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Identification of Amino Acid Residues Involved in Dendrotoxin Block of Rat Voltage-Dependent Potassium Channels

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SUMMARY

α-Dendrotoxin (DTX) is a 60-amino acid peptide belonging to the family of mamba snake neurotoxins; it is a potent blocker of some but not all voltage-gated potassium currents. Potassium currents recorded from oocytes injected with cloned potassium channel RNAs also vary in sensitivity to DTX. Expression of channels that were chimeras of the DTX-sensitive channel RBK2 and the DTX-insensitive channel RGK5 showed that the putative extracellular loop between transmembrane domains S5 and S6 contributes strongly to DTX sensitivity. Mutation of two residues ($Ala^{352}Glu^{353}$) in this region of RBK1 to conform to those at equivalent positions in RGK5 ($Pro^{374}Ser^{375}$) reduced the potency of DTX about 70-fold, and the substitution of Tyr^{379} in RBK1 by its counterpart in RGK5 (His^{401}) caused an additional 2.5-fold decrease in sensitivity. Converse substitutions in RGK5 significantly increased sensitivity to DTX. The results suggest that these residues contribute significantly to the channel-toxin interaction, providing further evidence that the S5-S6 loop lies at or near the external mouth of the channel, where DTX binding leads to channel occlusion. They offer a molecular explanation for the differences in DTX sensitivity observed among native potassium channels.

DTX is one of a family of basic peptide neurotoxins isolated from mamba snake venom (1). Initially, mamba venom neurotoxins were shown to cause muscle paralysis, by either blockade of acetylcholine receptors or reduction in transmitter release (reviewed in Refs. 2 and 3). In contrast, venom from one particular member of the family, *Dendroaspis angusticeps*, was found to enhance rather than block neuromuscular transmission. Harvey and co-workers (4, 5) demonstrated that the observed effects were due to increased acetylcholine release from nerve terminals. The increase of neurotransmitter release is now believed to result from a block of voltage-dependent potassium conductance, leading to action potential broadening. For example, peripheral administration of nanomolar concentrations of DTX induces repetitive firing in rat visceral sensory neurons by inhibiting a slowly inactivating outward potassium current (6). Intracerebral administration at doses 10,000 times lower than the lethal peripheral dose induces convulsions and death; these actions have been ascribed to facilitation of transmitter release in the hippocampus (7).

Dendrotoxins share significant homology with the Kunitz family of protease inhibitors. The peptides are believed to form stable, pear-shaped structures of about 3 × 2 nm. Most of the molecule is thought to comprise a twisted antiparallel β-sheet with short α-helical regions at the amino and carboxyl termini. All members of the family are about 60 amino acids in length with six conserved cysteines, which might stabilize the structure through the formation of disulfide bonds (1). All carry net positive charges, due to up to nine positively charged residues. In particular, those toxins that facilitate transmitter release bear four conserved lysine residues. Because DTX blocks some but not all potassium conductances, it has been useful in the pharmacological classification of channels (8–10). Its high affinity and selectivity also led to its use as a probe for channel isolation, and recently such a protein was purified from rat brain (11).

The isolation and functional expression of cloned voltage-dependent potassium channels from *Drosophila* (12–15) and mammals (16–20) offer a more direct approach to study the mechanism of action of DTX. The proteins encoded by the cloned DNAs display significant primary sequence homology and have been predicted to cross the membrane six times, with both the amino and carboxyl termini residing intracellularly (21); the functional channel is believed to be a multimer, probably comprising four subunits, which may be either the same or different (22–24). The variation in DTX sensitivity exhibited by the cloned and expressed potassium channels provides the opportunity to correlate primary sequence with DTX sensitivity and thereby determine regions of the channel involved in toxin binding. The present paper reports experi-

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ABBREVIATIONS: DTX, α-dendrotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TEA, tetraethylammonium.

ments in which such studies were carried out by expressing channel proteins having amino acid sequences altered by mutagenesis.

Experimental Procedures

Chimeric molecules were constructed using standard recombinant DNA techniques. Restriction endonucleases and T4 DNA polymerase were from Bethesda Research Laboratories (Gaithesburg, MD). Briefly, appropriate restriction fragments were purified by electrophoresis through GTG agarose gels (FMC, Rockland, ME). After electroelution and ethanol precipitation, these fragments were ligated and the reaction products were transformed into *Escherichia coli* host strain JM101. Transformants were analyzed by restriction digestion.

