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Four Polypeptide Components of Green Mamba Venom Selectively Block Certain Potassium Channels in Rat Brain Synaptosomes

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SUMMARY

Venom from the green mamba (*Dendroaspis angusticeps*) blocked ^{86}Rb efflux through voltage-gated K channels in rat brain synaptosomes. Crude venom inhibited both rapidly inactivating, 4-aminopyridine-sensitive K channels, and noninactivating, phencyclidine-sensitive, K channels. Fractionation of the venom by size exclusion chromatography and cation exchange high performance liquid chromatography yielded four 7000-dalton polypeptides (designated α-, β-, γ-, and δ-DaTX) that blocked synaptosome K channels. Two of these toxins, α- and δ-DaTX (10–100 nM), preferentially blocked the inactivating voltage-gated K channels. The other two toxins, β- and γ-DaTX, preferentially blocked the noninactivating voltage-gated K channels. The amino acid composition of these four toxins indicated that α-DaTX is identical to dendrotoxin [*Br. J. Pharmacol.* **77**:153–161 (1982)] and toxin $C_{13}S_2C_3$ [*Hoppe-Seyler's Z. Physiol. Chem.* **361**:661–674 (1980)]; the composition and partial sequence analysis indicate that δ-DaTX is identical to toxin $C_{13}S_1C_3$ [Hoppe-Seyler's Z. Physiol. Chem. **361**:661–674 (1980)]. β- and γ-DaTX have not previously been identified. Partial amino acid sequences of β- and γ-DaTX and the published sequences of α- and δ-DaTX reveal that the *C*-terminal segments of all four toxins are homologous. The *C*-terminal segments are also homologous with a number of nontoxic proteinase inhibitors. This raises the possibility that the *N*-terminal rather than the *C*-terminal regions are more likely responsible for the K channel blocking activity. The *N*-terminal portions of α- and δ-DaTX have some sequence homologies, but they have no obvious homologies with either β- or γ-DaTX. The finding of structurally similar peptide toxins with preferential activities toward different K channels may lead to the development of useful probes of K channel structure and may provide the means to distinguish among different K channels biochemically as well as physiologically.

Potassium channels appear to be the most diverse group of ion channels in biological systems (1). This group includes K channels modulated by membrane potential, by hormones and neurotransmitters, and by intracellular calcium ions and other "second messengers." They are known to be present in the plasma membranes of excitable tissues such as nerve and muscle, as well as in membranes of nonexcitable cells and intracellular organelles. It is not uncommon to find several different types of K channels in the same cell, although their spatial distributions within a given cell (e.g., between soma and processes) may vary greatly. Despite some significant differences in their properties, such as mode of activation and/or inactivation, many types of K channels share similar mechanisms of ion permeation and selectivity (2, 3).

In the nervous system, K channels play key roles in the control of resting membrane potential, action potential repolarization, firing frequency, and higher neuronal functions such as learning and memory (3, 4). The availability of selective blockers for these channels would aid greatly in the study of the role of K channels in cellular function. The blockers could be used to dissect complex physiological responses, involving many K channel types, so that the contributions of individual channel types could be identified. Highly specific probes might also prove useful for the identification and purification of the channel proteins.

Certain vertebrate polypeptide toxins ("dendrotoxins"), from the venom of elapid snakes of the genus *Dendroaspis*, facilitate synaptic transmission at the neuromuscular junction (5). This facilitation is probably the result of selective inhibition of a current carried by voltage-gated K channels in the motor

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ABBREVIATIONS: 4-AP, 4-aminopyridine; PCP, phencyclidine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DaTX, *Dendroaspis angusticeps* toxin; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

neurons. In mammalian central neurons, dendrotoxin selectively blocks an inactivating voltage-gated K current that apparently corresponds to the 4-AP-sensitive "A current" (6, 7). In mammalian peripheral neurons, however, dendrotoxin blocks slowly inactivating or noninactivating K currents [in autonomic ganglia (8, 9), sensory ganglia (10), and motor nerve terminals (11)]. A related toxin from the black mamba (toxin I or DpI) blocks a rapidly inactivating, voltage-gated, 4-AP-sensitive current in frog node of Ranvier (12). Dendrotoxin also blocks the noninactivating, delayed rectifier K current in axons from the marine annelid *Myxicola* (13).

We have purified a number of peptides from the venom of the Eastern green mamba, *Dendroaspis angusticeps* and have examined their effects on K channels in rat forebrain synaptosomes using ^{86}Rb efflux methods. These flux methods have previously been used to identify several different types of K channels in synaptosomes, including Ca-activated K channels and two types of voltage-gated Ca-independent channels (14, 15). In this report, we describe the structural and pharmacological characterization of four homologous *D. angusticeps* polypeptide toxins that block Ca-independent voltage-gated K channels in the synaptosomes. Two of the toxins preferentially block an inactivating channel, and the other two preferentially block a noninactivating channel. A preliminary report of some of these results has appeared in abstract form (16).

Methods

Synaptosome ^{86}Rb Efflux

^{86}Rb efflux from either crude (P_2) or purified synaptosomes was measured as described (14). Previous studies indicate that the ^{86}Rb efflux method is a reliable measure of K conductances in synaptosomes (14, 15); this is tested further in the present study.

Rat forebrain synaptosomes were prepared according to the procedure of Hajos (17), as modified by Krueger *et al.* (18). In some experiments the crude synaptosome preparation (the pellet, P_2, resulting from the second 10,000 × *g* centrifugation) was used without further purification. In other instances the synaptosomes were purified in a one-step sucrose density gradient and were recovered from the 0.8 M sucrose layer of the gradient (17, 18). Identical results were obtained with both preparations.

