 mM K^+-stimulated Rb efflux in the 5-sec incubation assay. Because this screening assay does not distinguish between block of the inactivating and noninactivating efflux components, the four most potent fractions (see below) were also tested in the time course assay (Fig. 11). All four fractions reduced the slope of the Rb efflux curve in K^+-rich media, without altering the ordinate intercept. This indicates that, in contrast to the results obtained with the *Leiurus* toxins, these four toxins all selectively block the noninactivating (delayed rectifier) voltage-gated K^+ channel.

The dose-response curves for all five *Tityus* toxins on the 100 mM K^+-stimulated ^{86}Rb efflux are shown in Fig. 12A, and the results are summarized in Table 1. Two of the toxins, Ts_{K2}

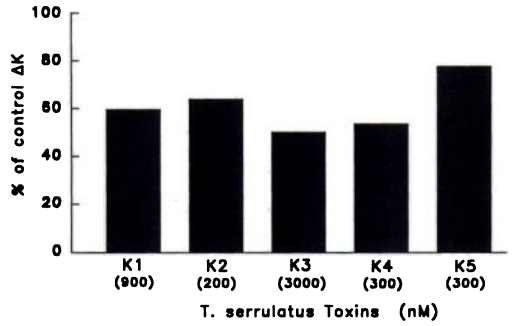

Fig. 10. Effects of various fractions from *Ts* venom (see Fig. 1B) on the Ca^{2+}-independent, 100 mM K^+-stimulated ^{86}Rb 5-sec efflux (ΔK) from rat brain synaptosomes. Six replicate determinations were made for each condition. The *bars* indicate the percentage of control ΔK observed in the presence of each of the fractions at the estimated concentrations indicated below the respective bars (concentrations were calculated as described in Materials and Methods). Control ΔK was 13.5 ± 0.3%. All of these fractions significantly inhibited ΔK ($p < 0.01$ in all cases).

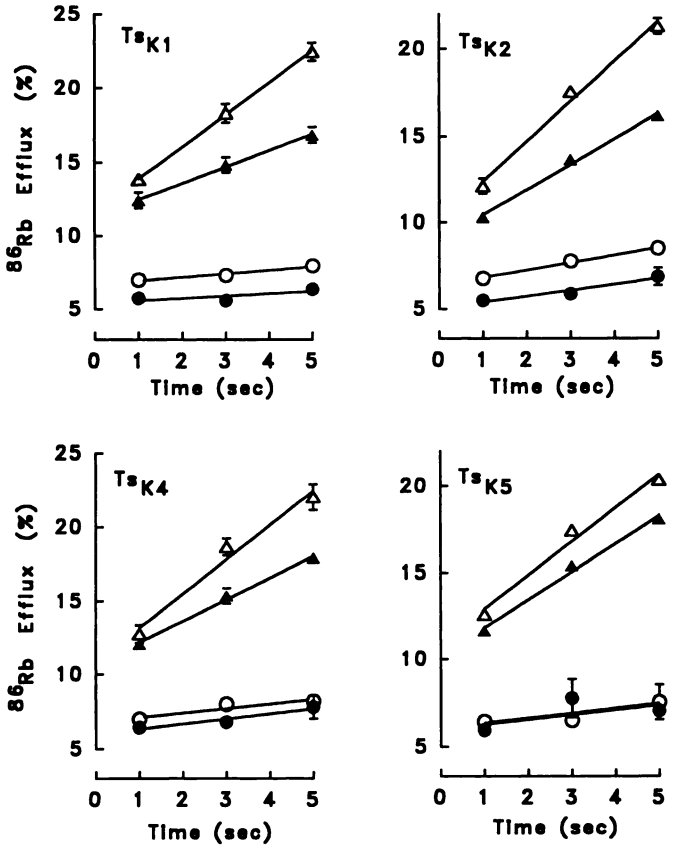

Fig. 11. Effects of four toxins purified from *Ts* venom (Ts_{K1}, Ts_{K2}, Ts_{K4}, and Ts_{K5}) on the time course of ^{86}Rb efflux from rat brain synaptosomes in the absence of extracellular Ca^{2+}. *Open symbols*, data obtained in the absence of toxin; *circles*, efflux in 5K; *triangles*, efflux in 100K. The estimated toxin concentrations in the preincubation media (*solid symbols*) were Ts_{K1}, 900 nM; Ts_{K2}, 200 nM; Ts_{K4}, 30 nM; and Ts_{K5}, 300 nM. Each *symbol* indicates the mean of five or six replicate determinations; *standard error bars* are shown where they extend beyond the *symbols*. The *lines* were fitted by the method of least squares.