Site-directed mutants were constructed using a phagemid, pSelect (Promega, Madison, WI), or a derivative, pSelect(−), which allows rescue of the (+)-strand.[1] Channel-encoding sequences were subcloned, and single-stranded DNA was rescued by superinfection with M13K07 helper phage. Mutagenic oligonucleotides were annealed to the single-stranded DNA in the presence of an oligonucleotide that corrects a frameshift mutation in the β-lactamase gene of the plasmid. Complementary DNA was synthesized using T4 DNA polymerase and ligase (BRL). The reaction products were transformed into BMH 71-18 *mutS* and grown in liquid culture containing ampicillin; miniprep DNA was isolated and used to retransform JM101. Individual colonies were isolated, single-stranded DNA was rescued, and the nucleotide sequences of the mutagenized regions were determined. Oligonucleotides were synthesized on an Applied Biosystems PCRmate, and DNA sequencing was performed by dideoxy chain termination, according to published methods (25). RNA was synthesized *in vitro* from appropriate templates using our published methods (16).

Xenopus laevis were housed and oocytes were prepared and injected as previously described (16). Currents were recorded using a two-electrode voltage-clamp 24–96 hr after injection. Cells were constantly perfused in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6, with 0.1% bovine serum albumin. Cells were clamped at −80 mV, and potassium currents were recorded during 250-msec depolarizing commands to 0 mV every 20 sec. In most experiments, the DTX concentration was increased cumulatively (see Fig. 1). Concentrations of DTX giving 50% inhibition (EC_{50}) were estimated by fitting a logistic function ($I = 100 \cdot [DTX]^n/(EC_{50}^n + [DTX]^n)$), where I was the mean percentage of inhibition observed in three to six oocytes. The coefficient n was not significantly different from 1. Three batches of DTX (two from Sigma; one the gift of Dr. J. O. Dolly) were used in these studies; they were kindly purified by Dr. R. Hartshorne (The Ohio State University) by gel filtration and ion exchange high performance liquid chromatography. The EC_{50} for inhibition of current through RBK1 channels ranged from 0.3 to 1.5 nM among different batches, but the quantitative comparisons reported are from experiments with the same batch.

Results

We have previously reported the functional characterization of three cloned rat potassium channels in *Xenopus* oocytes, RBK1 (16) (also called RCK1) (26), RBK2 (22) (also called BK2) (27), and RGK5 (17) (this differs by two amino acids from RCK3) (18). These channels display delayed rectifier properties, with the main difference between RGK5 and the other two being its inactivation during depolarizations lasting several seconds. The amino acid sequences of the predicted channel proteins are highly homologous, yet they differ markedly with respect to DTX sensitivity. Fig. 1a shows the current through RBK1 channels; it was blocked approximately 50% by 1 nM DTX. The block occurred rapidly, and the current quickly recovered its control value when the DTX application was discontinued. RBK2 channels had a DTX sensitivity similar to that of RBK1. Block of RBK1 by 1 nM DTX showed little or no voltage dependence; the block was not more than 1.2 times greater at +20 mV than at −20 mV. Similar inhibition of the current was observed whether increasing concentrations of DTX were applied sequentially or cumulatively (Fig. 1a). In contrast, currents in oocytes injected with RGK5 RNA were virtually insensitive to DTX (Fig. 1b).

As an initial step to localize residues that contribute to DTX sensitivity, chimeric subunits were constructed. RBK2 and RGK5 were used because they both have a *PstI* restriction site located 5′ to the region encoding the S4 transmembrane domain, dividing the coding region approximately in half. The chimera containing the amino terminus of RBK2 and the carboxyl terminus of RGK5 was expressed; the currents were unaffected by 10 nM DTX. In contrast, the channel composed of the amino terminus of RGK5 and the carboxyl terminus of RBK2 was about as sensitive to DTX (EC_{50}, 0.21 ± 0.15 nM; n = 3) as the wild-type RBK2 (EC_{50}, 0.38 ± 0.02 nM; n = 3).

The rapid onset and offset of the action of DTX indicates an extracellular site of action. The carboxyl-terminal portion of the chimeric channel protein is thought to be intracellular except for a 40-residue sequence between membrane-spanning regions S5 and S6 (21). A well conserved cluster of negative charges is present in the first 10 residues of this region in all homologous cloned potassium channel subunits. Thus, through-space electrostatic interactions between charged residues in RBK1 and DTX were examined by measuring the effectiveness of DTX to inhibit current through RBK1 channels in solutions of different ionic strength. In one series of experiments, the EC_{50} for DTX was 1.5 ± 0.06 nM (n = 3) in normal solution (96 mM NaCl) and 0.36 ± 0.03 nM (n = 5) in an iso-osmotic solution containing only 48 mM NaCl (sucrose substitution). This 3.5-fold increase in sensitivity to DTX suggests that through-space electrostatic forces play a role in DTX binding, similar to that observed for charybdotoxin binding to Shaker potassium channels (28).