Synaptosomes (either P_2 or the purified preparation) were slowly equilibrated with a physiological salt solution (5K, containing, in mM: NaCl, 145; KCl, 5; RbCl, 0.1; $MgCl_2$, 2; glucose, 10; NaH_2PO_4, 0.5; and HEPES, 10, buffered to pH 7.4 with Tris). The synaptosomes (approximately 40 mg of protein/ml) were then incubated in 5K containing ^{86}Rb (10–20 μCi/ml) for 30 min at 30°. A 50-μl aliquot of tracer-loaded synaptosomes was pipetted onto a 13-mm diameter glass fiber filter (No. 25; Schleicher and Schuell, Keene, NH) and washed five times at 30° by vacuum filtration (wash solution was 5K with 0.1% bovine serum albumin) to remove extracellular tracer. The wash solution also contained toxin, where appropriate, in order to expose the synaptosomes to the toxin for 12 to 15 sec before initiating the efflux measurement. The synaptosomes were then exposed for 1–6 sec to one of the following efflux solutions at 30°, all with or without toxin: 5K, 50K or 100K, or 50K/Ca or 100K/Ca. The 50K and 100K efflux solutions were similar to 5K but contained 50 or 100 mM KCl and only 100 or 50 mM NaCl, respectively; the 50K/Ca and 100K/Ca efflux solutions also contained 1 mM $CaCl_2$ and only 1 mM $MgCl_2$.

Efflux incubations were terminated with a stop solution containing (mM): tetraethylammonium chloride, 145; tetrabutylammonium chloride, 5; RbCl, 0.1; $NiCl_2$, 10; $MgCl_2$, 10; and HEPES, 10, buffered to pH 7.4 with Tris. The synaptosomes were rapidly filtered, and the filtrate (efflux solution plus stop solution) was collected in a scintillation vial. The ^{86}Rb in the filtrate and on the filters (i.e., in the synaptosomes) were both determined by liquid scintillation counting. The percentage of total radioactivity content released during the incubation was then calculated:

$$^{86}\text{Rb efflux (\% of content)} = \frac{\text{(cpm in filtrate)}}{\text{(cpm in filtrate) + (cpm on filter)}} \times 100\%$$

In most experiments, the efflux for each condition was measured as a function of efflux time; each determination was made in quadruplicate. The time course line for each condition was then calculated by linear regression analysis.

A representative time course experiment, illustrated in Fig. 1A, shows the various components of the ^{86}Rb efflux measured in the absence of toxin (14). The slope of the *5K line* corresponds to the resting Rb efflux; the residual tracer in the 5K solution at zero time includes the efflux between the end of the wash period and the introduction of the efflux solution, as well as incompletely removed extracellular tracer that was washed off with the efflux and stop solutions.

Component T is the increment in the ordinate-intercept of the linear regression line produced by elevation of the extracellular K concentration (i.e., depolarization) in the absence of Ca. This transient component is most sensitive to 4-AP and probably represents Rb efflux through the voltage-gated inactivating (within 1 sec) K channel that is responsible for the A current (14).

Component S is the increase in the slope of the linear regression line that occurs when extracellular K is increased in the absence of Ca (i.e., the difference between the *dashed line* and the *50K* line in Fig. 1A). At the K concentrations used in these experiments, approximately one third to one half of this increment (labeled S_V) was sensitive to inhibition by low micromolar concentrations of PCP (19) and high concentrations (>1 mM) of 4-AP (14). Component S_V, the PCP-sensitive component, appears to correspond to Rb efflux through a voltage-gated noninactivating K channel (14, 19). The remainder of S (termed S_R) is attributable to increased Rb efflux through the "resting K conductance" as a result of the increase in electrical driving force during depolarization (14).

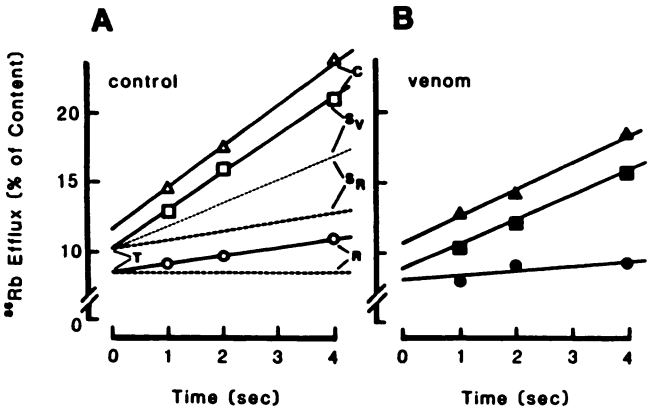

Fig. 1. A, The components of ^{86}Rb efflux from rat forebrain synaptosomes. ^{86}Rb efflux was measured in the presence of 5K (○), 50K (□), or 50K/Ca (△) as described in Methods. The data are from a single experiment in which each data point was measured in quadruplicate. The *lower thick broken line* is drawn parallel to the *abscissa*; the *upper thick broken line* is drawn parallel to the *5K line*, with the same ordinate intercept as the *50K line*. Components R, T, S_R, S_V, and C are indicated; S is the sum of S_R + S_V (the *thin broken line* is arbitrarily positioned so that S_V = S_R because S_V, the PCP-sensitive component, was not measured in this experiment; see Ref. 19). B, The effect of crude *D. angusticeps* venom on the components of ^{86}Rb efflux from rat forebrain synaptosomes. The efflux was measured in the 5K (●), 50K (■), and 50K/Ca (▲) solutions containing 0.35 mg/ml *D. angusticeps* venom. The venom had no effect on the Ca-stimulated efflux (component C; see A). Each *point* is the average from three experiments. The *lines* in both A and B were determined by the methods of least squares.