and Ts_{K4}, appeared to be particularly potent; they inhibited the 100 mM K^+-stimulated ^{86}Rb efflux with IC_{50} values of about 30 nM and 8 nM, respectively (Fig. 12B). These two were purified to near-homogeneity by reverse phase HPLC (Fig. 13). The apparent decrease in potency of Ts_{K2} after reverse phase HPLC purification may be attributable to protein unfolding (20). The block of the delayed rectifier channel by these toxins fit the Hill equation with Hill coefficients of about 1.3–1.9 (see Fig. 12 legend).

Unfractionated *Ts* venom had little effect on the Ca^{2+}-activated Rb efflux (Fig. 3). Therefore, the purified polypeptides from this venom were not tested individually for their actions on this component of Rb efflux (ΔC).

NTX, from the New World scorpion *Cn*, has been reported to block delayed rectifier K^+ channels in squid axons with an IC_{50} of about 300 nM (21) and to augment transmitter release from rat brain synaptosomes with an ED_{50} of about 2 nM (22). When tested in our ^{86}Rb efflux assay, NTX preferentially inhibited the noninactivating component of ΔK (Fig. 14A). The IC_{50} for this effect was about 15 nM (Fig. 14B); thus, it is slightly less potent than Ts_{K4}. NTX is also reported to inhibit the Ca^{2+}-activated K^+ conductance in skeletal muscle, but with very low affinity (apparent K_d = 450 nM) (6); likewise, in synaptosomes, 300 nM NTX had only a slight inhibitory effect on ΔC (Fig. 14C).

Additive Effects of Various Toxins on the Inactivating and Noninactivating Components of ΔK

The additive effects of pairs of K^+ channel toxins were tested in order to provide further evidence that they block different K^+ channels. In these experiments, maximally effective concentrations of the toxins were tested, both alone and in pairs, on the 5-sec K^+-stimulated Rb efflux (ΔK). Fig. 15A shows that two toxins that both block the inactivating voltage-gated K^+ channel in synaptosomes, ChTX (3) and α-DaTX from the venom of the green mamba (14), each inhibited ΔK by about 35%. However, as expected for two toxins that block the same K^+ channel, their effects were not additive (Fig. 15A, *right bar*). Likewise, the effects of Ts_{K2} and NTX, which both block the noninactivating voltage-gated K^+ channel, were not additive (Fig. 15B).

In contrast, the inhibitory effects of Ts_{K2} and three blockers of the inactivating voltage-gated K^+ channel, ChTX, α-DaTX, and Lq_{K4}, were additive when maximally effective toxin concentrations were used (Fig. 15, C, D, and E, respectively).

Discussion

K^+ channel toxins from New and Old World scorpions. Previous studies have demonstrated that synaptosomes are a convenient preparation in which to explore some of the properties of ion channels and are especially useful for studies of K^+ channel pharmacology. The present report shows how synaptosomes can be used to screen a number of samples rapidly for their ability to block three different types of K^+ channels. The results of these preliminary experiments (Figs. 2 and 3) demonstrate that venoms from two New World scorpions, *Ts* and *Cs*, contain one or more components that selectively block delayed rectifier K^+ channels (Fig. 2B). In contrast, venoms from five Old World scorpions, *Lqh*, *Lqq*, *Aa*, *Bj*, and *Pi*, contain one or more components that selectively block the A-type K channel (Fig. 2A). In addition, only *Lqh* venom was found to contain substantial amounts of one or more components that block the maxi Ca^{2+}-activated K^+ channel (Fig. 3). It seems noteworthy that, even though all of the venoms contain components that block K^+ channels, there is negligible overlap in the specificities of the Old and New World scorpion venoms. This suggests that evolutionary pressures subsequent to the

