

Glycine 30 in iberitoxin is a critical determinant of its specificity for maxi-K versus K_V channels

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Abstract Iberitoxin (IbTX) is a remarkably selective α -K toxin peptide (α -KTx) inhibitor of the maxi-K channel. In contrast, the highly homologous charybdotoxin inhibits both the maxi-K and K_V 1.3 channels with similar high affinity. The present study investigates the molecular basis for this specificity through mutagenesis of IbTX. The interactions of mutated peptides with maxi-K and K_V 1.3 channels were monitored through dose-dependent displacement of specifically bound iodinated α -KTx peptides from membranes expressing these channels. Results of these studies suggest that the presence of a glycine at position 30 in IbTX is a major determinant of its specificity while the presence of four unique acidic residues in IbTX is not. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Iberitoxin; Maxi-K channel; K_V 1.3 channel; α -K peptide; Charybdotoxin

1. Introduction

Large-conductance calcium-activated (maxi-K) potassium channels represent a unique class of potassium channels that are synergistically activated by depolarizing membrane potentials and intracellular calcium. Thus, the maxi-K channel provides an important link between calcium- and voltage-dependent signaling processes. Because of these unique properties, maxi-K channels play critical roles in a variety of physiological processes. In smooth muscle, maxi-K channels regulate uterine contractions [1], and they play a critical role in regulating arterial tone and blood pressure [2,3]. Maxi-K channels also regulate electrical tuning in cochlear hair cells [4], hormone release in pituitary cells [5] as well as spike broadening and neurotransmitter release in neurons [6]. Iberitoxin (IbTX), a highly selective α -K peptide (α -KTx) inhibitor of the maxi-K channel [7], has proved invaluable in elucidating the diverse physiological roles of these channels.

The α -KTx peptides inhibit the flow of K^+ ions through the K^+ channel pore and are powerful tools for probing the structure and function of K^+ channels [8] as well as for understanding their physiological function. The different α -KTx subfamilies, shown in Fig. 1, display an extraordinary ability to distinguish between the large family of voltage-gated potassium (K_V) channels and the maxi-K channel. Peptides from the α -KTx 2.x and 3.x subfamilies block K_V channels with high affinity but not the maxi-K channel [8,9]. Conversely, peptides from the α -KTx 1.x subfamily display high affinity interactions with the maxi-K channel. Most α -KTx 1.x peptides, such as charybdotoxin (ChTX or α -KTx 1.1), also inhibit K_V 1 channels with high affinity [10,11]. However, two of these toxins, IbTX (α -KTx 1.3) and limbatus toxin (LbTX or α -KTx 1.4) are highly selective for the maxi-K channel. Despite its widespread use as a selective maxi-K channel inhibitor, the molecular basis for IbTX specificity is poorly understood. In this work we examine the interactions of IbTX and noxiustoxin (NxTX or α -KTx 2.1) mutants with maxi-K and K_V 1.3 channels to reveal that a single residue in IbTX, glycine 30, is a major determinant of its specificity for the maxi-K channel.

2. Materials and methods

2.1. Peptide

The plasmids pG9IbTX and pG9NxTX, encoding T7 gene 9, six histidines, a factor Xa cleavage site and either the IbTX gene [12] or the NxTX gene [9], were used to generate toxin mutants using a two-step polymerase chain reaction mutagenesis strategy as described [12]. The identity of all DNA constructs was verified using dideoxy sequencing [13]. The *Escherichia coli* strain BL21 (DE3), harboring the pG9IbTX or pG9NxTX plasmids, was cultured and induced with isopropyl 1-thio- β -galactopyranoside, and the T7 gene 9 toxin fusion protein was purified by DEAE ion exchange chromatography as described [12]. After folding, the fusion protein was cleaved from the toxin with TPCK-treated trypsin and purified by FPLC ion exchange [9] and by reverse phase high performance liquid chromatography (HPLC) as described [12]. The identity of each peptide was confirmed by either MALDI-MS mass spectrometric analysis or by N-terminal sequencing of the first six amino acids (Commonwealth Biotechnologies). Cyclization of the N-terminal glutamine in IbTX mutants, performed as described [12], was verified either by MALDI-MS mass spectrometric analysis or N-terminal sequencing of the first six amino acids before and after cyclization. The predicted and measured masses for each peptide were IbTX-S10A (4232, 4231), IbTX-S10A-D4N-D6N (4230, 4229.2), IbTX-S10A-D24N (4231, 4229.2), IbTX-S10A-D4N-D6N-D19N-D24N (4211, 4211), IbTX-S10A-G30N (4289, 4287), NxTX- Δ Thr1- Δ Asn39 (3980.7, 3978.4), NxTX-N31G- Δ Thr1- Δ Asn39 (3923.7, 3924.8). Purification and char-