Component *C* is the Ca-dependent increase in Rb efflux that occurs when Ca is added to the K-rich solutions. This efflux component is blocked by tetraethylammonium ions and quinine sulfate (15) and by charybdotoxin (20). Thus, it corresponds to Rb efflux through a large conductance ("maxi") Ca-activated K channel (15, 21; see also Ref. 22).

Venom Fractionation

D. angusticeps venom was purchased from Sigma Chemical Co. (St. Louis, MO). The venom was fractionated by a modification of the method of Harvey and Karlsson (23). One gram of the venom was dissolved in 5 ml of 0.1 M ammonium acetate buffer (pH 6.8), filtered through a 0.45-μm pore filter (Millipore, Bedford, MA), and loaded onto a Sephadex G-50 (fine) column (92 × 2.5 cm) equilibrated with the same buffer. The material was eluted with the ammonium acetate buffer at a flow rate of about 0.5 ml/min. Absorbance of the eluate was monitored at 280 nm. Five major peaks of UV-absorbing material was obtained (Fig. 2).

Only the third peak from the Sephadex column (Fig. 2, *Fraction III*) contained material that inhibited the ^{86}Rb efflux in synaptosomes (see Results). Fraction III was lyophilized and redissolved in 0.1 M ammonium acetate, and aliquots containing 30–40 mg of protein were loaded onto a CX-300 HPLC cation exchange column (Brownlee Labs, Santa Clara, CA). The material retained on the column was eluted at a rate of 1 ml/min with a linear gradient of ammonium acetate (0.1 to 0.8 M; Fig. 3); elution was monitored at 280 nm. As illustrated in Fig. 3, more than 12 protein peaks were recovered; these fractions were collected and lyophilized.

Protein was determined by the bicinchoninic acid assay (24) (Pierce, Rockford, IL). Urea-SDS-PAGE was performed as described (25).

Amino Acid Analysis and Peptide Sequences

Amino acid analysis. Peptides were hydrolyzed for 24 and 48 hr in 6 N HCl at 110° *in vacuo*. The composition of each sample was determined on a Durrum D-500 amino acid analyzer (Dionex, Sunnyvale, CA). Corrections for loss of threonine and serine were made by linear extrapolation to zero time. Cysteine was determined as cystine, after hydrolysis, and is thus subject to substantial error.

Primary sequence analysis. Twenty-one cycles of automated Edman sequencing were performed directly on δ-DaTX. β- and γ-DaTX were oxidized with performic acid according to the method of Hirs (26) and digested with 1% (w/w) diphenylcarbamyl chloride-treated trypsin (Sigma). Tryptic peptides were separated on a C_{18} reverse phase HPLC column (Alltech Associates, Deerfield, IL) using aqueous 0.1% trifluoroacetic acid in a gradient from 0 to 60% acetonitrile for elution. Partial sequencing was performed directly on the lyophilized HPLC fractions. In all cases, peptides were applied to a Biobrene Plus (Applied Biosystems, Inc., Foster City, CA)-treated filter on an Applied Biosystems gas phase sequencer (27). Initial recoveries were between 40 and 50% for each peptide, with repetitive yields averaging 94%. Phenylthiohydantoin amino acids were generated with trifluoroacetic acid on the sequencer and identified by the method of Hunkapiller and Hood (28).

Results

Effect of *D. angusticeps* venom on synaptosome K channels

Fig. 1B shows the effect of unfractionated *D. angusticeps* venom (0.35 mg/ml) on ^{86}Rb efflux from purified synaptosomes. The venom markedly reduced both the slope of the efflux into 50K solution (i.e., component *S*) and the K-stimulated increment in ^{86}Rb efflux at 0 time (i.e., component *T*); *D. angusticeps* venom had virtually no effect on the Ca-dependent ^{86}Rb efflux (i.e., component *C* in Fig. 1A). The unfractionated venom also reduced the slope of the efflux into 5K solution, suggesting that it may have inhibited the resting K conductance, but this was a small and inconsistent finding.

Chromatography of *D. angusticeps* Venom

The components of *D. angusticeps* venom responsible for inhibiting the ^{86}Rb efflux were purified by a two-step chromatographic procedure. Five venom fractions were obtained from the Sephadex G-50 column (labeled I–V in Fig. 2). Only fraction III exhibited any ^{86}Rb efflux inhibitory activity when the lyophilized and redissolved material was used at a concentration comparable to that employed for the unfractionated venom (~0.35 mg/ml). The peptides in fraction III had an average molecular weight of approximately 5000 (see Fig. 2, *inset*).