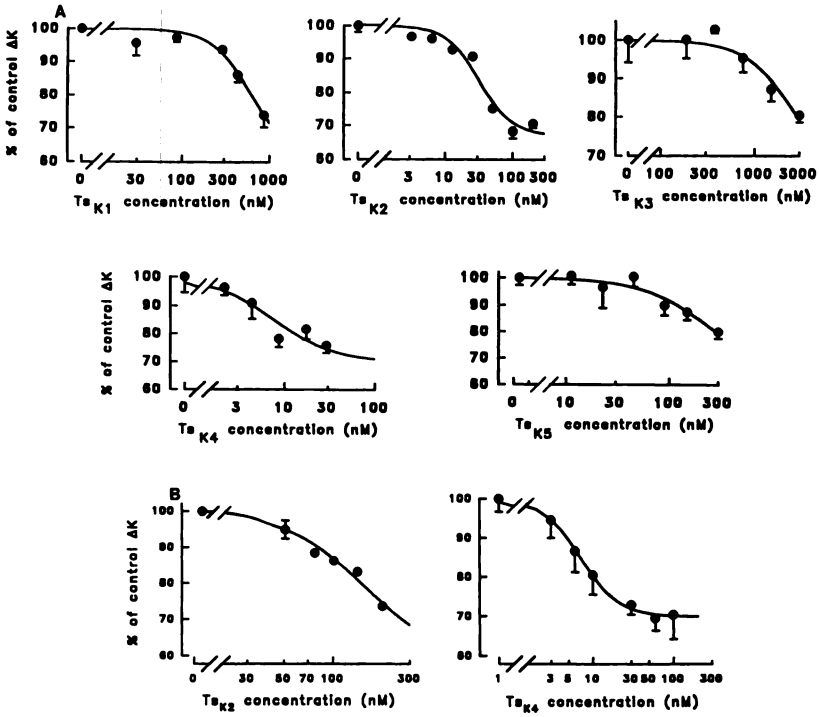

Fig. 12. Dose-response curves for block of the Ca^{2+}-independent, 100 mM K^+-stimulated ^{86}Rb efflux (ΔK) from rat brain synaptosomes by five polypeptide toxins from *Ts* venom (Ts_{K1}, Ts_{K2}, Ts_{K3}, Ts_{K4}, and Ts_{K5}). A, Data obtained with fractions separated by ion exchange HPLC. B, Data obtained with Ts_{K2} and Ts_{K4} purified by reverse phase HPLC. ΔK was calculated as the Rb efflux into Ca^{2+}-free 100K minus the efflux into 5K, either in the absence (*0*) or in the presence of toxin. Each *panel* was obtained with a different synaptosome preparation. The *symbols* correspond to the differences between the means of five or six replicate determinations in 5K and in 100K. *Standard error bars* are shown where they extend beyond the *symbols*; standard errors for the ΔK values were calculated as described (34). The toxin concentrations were estimated as described in Materials and Methods. The calculated Hill coefficients were Ts_{K1}, 1.9; Ts_{K2}, 1.8 (1.7 after reverse phase HPLC); Ts_{K3}, 1.6; Ts_{K4}, 1.3 (1.8 after reverse phase HPLC); and Ts_{K5}, 1.6.

geographical isolation of these related genera may have caused the divergence in specificity.

These assay methods were extended to the examination of purified fractions from the venoms of *Lqh* and *Ts*, which revealed a number of minor venom components with potent K^+ channel-blocking activity. Seven fractions from *Lqh* venom were found to have K^+ channel-blocking activity. Only two of these fractions, ChTX (1, 3) and Lq_{K4}, blocked the 4-AP-insensitive (maxi) Ca^{2+}-activated K^+ channel in synaptosomes. Both ChTX and Lq_{K4} also blocked the A-type K^+ channel, but with slightly higher IC_{50} values than the IC_{50} values for block of the Ca^{2+}-activated K^+ channel. Lq_{K4} may be identical to *Lqh* toxin ChTX-Lq2 described by Lucchesi *et al.* (24).

Five other fractions from *Lqh* venom were examined in detail. All five selectively blocked the A-type K^+ channel but had neglible effects on either the Ca^{2+}-activated or the delayed rectifier K^+ channels. Whether one of these five is the blocker of apamin-sensitive (small conductance) Ca^{2+}-activated K^+ channels ("leiurotoxin I") identified in *Lqh* venom (25–27) remains to be determined. This blocker of the apamin-sensitive K^+ channel has some sequence homology with ChTX, ChTX-Lq2, and NTX (27).

Five fractions with K^+ channel-blocking activity were isolated from *Ts* venom. Four of these fractions were studied in detail and were found to block the delayed rectifier K^+ channel selectively. In contrast to the effects of the toxins from *Lqh* venom, these *Ts* toxins had no effect on the A-type K^+ channel, as expected from preliminary screening of the venom (Figs. 2 and 3). The observation that maximally effective concentrations of the toxins from these two scorpions have additive effects on the K^+-stimulated Rb efflux (Fig. 15) provides further evidence that they block different types of voltage-gated K^+ channels.