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Abbreviations: α -KTx, α -K peptide; ChTX or α -KTx 1.1, charybdotoxin; HgTX-1 or α -KTx 2.3, hongotoxin-1; IbTX or α -KTx 1.3, iberitoxin; [¹²⁵I]HgTX-1-A19Y/Y37F, mono-iodinated HgTX-1-A19Y/Y37F; [¹²⁵I]IbTX-D19Y/Y36F, mono-iodinated IbTX-D19Y/Y36F; NxTX or α -KTx 2.1, noxiustoxin; K_i , toxin inhibition constant

acterization of recombinant NxTX and NxTX-S14W were as described [9]. The peptides were >99% pure as judged by HPLC elution profiles and mass spectrometric analysis. ChTX and IbTX were from Peninsula Laboratories.

2.2. Transfection of TsA-201 cells and membranes

Maxi-K channels were transiently transfected into a TsA201 cell line using the human α clone huR2(+) subcloned into a pCI-neo vector and membrane preparation were as described [14]. Membranes from HEK-293 cells stably transfected with the Kv1.3 channel were prepared as described [15].

2.3. Binding assays

The interaction of mutant peptides with the Kv1.3 channel was monitored by dose-dependent displacement of mono-iodinated honogotoxin-1-A19Y/Y37F ($[^{125}\text{I}]\text{HgTX-1-A19Y/Y37F}$) specifically bound to HEK membranes expressing Kv1.3 channels as described [16]. Membranes were incubated with $[^{125}\text{I}]\text{HgTX-1-A19Y/Y37F}$ (2200 Ci/mmol) in the absence or presence of unlabeled mutant peptide until equilibrium was achieved [16]. Non-specific binding, determined from the amount of radioactivity remaining after incubation with excess unlabeled with NxTX-S14W [16], represented <10% of total binding. At the end of the incubation period, samples were diluted with ice-cold buffer and rapidly filtered through Whatman GF/C filters presoaked with 0.5% polyethyleneimine and washed with ice-cold medium as described [17]. Assays were performed in triplicate for each experimental condition.

The interaction of peptides with maxi-K channels was monitored from the dose-dependent displacement of the mono-iodinated IbTX-D19Y/Y36F ($[^{125}\text{I}]\text{IbTX-D19Y/Y36F}$) specifically bound to membranes expressing maxi-K channel alpha subunits as described [18]. Membranes were incubated with $[^{125}\text{I}]\text{IbTX-D19Y/Y36F}$ (2200 Ci/mmol) in the absence or presence of unlabeled peptide until equilibrium was achieved as described [18]. Non-specific binding, determined from excess unlabeled IbTX, represented <15% of total binding. Assays were performed in triplicate for each experimental condition.

Data were computer-fitted to the general dose-response equation,

and then analyzed for toxin inhibition constant (K_i) values using the method described [19]. Measurements were made a minimum of three times and errors are described as standard errors of the mean.

3. Results and discussion

IbTX and ChTX display a remarkable difference in their specificity for maxi-K versus Kv1.3 channels and yet their three-dimensional solution 1H NMR structures [20,21] reveal nearly super imposable alpha carbon backbone structures, see Fig. 2. Thus, IbTX specificity is likely defined by specific amino acids. Comparison of IbTX and ChTX reveals differences in sequence identity are localized to five structurally discrete domains, I through V, Figs. 1 and 2. Domains I, III, IV and V contribute to the peptide:channel binding surface [22,23] and thus are likely to influence specificity.