Fraction III was subjected to further purification on an HPLC cation exchange column. More than 12 UV-absorbing peaks were resolved (Fig. 3). These fractions were collected, lyophilized, dissolved in water, and tested for their ability to inhibit K-stimulated ^{86}Rb efflux during a 5-sec incubation. Only four fractions (peaks designated as α-, β-, γ-, and δ-DaTX in

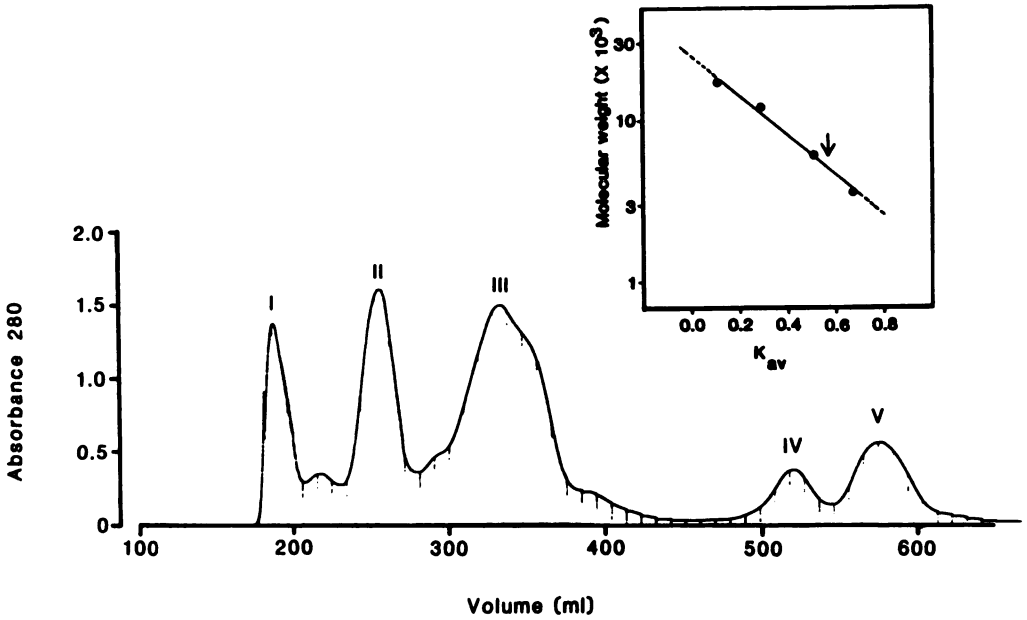

Fig. 2. Sephadex G-50 gel filtration chromatography of crude *D. angusticeps* venom. Protein fractions were eluted from a 2.5 × 92.5 cm gel column as described in Methods. Flow rate was 0.5 ml/min. *Inset*, calibration of the Sephadex G-50 column, using myoglobin, cytochrome *c*, aprotinin, and β-chain of insulin (*data points*, in order of decreasing molecular weights). K_{av} = (elution volume of the solute − void volume)/(total volume − void volume). The *arrow* indicates the position of peak III.

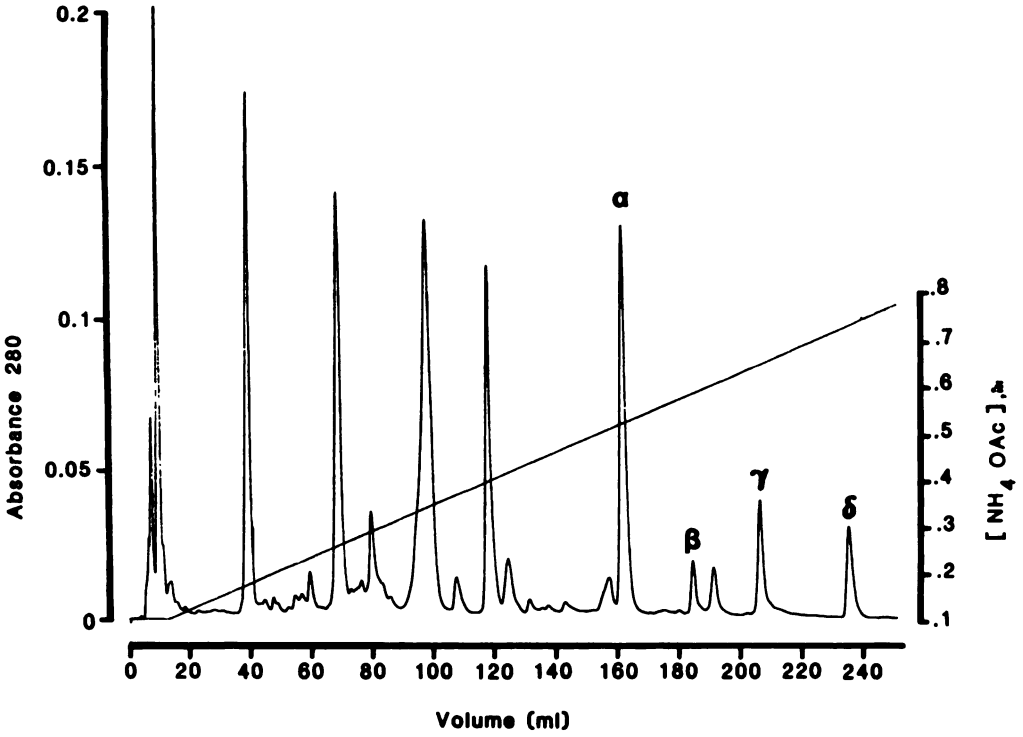

Fig. 3. HPLC cation exchange chromatography of fraction III. Adsorbed proteins were eluted with a linear gradient of ammonium acetate. Flow rate was 1 ml/min. The four peaks that were found to inhibit ^{86}Rb efflux from synaptosomes (see Results) are indicated by α, β, γ, and δ, in the order of their elution from the column.

Fig. 3) caused significant inhibition of the K-stimulated ^{86}Rb efflux.