The IC_{50} values obtained in this study probably underestimate the true K_I values. The Rb fluxes were not measured during steady state toxin binding conditions because only very small amounts of the toxins were available. The synaptosomes were, therefore, equilibrated with the toxins in a small volume, and the toxins were then washed out for 12–15 sec (at the time extracellular ^{86}Rb was washed away; see Materials and Methods) before the 5-sec timed efflux was started; neither the wash solution nor the efflux solution contained toxin. Some toxin molecules may have dissociated from the channels during this washout and efflux period; for example, preliminary data on the dissociation rate of toxin Lq_{K7} suggest that the IC_{50} for this toxin may have been overestimated by a factor of about 2. Furthermore, impure preparations from the ion exchange HPLC separation and partial denaturation during reverse phase HPLC (see Results) also contributed to underestimation of at least some of the IC_{50} values.

Electrophysiological correlates of the synaptosome Rb efflux assay. A key concern in interpreting the data reported here is the relationship between the components of the Rb efflux and the various types of K^+ channels that have been identified in electrophysiological experiments. Unfortunately, few of the components of *Lqh* and *Ts* venoms have yet been tested on K^+ channels in electrophysiological experiments. Where such data are available, however, the results fit with our interpretation of the correspondence between specific types of K^+ channels (A-type, delayed rectifier, and maxi Ca^{2+}-activated K^+ channels) and three components of the Rb efflux (K^+-stimulated increases in the ordinate intercept and in the slope of the time-course curve and the Ca^{2+}-dependent component, respectively). For example, the fact that ChTX reduces the increment in ordinate intercept as well as the Ca^{2+}-dependent component of the Rb efflux (3) is consistent with its block of A-type K^+ channels (2, 28) as well as maxi Ca^{2+}-activated K^+ channels. α-DaTX, which also selectively blocks A-type K^+ channels (29), preferentially inhibits the K^+-stimulated increase in Rb efflux ordinate intercept (14). Moreover, unfractionated venom from *Pi* appears to act on the same K^+ channels (A-type) as α-DaTX (8). This is consistent with our observation