ChTX and IbTX exhibit a striking difference in electrostatic structure [8] that is derived in part from four extra acidic residues in IbTX (D4, D6, D19 and D24) that reside in domains I, II and III, Fig. 1. To test whether these Asp residues prevent a high affinity interaction we generated four IbTX-S10A mutants (D4N, D4N-D6N, D24N, D4N-D6N-D19N-D24N) and examined their interactions with Kv1.3 channels, see Fig. 3 and Table 1. The S10A mutation, in both ChTX [23] and IbTX [24], displays a ~10-fold weaker interaction that is manifested as a faster dissociation rate constant. The faster dissociation kinetics seen with the S10A mutation will facilitate future mechanistic characterization of mutant toxin block of single maxi-K channels. This S10A mutation in ChTX also causes a 10-fold weaker interaction with the *Shaker* Kv channel [22] and thus does not influence specificity.

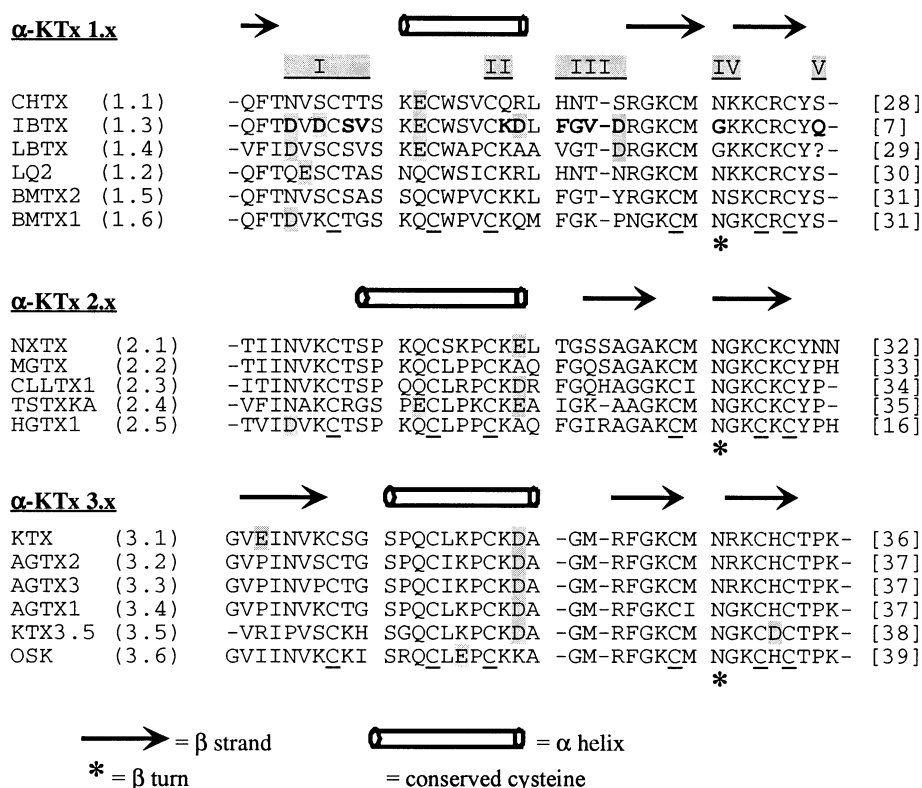


Fig. 1. Amino acid sequences for the α -KTx. Domains I, II, III, IV and V that define sequence differences between ChTX and IbTX are indicated. Residues in IbTX unique from ChTX are in bold. Acidic residues are shaded. Secondary structures, position of the beta turn and strictly conserved cysteines are annotated. (See also Refs. [7,16,28–39].)

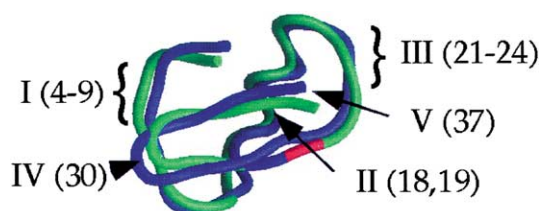


Fig. 2. Sequence differences between IbTX and ChTX are defined by five structurally discrete domains. Superimposed α -carbon backbone structures for IbTX [21] are in green, and for ChTX [20] in blue.