Selectivity of Block of ^{86}Rb Efflux produced by α-, β-, γ-, and δ-DaTX

Each of the four active fractions from the cation exchange column was determined to be a single polypeptide by urea-SDS-PAGE. The effect of each polypeptide on the time course of ^{86}Rb efflux was determined; the results are illustrated in Figs. 4–7. P_2 synaptosomes, rather than purified synaptosomes, were used in most of these experiments in order to shorten the preparation time. The time course lines (e.g., compare Figs. 1 and 4) and the effects of the toxins were very similar when the two types of synaptosome preparations were directly compared (not shown).

α-DaTX. Fig. 4 shows that 100 nM α-DaTX had virtually no effect on the ^{86}Rb efflux into 5 mM K solution. However, it substantially reduced the efflux into 100 mM K solution at all the time points tested. This corresponds to a selective reduction in efflux component T, with no inhibition of component S, because the ordinate intercept was reduced while the slope of the line in the K-rich solution was unaffected. Based on the kinetic and pharmacologic properties of T, including its sensitivity to 4-AP (14), it appears that α-DaTX selectivity inhibits an inactivating (within 1 sec), voltage-gated K channel in synaptosomes. This channel may be similar to the inactivating K channel in hippocampal Ca_1 neurons that is also blocked by α-DaTX (7).

The observed specificity of α-DaTX cannot be explained by a faster block of component T than component S_V. Although the synaptosomes were exposed to the toxin for only 15 sec in these experiments, a maximal effect had probably been achieved because nearly identical results were obtained with a much longer incubation (16 min) with α-DaTX.[2]

[2] M. J. Scheider, personal communication.

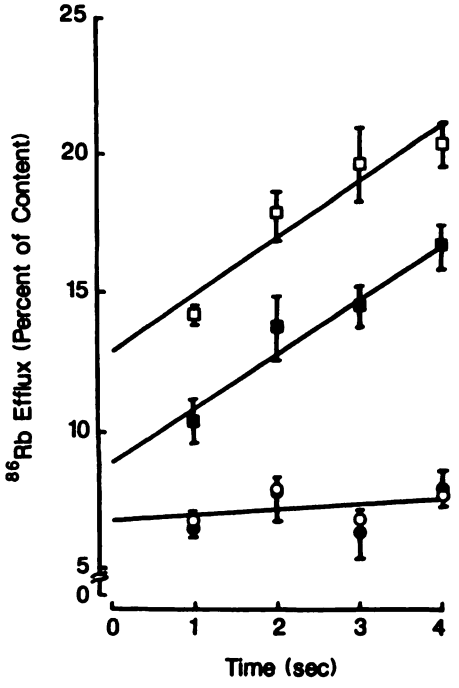

Fig. 4. Effect of 100 nM α-DaTX on the efflux of ^{86}Rb from rat forebrain P_2 fraction. The efflux into 5K (*circles*) and 100K (*squares*) solutions was measured in the absence (*open symbols*) and presence (*closed symbols*) of the toxin. Each *point* is the mean (± standard error) of four determinations; the *lines* were determined by the method of least squares.

Lower concentrations of toxin were also tested. With incubation for only 15 sec, 10 nM α-DaTX inhibited component T by 42 ± 7% (n = 4). The IC_{50} (concentration producing 50% inhibition of T) was about 15–20 nM under these conditions. This is considerably higher than the concentration of α-DaTX required for half-maximal saturation of the synaptic membrane binding sites, about 1 nM in equilibrium binding studies (29,

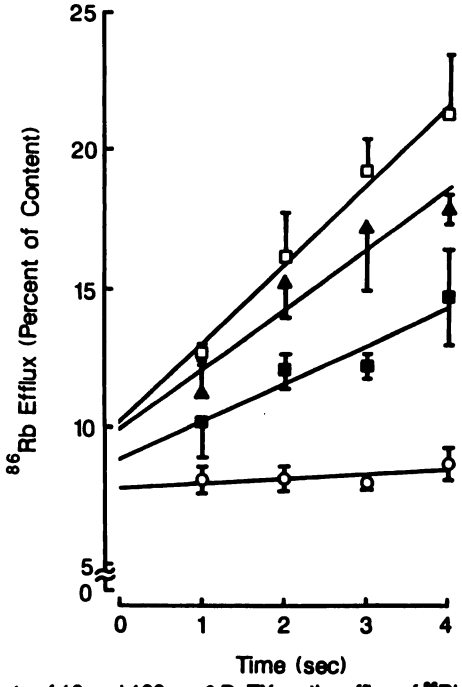

Fig. 5. Effects of 10 and 100 nM β-DaTX on the efflux of ^{86}Rb efflux from rat forebrain P_2 fraction. Efflux into 5K (*circles*) and 50K (*squares* and *triangles*) solutions was measured in the absence (*open symbols*) and presence (*solid symbols*; *triangles*, 10 nM; *squares*, 100 nM) of the toxin. Each *symbol* represents the mean ± standard error of four determinations.

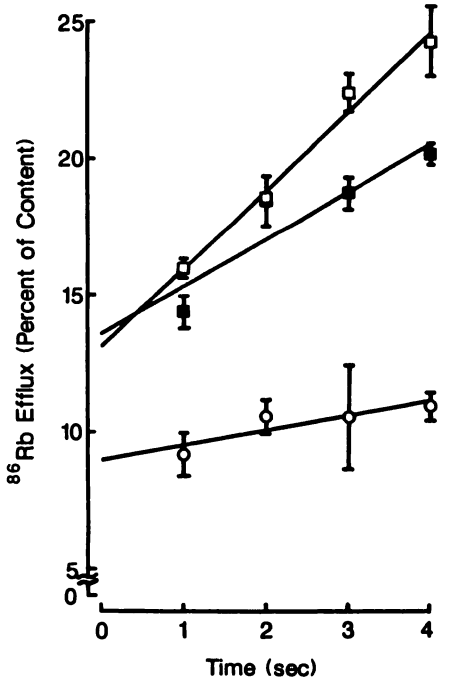

Fig. 6. Effect of 100 nM γ-DaTX on the efflux of ^{86}Rb from rate forebrain P_2 fraction. See legend to Fig. 4 for explanation of symbols.