Although many of the negatively charged residues in the region between S5 and S6 are conserved among the various cloned potassium channel subunits, there is a correlation between DTX sensitivity and the presence of a negatively charged residue in the position corresponding to Glu^{353} in RBK1 (Table 1) (see also Ref. 18); this position is occupied by neutral residues in DTX-insensitive subunits such as RGK5. Site-directed mutants were constructed by replacing the targeted amino acids within each parental backbone. Replacement of RBK1 Glu^{353} with Ser [RBK1(E353S)] resulted in a channel 30-fold less sensitive to DTX than wild-type. Many of the cloned subunits that are insensitive to DTX contain a Pro immediately amino-terminal to the position corresponding to Glu^{353} in RBK1; this residue was also changed. Replacement of Ala^{352} with Pro [RBK1(A352P)] produced a 2.5-fold reduction in DTX sensitivity (Fig. 2; Table 1). The effects of the two mutations were multiplicative; the double-mutant RBK1(A352P,E353S) was 70-fold less sensitive to DTX than native RBK1 (Fig. 2; Table 1). The action of DTX on the mutants lacking the negative charge, although weaker than for RBK1, was still sensitive to ionic strength. RBK1(E353S) was tested with 25 nM DTX, and the inhibition increased from 37.7 ± 1.5% to 71.0 ± 2.2% (n =

[1] J. P. Adelman, unpublished observation.

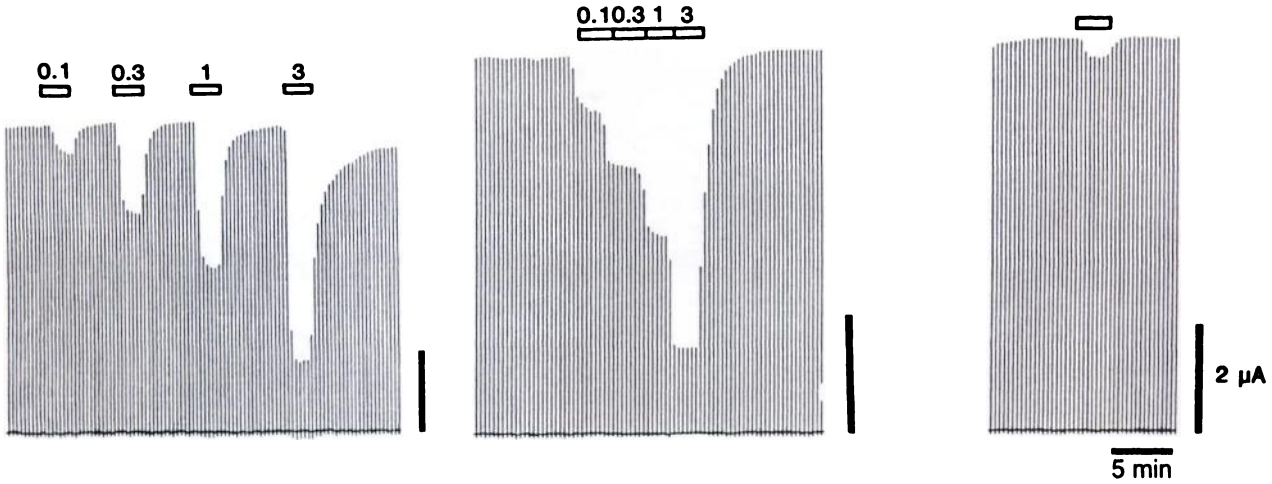

Fig. 1. DTX inhibits potassium current in oocytes injected with RBK1 RNA but has little effect in oocytes expressing RGK5 RNA. Upward deflections are membrane currents evoked by depolarizing commands from −80 mV to 0 mV for 250 msec at intervals of 20 sec. a, Two representative records to show the effects of discrete and cumulative applications of DTX. The inhibition occurs rapidly and reverses readily with washing. b, RGK5 current is almost unaffected by 100 nM DTX.