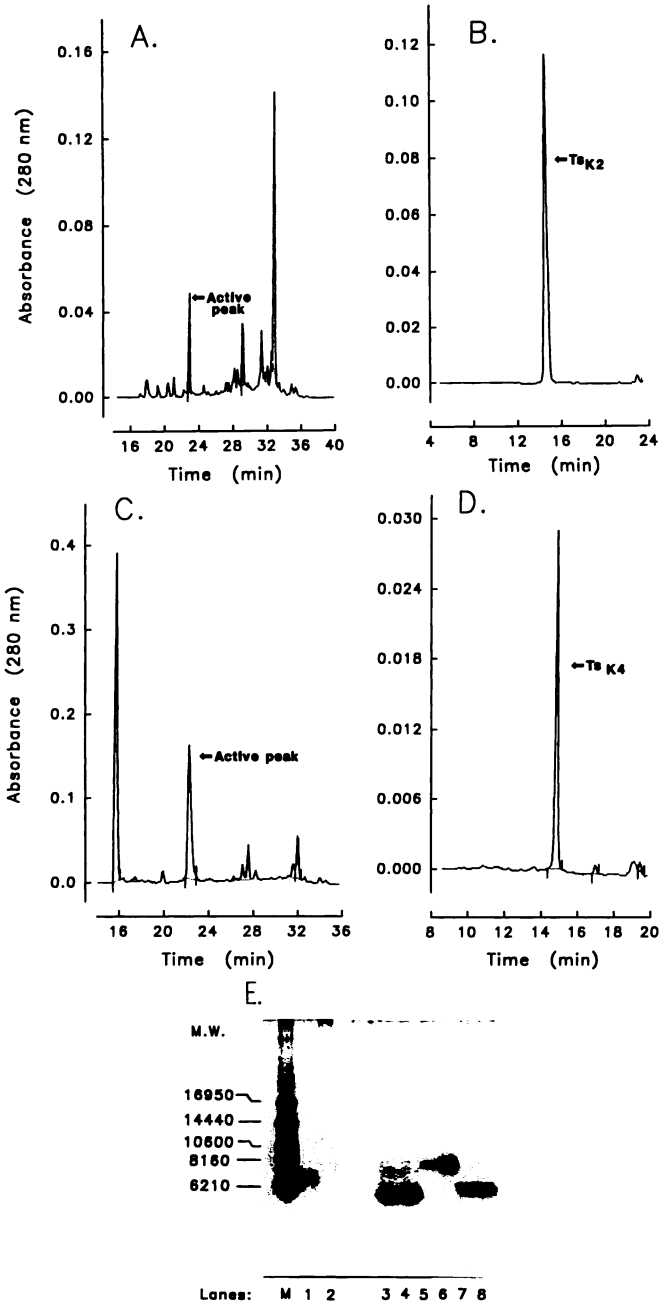

Fig. 13. Purification of Ts_{K2} (A and B) and Ts_{K4} (C and D) from *Ts* venom by reverse phase HPLC. A and C, reverse phase HPLC purification (60-min gradients; see Materials and Methods) of the two toxins from the respective ion exchange HPLC fractions; the active peaks are indicated by the *arrows*. B and D, rechromatographed pure toxins, Ts_{K2} and Ts_{K4}, respectively (30-min gradients). The latter were used for SDS-PAGE analysis and Rb flux assays. E, SDS-PAGE separation of purified *Ts* toxins (see Fig. 8 legend for molecular weight calculations). *Lane 1*, 10 ng of Ts_{K1} (purified by reverse phase HPLC), M_r 6900. *Lane 2*, 10 ng of Ts_{K2} (purified by reverse phase HPLC), M_r 8160. *Lanes 3* and *4*, 25 and 50 ng, respectively, of Ts_{K4} (purified by reverse phase HPLC), M_r 3900. *Lanes 5* and *6*, 10 and 25 ng, respectively, of α-DaTX; *lanes 7* and *8*, 25 and 50 ng, respectively, of ChTX.

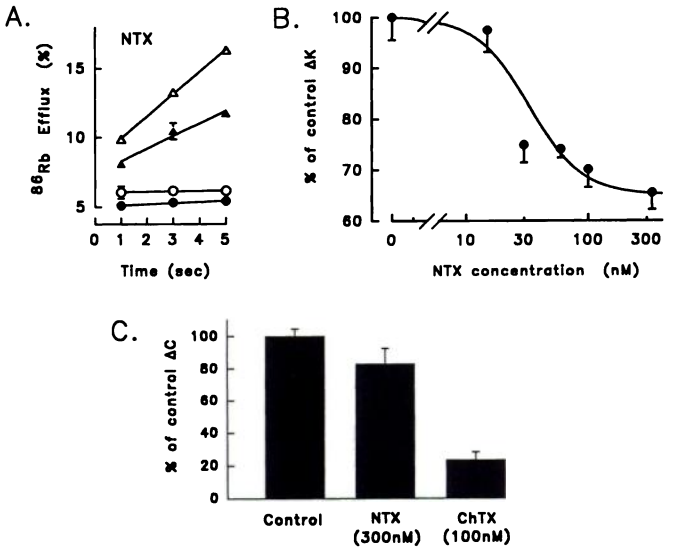

Fig. 14. Effects of NTX on the Ca^{2+}-independent K^+-stimulated Rb efflux (A and B) and the Ca^{2+}-dependent Rb efflux (C) in synaptosomes. A, Effect of 100 nM NTX on the time course of Rb efflux. Each *symbol* indicates the mean of five replicate determinations. *Open symbols*, data obtained in the absence of toxin; *closed symbols*, data obtained in the presence of toxin; *circles*, efflux in 5K; *triangles*, efflux in 100K. B, Dose-response curve illustrating the effect of NTX on ΔK. The *symbols* correspond to the differences between the means of six replicate determinations in 5K and in 100K. Control ΔK was 10.1 ± 0.5%. C, The effect of NTX on ΔC (see Fig. 9 legend for experimental details). Control ΔC was 7.2 ± 0.6%. *Standard error bars* are shown where they extend beyond the *symbols*; standard errors for the ΔK and ΔC values were calculated as described (34).

that this unfractionated venom selectively reduces the K^+-stimulated increase in Rb efflux ordinate intercept (Fig. 2A).

Polypeptides from the venoms of two New World scorpions, NTX from *Cn* and toxin II-9 from *Ts* (which have 18 amino acids of 31 in common), block the delayed rectifier K^+ channels in squid giant axons (4, 21, 30). There are no published reports about the effects of these toxins on A-type K^+ channels. Nevertheless, the fact that NTX and the unfractionated venoms from *Cs* and *Ts*, as well as the purified fractions from the latter venom, all selectively inhibited the depolarization-induced, noninactivating component of the synaptosome Rb efflux is consistent with the idea that the venoms of the New World scorpions, genera *Centruroides* and *Tityus*, contain selective blockers of the delayed rectifier. The weak inhibitory effect of NTX on the Ca^{2+}-activated K^+ channel in synaptosomes (Fig. 14C; Table 1) is consistent with the electrophysiological data from skeletal muscle (6).