30). The high IC_{50} may, however, reflect the very short exposure to the toxin in the flux experiments.

β-DaTX. The effects of β-DaTX were quite different: as shown in Fig. 5, 10 nM β-DaTX reduced the ^{86}Rb efflux (slope) into 50 mM K solution (i.e., it partially blocked component S) but had virtually no effect on the ordinate intercept (i.e., component T). However, a 10-fold higher concentration of this

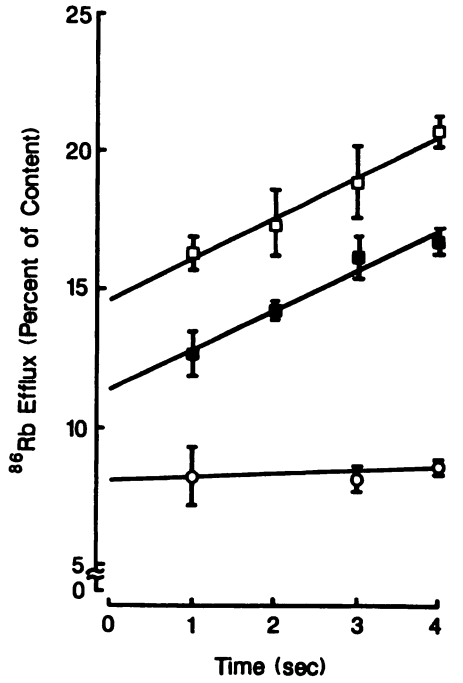

Fig. 7. Effect of 100 nM δ-DaTX on the efflux of ^{86}Rb from rat forebrain P_2 fraction. See legend to Fig. 4 for explanation of symbols.

toxin did reduce the ordinate intercept by about 50% but had little further effect on the slope of the efflux curve in K-rich media. These effects are identical to those observed with PCP, which preferentially blocks component S_V in ^{86}Rb efflux experiments (19) and the noninactivating K current in electrophysiological experiments (31). This partial inhibition of S probably represents complete block of the PCP-sensitive, noninactivating, voltage-gated K channel, component S_V (19). It is component S_V that appeared to be sensitive to β-DaTX, rather than S_R (the K-stimulated ^{86}Rb efflux through the resting K conductance; see Methods) because the efflux into 5 mM K media was not affected (not shown). β-DaTX also had no effect on the Ca-dependent ^{86}Rb efflux in K-rich solution (data not shown).

γ- and δ-DaTX. Fig. 6 shows that 100 nM γ-DaTX inhibited only ^{86}Rb efflux component S and thus had an action very similar to that of β-DaTX. In preliminary experiments (not shown), γ-DaTX blocked the same fraction of S as did PCP, namely S_V; in the presence of PCP, γ-DaTX had no further effect on S. On the other hand, δ-DaTX behaved very much like α-DaTX; 100 nM δ-DaTX inhibited only ^{86}Rb efflux component T (Fig. 7).

Amino Acid Analysis and Peptide Sequences

Each of the four peptide toxins, α-, β-, γ-, and δ-DaTX had a unique amino acid composition (Table 1). Three of the four active peptides (α-, β-, and γ-DaTX) have blocked *N*-terminals, presumably due to pyroglutamate, by analogy with several other known *Dendroaspis* toxin sequences (e.g., Ref. 32).

The amino acid composition of α-DaTX (Table 1) is identical to the published composition of dendrotoxin (from *D. angusticeps*), which has been sequenced by Joubert and Taljaard (called toxin $C_{13}S_2C_3$; Ref. 32) and by Harvey and Karlsson (5). Based on the HPLC elution pattern and the amino acid composition, we assume that our α-DaTX is the same as dendrotoxin (5) and $C_{13}S_2C_3$ (32). Our nomenclature may be helpful

in distinguishing this toxin from other dendrotoxins that have been purified from various species of mamba (cf. Ref. 33).

The composition (Table 1) and the sequence of the first 21 amino acids of our fourth peptide, δ-DaTX, are identical to those of *D. angusticeps* toxin $C_{13}S_1C_3$ of Joubert and Taljaard (32). Therefore, we assume that our δ-DaTX corresponds to $C_{13}S_1C_3$.

Alignment of the partial sequences of β- and γ-DaTX with the other toxins (see Fig. 8) indicates that the carboxyl terminal half of each of the peptides is highly conserved. However, considerable variability is observed in the sequences of the first 30 amino acids of all four *D. angusticeps* peptides.