TABLE 1

S5–S6 region of RBK1 (beginning at F^{346}), RBK2 (beginning at F^{346}), and RGK5 (beginning at F^{368})

Regions denoted as SS1 and SS2 by Guy and Conti (21) are indicated beneath the sequences. Numbers to the right (mean ± standard error of three to six oocytes) are DTX EC_{50} or the inhibition produced by 100 nM DTX.

	EC_{50}	Inhibition by 100 nM DTX
	nM	%
RBK1		
FAEAEEAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGK	0.96 ± 0.04	
.....P.....	2.4 ± 0.3	
.....PS.....	69 ± 11	
.....S.....	26 ± 0.2	
.....H.....	2.5 ± 0.1	
.....PS.....H.....		41 ± 2
RBK2		
FAEADERDSQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGK	0.38 ± 0.02	
RGK5		
FAEADDPSSGFNSIPDAFWWAVVTMTTVGYGDMNPVTIGGK		3.1 ± 0.2
.....A.....		2.8 ± 0.8
.....E.....		35 ± 1.1
.....AE.....		32 ± 1.9
.....Y.....		23 ± 1.6
.....E.....Y.....		40 ± 2.2
SS1 SS2		

3) when ionic strength was halved; for RBK1(A352P,E353S), 60 nM DTX was used and the respective values were 43.9 ± 3.4% and 76.2 ± 1.4% (n = 3).

Converse mutants were next constructed in the DTX-insensitive channel RGK5; because the sensitivity is so low, the results are expressed as inhibition caused by 100 nM DTX, rather than as EC_{50}. RGK5(S375E) was inhibited 10-fold more than native RGK5 at 100 nM toxin. Changing Pro^{374} to Ala [RGK5(P374A)] affected sensitivity very little, and the double-mutant RGK5(P374A,S375E) was no more sensitive to DTX than the single-mutant RGK5(S375E) (see Table 1). Thus, the addition of a negative charge to RGK5 increases DTX sensitivity, but the effect is not as great as the decrease in sensitivity produced by removal of the negative charge from RBK1.

A Tyr in the SS2 region of the S5/S6 loop of voltage-gated potassium channels (Tyr^{379} in RBK1) has recently been shown to lie at the external mouth of the channel, affecting single-channel rectification and TEA binding (29–31). Further, computer modeling (21) has predicted that this residue lies at the external mouth of the channel; therefore, possible involvement of this amino acid in mediating DTX sensitivity was investigated. Substitution of Tyr by its counterpart in RGK5 [RBK1(Y379H)] resulted in a 2.5-fold decrease in DTX potency. When this change was introduced together with the two changes close to S5 described above, the triple mutant was >100-fold less sensitive to DTX than was RBK1. Conversely, inhibition of the mutant RGK5(H401Y) by 100 nM DTX was increased 7-fold, compared with wild-type, and this same sub-

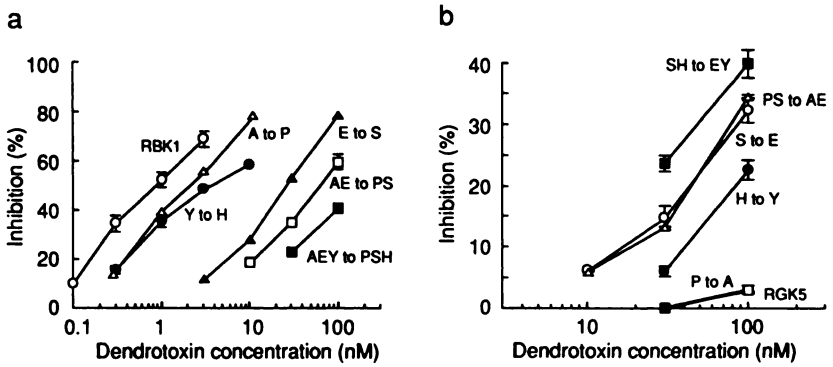

Fig. 2. Concentration-response curves for the inhibition by DTX of currents in oocytes previously injected with RBK1 RNA and mutants thereof (a) and RGK5 RNA and mutants thereof (b). Each symbol indicates the mean effect of DTX on three to six oocytes; in some cases, the standard error is less than the size of the symbol.

stitution in concert with the introduction of Glu^{375} increased DTX inhibition 13-fold, relative to wild-type RGK5.