Significance of the diversity of K^+ channel blockers. Polypeptide toxins with these selectivities should be useful for a variety of purposes. For example, they could be used as selective ligands to purify the various types of K^+ channel proteins by affinity chromatography. This approach may be an important complement to molecular biological methods that are based on homologies to the *Drosophila* Shaker (A-type) K channel (31). The purified toxins may also be useful for determining various aspects of channel structure at the toxin binding sites (e.g., see Refs. 32 and 33); presumably, these sites are located on the portions of the channels that are exposed to the extracellular fluid. The purified toxins may also prove useful for elucidating the specific roles of various types of K^+ channels in complex cellular activities, especially in cells whose activity depends upon more than one type of K^+ channel. As mentioned above, these toxins should be useful for determining toxin structure-activity relationships and for elucidating the mechanisms by which these molecules block the channels. The small sizes of the toxin molecules (30–37 amino acids) suggest that

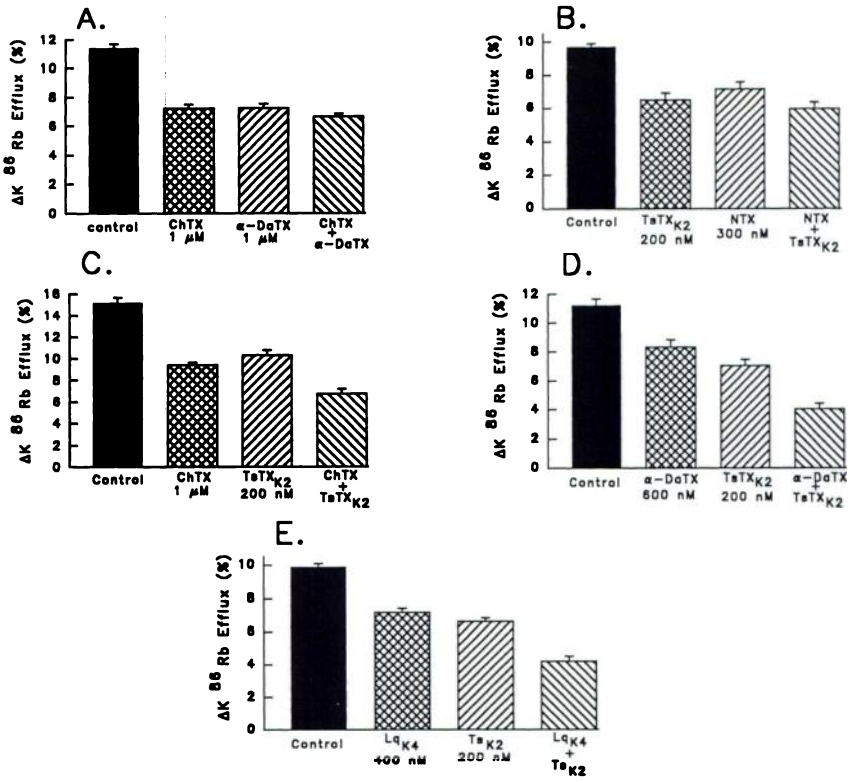

Fig. 15. Additive effects of various toxin pairs on the 5-sec K^+-stimulated ^{86}Rb efflux in synaptosomes. In each *panel*, the *left bar* corresponds to ΔK in the absence of toxins, the *two middle bars* show, respectively, the ΔK with each of the two toxins present alone (in both 5K and 100K media) at the indicated concentrations (estimated for Ts_{K2} and Lq_{K4}), and the *right bar* shows ΔK when both toxins are present together in the 5K and 100 K media. A, ChTX and α-DaTX. B, Ts_{K2} and NTX. C, Ts_{K2} and ChTX. D, Ts_{K2} and α-DaTX. E, Ts_{K2} and Lq_{K4}. The toxins were obtained from the ion exchange HPLC column step. In each experiment, six replicate determinations were made for each condition; the standard errors of the ΔK values were calculated as described (34).

they can be synthesized easily and that selectively modified molecules can also be made. The toxins may even be prototypes for endogenous ligands with similar activities.

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Send reprint requests to: Mordecai P. Blaustein, M.D., Department of Physiology, University of Maryland School of Medicine, 655 W. Baltimore Street, Baltimore, MD 21201.