TABLE 1

Amino acid composition of four polypeptide toxins purified from *D. angusticeps* venom

Amino acid[a]	α-DaTX[b]	$C_{13}S_2C_3$[c]	δ-DaTX[b]	$C_{13}S_1C_3$[c]	β-DaTX[b]	γ-DaTX[b]
AlA (A)	1.2	1	4.0	4	1.8	2.6
ArG (R)	8.4	8	4.6	4	5.0	4.2
AsX[d] (D + N)	5.5	6	3.2	3	3.8	3.0
CyS (C)	5.8	6	5.3	6	5.9	4.9
GlX[e] (E + Q)	6.0	6	3.3	3	4.5	5.2
GlY (G)	5.1	5	5.2	5	4.9	6.1
HiS (H)	1.1	1	0.3	0	0.9	0.5
IlE (I)	3.9	4	2.1	2	3.7	2.4
LeU (L)	2.2	2	2.2	2	4.4	3.1
LyS (K)	6.0	6	8.5	9	6.7	8.1
MeT (M)	0	0	0	0	0.0	0.2
PhE (F)	3.0	3	3.0	3	3.1	5.3
PrO (P)	3.4	3	4.0	4	4.1	4.0
SeR (S)	2.4	2	2.1	2	4.4	4.2
ThR (T)	2.2	2	2.1	2	3.7	2.5
TrP (W)	ND[f]	1	ND[f]	1	ND[f]	ND[f]
TyR (Y)	2.7	3	4.2	5	1.7	1.9
VaL (V)	0	0	1.8	2	0.4	1.0

[a] Standard one-letter notation from Ref. 39.
[b] Average of 24 and 48 hr hydrolysate analysis. THR and SER values are derived from linear extrapolation to zero time of hydrolysis. Compositions based on recovery of 59 amino acids for α-, γ-, and δ-DaTX and 57 amino acids for β-DaTX.
[c] From Ref. 32.
[d] Composition analysis for AsX represents the sum of AsP + AsN.
[e] Composition analysis for GlX represents the sum of GlU + GlN + pyroGlU.
[f] Not determined because these residues were destroyed by acid hydrolysis.

Discussion

Our preliminary experiments revealed that crude *D. angusticeps* venom blocked not only the inactivating, voltage-gated, 4-AP-sensitive K channel (component T), as expected for dendrotoxin (6, 7) but also the noninactivating, voltage-gated K channel (component S_v; see Fig. 1 and Refs. 14 and 19). Using a modification of the method of Harvey and Karlsson (23), in which their cation exchange fractionation was replaced by an HPLC cation exchange step, we identified four polypeptide components of the venom that exhibit K channel blocking activity: α- and δ-DaTX, which preferentially blocked component T, and β- and γ-DaTX, which preferentially blocked component S_v (Figs. 4–7). The amino acid compositions of all four toxins were determined (Table 1). We verified the structures of the two previously known toxins and obtained partial sequences for the two newly identified toxins (Fig. 8).

α- and δ-DaTX: Structure and Function

Five presynaptically acting toxins that have previously been isolated from *Dendroaspis* venoms, $C_{13}S_2C_3$ (dendrotoxin or α-DaTX) and $C_{13}S_1C_3$ (δ-DaTX) from *D. angusticeps*, DpI and DpK from *Dendroaspis polylepsis polylepsis*, and DV14 (identical to $C_{13}S_1C_3$) from *Dendroaspis viridis*, have been sequenced (Refs. 5, 23, and 32–34; see Fig. 8). All of these toxins facilitate neurotransmitter release and thereby enhance the indirectly elicited muscle twitch without altering direct muscle responses to agents such as acetylcholine, carbamylcholine, or KCl in chick neuromuscular preparations (e.g., Ref. 5). One of these toxins, α-DaTX (7, 8), has been tested on mammalian hippocampal neurons in voltage-clamp experiments and has been shown to block a 4-AP-sensitive, inactivating, voltage-gated K current. We found that both α- and δ-DaTX blocked the inactivating 4-AP-sensitive component of the ^{86}Rb efflux in synaptosomes. Block of a K current should enhance Ca entry and transmitter release (6, 23) by inhibiting repolarization and prolonging the action potential at nerve endings.

It should be useful to try to relate these selective actions to toxin structure. Two of the toxins, α-DaTX and DpI, have a striking sequence homology, with differences in only six amino acids (Refs. 5 and 33; and see Fig. 8). Also, δ-DaTX is nearly