The mutant channels examined did not obviously differ in voltage dependence from the parent wild-type channels, and the action of DTX was also not voltage dependent. In the case of RBK1, RBK1(A352P,E353S), and RGK5(P374A,S375E), the inhibition caused by DTX was not significantly different at −20 mV and at +20 mV. RBK1 mutants activated more slowly than wild-type RBK1, and RGK5 mutants activated more rapidly than RGK5, but the activation was in all cases intermediate between the values for RBK1 (<5 msec at 0 mV) (16) and RGK5 (about 20 msec at 0 mV) (17).

Discussion

The present study has identified RBK1 Ala^{352}, Glu^{353}, and Tyr^{379}, three residues located in the loop between transmembrane domains S5 and S6, as being capable of influencing DTX block of the channel. Cation-selective channels in general are thought to have a permeation pathway that is preceded by a cloud of negative charge, which contributes to ion selectivity (32). Experimental evidence for this has been provided by chemical modification studies in which carboxyl-modifying agents have been applied to sodium channels or calcium-activated voltage-sensitive potassium channels, resulting in altered ion permeation and gating; such treatments also inhibit binding of saxitoxin and tetrodotoxin to some sodium channels and charybdotoxin binding to some potassium channels (33–36). Site-directed mutagenesis allows the carboxyl residues that are important in selectivity and toxin binding to be identified more specifically.

MacKinnon and Miller (28) found that substitution of Glu^{422} by Gln reduced the inhibition of current through ShB channels by charybdotoxin 3.5-fold, and substitution by lysine reduced the inhibition 12-fold. In the present study, replacement of Glu^{353} by Ser caused about a 30-fold reduction in sensitivity of RBK1 to DTX. Although not in exactly analogous positions by sequence alignment, these two residues are in close proximity in the region between S5 and S6. Thus, it is likely that, despite differences in primary sequence and structure, these two toxins bind to a very similar region of voltage-dependent potassium channels. This is consistent with the observation that charybdotoxin can inhibit ^{125}I-DTX binding to rat brain synaptosomal membranes (37).

Based upon computer modeling of potassium channel subunit structure, Guy and Conti (21) have presented a model in which the extracellular loop between transmembrane regions 5 and 6 (about 40 amino acid residues) contains two subsections, termed SS1 and SS2 (see Table 1). These are thought to dip back into the membrane, contributing to the extracellular mouth of the channel and the permeation pathway (21). Mammalian potassium channels thus far cloned and tested fall into two classes, sensitive (EC_{50}, <10 nM) or insensitive (EC_{50}, >200 nM) to DTX (16, 18, 20, 26). A Tyr residue in SS2 (Tyr^{379} in RBK1) has been identified as a major determinant of the binding of TEA (29, 30). The present results indicate that it also participates in DTX binding. Replacement of this Tyr in RBK1 with its counterpart (His) in RGK5 reduced the affinity for DTX by a factor of about 2.5. The effect appeared to be relatively independent of the substitutions discussed above in the SS1 region (Ala^{352} and Glu^{353}), and the converse substitutions in RGK5 had the expected effects (Table 1).

The mutations made in this study resulted in the expression of channels that had altered sensitivities for DTX of up to 150-fold, a change in binding energy equivalent to about 3 kcal/mol. Although this contribution is very significant, other residues must also play a role, because the changes in apparent K_D observed for single amino acid substitutions never amounted quantitatively to the difference between RGK5 and RBK1. The RBK1 mutant with all three substitutions [RBK1(A352P, E353S,Y379H)] had about the same sensitivity to DTX as the most sensitive RGK5 mutant tested [RBK1(S375E,H381Y)]. Some of the other, as yet unidentified, residues that contribute to DTX binding likely participate in ionic interactions, because the blocking potency of DTX was still increased by lowering the ionic strength in the Glu to Ser mutant. Given the size of the DTX molecule and the high affinity with which it binds, it seems likely that the bound complex is stabilized by a number of interactions, unlike the case with the structurally much simpler molecule TEA (29, 30).

Although first thought to be a presynaptic toxin, because of its action to increase transmitter release (1, 4, 5), DTX is now known to block noninactivating, voltage-gated potassium currents in the membrane of neuronal somata such as dorsal root ganglion cells and hippocampal pyramidal neurons (6, 9, 38, 39). Action potential broadening in presynaptic fibers is, therefore, thought to account for the enhanced transmitter release. The effective concentrations in guinea pig dorsal root ganglion cells are the same as those that block RBK1 and RBK2 in *Xenopus* oocyte. The present finding that DTX sensitivity can be attributed in large measure to the presence of discrete amino acid residues may eventually help determine the molecular species of channels that are expressed in presynaptic nerve terminals.

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