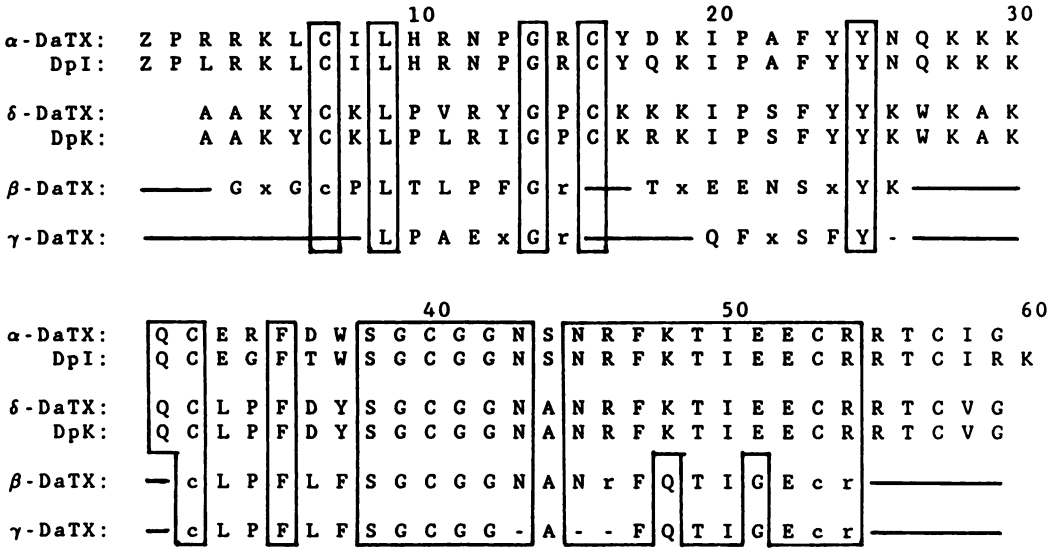

Fig. 8. Amino acid sequences of α-DaTX (Refs. 5 and 32) and δ-DaTX (Ref. 32 and this report) and partial sequences of β-DaTX and γ-DaTX (this report). The sequences for DpI and DpK, from *D. polylepsis polylepis* (33) are also shown. Standard one-letter notation is used for the amino acids (39); *lower case letters* indicate some degree of uncertainty in the identity of the amino acid. *x*, Unidentified residue; –, uncertainty due to a low yield; *solid line*, no peptide available for sequencing in this region. The three disulfide bridges are located between CyS_7 and CyS_{57}, CyS_{16}, and CyS_{40}, and CyS_{32} and CyS_{53} in α-DaTX (31). The *boxes* indicate the highly conserved amino acids in the elapid snake toxins and the proteinase inhibitors (5, 32, 33).

identical to DpK, with differences in only three amino acids (Refs. 5 and 33; and see Fig. 8). Furthermore, the four toxins all have three disulfide bridges and identical amino acids at 34 positions but the two pairs also have a substantial number of differences (Fig. 8). The most conserved portions of these toxin molecules are the *C*-terminal halves (amino acids 32–59; see Fig. 8), which we term the core. β- and γ-DaTX also share much of this core (Fig. 8). However, much of this homologous core is also shared by a number of peptides that exhibit antiproteinase activity and do not facilitate synaptic transmission (32, 33). Some of these proteinase inhibitors have been purified from snake venom, but many have been obtained from various other sources including cow pancreas (32, 33). Thus, it seems more likely that the specificity of the *D. angusticeps* and *D. polylepsis polylepsis* toxins is determined by the amino acid sequences in the *N*-terminal halves of the molecules. We cannot, however, exclude the possibility that single nonconservative amino acid substitutions in the *C*-terminal core (e.g., at positions 48 and 51; see Fig. 8) may substantially alter the pharmacological properties of these polypeptides (33).

β- and γ-DaTX Structure and Function

The other two polypeptide neurotoxins that we isolated from *D. angusticeps* venom, β- and γ-DaTX, have molecular weights of 6600–7000 (see Table 1; the molecular weights of α- and δ-DaTX are, respectively, 7054 and 6553) and contain six cysteines and seven or eight lysines (Table 1). Their amino acid compositions do not match any previously identified components of *Dendroaspis* venoms (see Refs. 5, 32, and 33); however, the composition and sequence analysis indicate the presence of the same core amino acid regions as observed in the four toxins that block the inactivating K channel (Fig. 8). When the available sequences are aligned, there do not appear to be any obviously similar patterns between the *N*-terminal ends of α- and δ-DaTX or between β- and γ-DaTX (Fig. 8). In the first 31 positions there are 15 differences between α- and δ-DaTX (Fig. 8), and at least 11 differences between β- and γ-DaTX (based on the data in Fig. 8 and Table 1).

These structural characteristics are noteworthy because of the differences in the actions of the two pairs of toxins. Whereas α- and δ-DaTX preferentially blocked the inactivating 4-AP-sensitive component of the K-stimulated ^{86}Rb efflux in synaptosomes, β- and γ-DaTX preferentially blocked the noninactivating component (S_V) that is also selectively blocked by PCP (19).

Polypeptide Toxins as Probes of K Channel Structure and Function

The fact that β-DaTX, at high concentrations, also blocked the voltage-gated, inactivating, PCP-sensitive K channels in synaptosomes (Fig. 5) may have interesting implications for the structures of the K channels. Overlapping selectivity was also observed for α-DaTX in synaptosomes; at very high concentrations it blocked the noninactivating as well as the inactivating voltage-gated K channels (data not shown). These findings, as well as some recent observations on the effects of certain scorpion toxins, raise the possibility that several different types of K channels (voltage-gated inactivating and noninactivating K channels and Ca-activated K channels) may all have binding sites for a group of snake and scorpion venom polypeptides and that the order of selectivity for binding of these peptides may be different for each of the channel types. For example, noxiustoxin, from the scorpion *Centruroides noxius*, and charybdotoxin, from the scorpion *Leiurus quinquestriatus*, both block voltage-gated noninactivating K channels as well as certain Ca-activated K channels, but their selectivities are different; noxiustoxin preferentially blocks the noninactivating K channels (35), whereas charybdotoxin preferentially blocks the Ca-activated K channels (36).[3] Our snake toxin data may be comparable; they indicate that α-DaTX and β-DaTX both block inactivating as well as noninactivating voltage-gated K channels in synaptosomes, but β-DaTX preferentially blocks the noninactivating channels whereas α-DaTX preferentially blocks the noninactivating channels. In this regard, it is noteworthy that a variety of voltage-gated, rapidly activating K channels ("A-channel variants") have been identified in different mammalian neurons; these channels differ in their rates of inactivation and their sensitivity to 4-AP and α-DaTX (37).

These findings raise the possibility that there may be structural homologies among the various types of K channels that can be recognized by the several types of snake and scorpion K channel toxins, including the two classes of DaTXs that we have identified. This would not be surprising, given the similarities among permeation mechanisms in various K channels (2, 3) and the evidence for some structural homology between the A current channel and the voltage-gated tetrodotoxin-sensitive Na channel (38).

These selective toxins may be the critical tools needed to identify and isolate the toxin receptor/putative K channel proteins. Isolation and characterization of the structure and function of these membrane proteins should resolve many of the uncertainties mentioned above and provide new insight into the relationship between the structure and function of ion channels.

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