HgTX-1 is a highly selective, high affinity blocker of $K_V1.3$ channels [16]. Thus, competition experiments with [125 I]HgTX-1-A19Y/Y37F provide a reliable means for monitoring the interaction of mutant peptides with $K_V1.3$ channels. Fig. 3 shows that ChTX displaces bound [125 I]HgTX-1-A19Y/Y37F from membranes containing $K_V1.3$ channels with an IC_{50} value of 140 pM. In contrast, IbTX-S10A (100 nM) and the four charge mutants (300 to 1,000 nM) had no effect on bound [125 I]HgTX-1-A19Y/Y37F, Fig. 3 and Table 1. Conversely, ChTX, IbTX-S10A and the same IbTX-S10A charge mutants displace [125 I]IbTX-D19Y/Y36F from membranes expressing maxi-K channels with K_i values ranging from 7 to 150 pM, Fig. 4 and Table 1. The results of these binding measurements suggest that charge alone cannot explain the remarkable specificity of IbTX for the maxi-K vs. K_V channels.

Among the five structural domains that distinguish IbTX and ChTX, the single glycine in domain IV of IbTX is unique, in that all α -KTx peptides known to block K_V channels contain an asparagine at this position, Fig. 1. To test the role of this Gly in IbTX specificity we generated the IbTX-S10A mutant, G30N, and examined its interaction with the $K_V1.3$ channel, Fig. 3 and Table 1. Binding of [125 I]HgTX-1-A19Y/Y37F was inhibited by IbTX-S10A-G30N with a K_i value of 43 nM. Thus, the G30N mutation in IbTX promotes a high affinity interaction with the $K_V1.3$ channel. In contrast, the IbTX-S10A-G30N (K_i 370 pM) mutation weakens binding to the maxi-K channel by about three-fold compared to IbTX-S10A (K_i 130 pM), Fig. 4 and Table 1. These data taken together strongly suggest that the nature of residue 30, in the peptide beta turn, is a major determinant of specificity.

To test whether 'Asn 30' in the beta turn is generally important for the interaction of α -KTx peptides with K_V channels we mutated this analogous 'Asn31' in NxTX (α -KTx 2.1). NxTX blocks with high affinity $K_V1.3$ but not maxi-K channels [9]. Some of the NxTX specificity for $K_V1.3$ results from the increased backbone length at the N- and C-termini [9,25]. The NxTX deletion mutant, NxTX- Δ Thr1- Δ Asn39, truncated

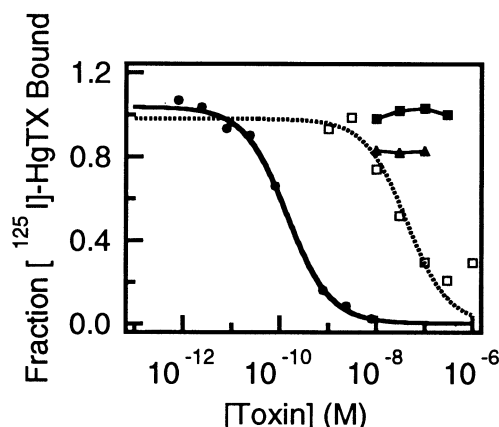


Fig. 3. Effects of ChTX and mutants of IbTX-S10A on [125 I]HgTX-1-A19Y/Y37F binding to $K_V1.3$ channels. Specific binding of [125 I]HgTX-1-A19Y/Y37F to HEK membranes transfected with $K_V1.3$ channels is plotted as a function of increasing concentrations of ChTX (filled circles), IbTX-S10A (filled triangles), IbTX-S10A-G30N (open squares) and IbTX-S10A-D4N-D6N-D19N-D24N (filled squares). Membranes were incubated in a medium containing 0.2 pM [125 I]HgTX-1-A19Y/Y37F, 100 mM NaCl, 5 mM KCl, 20 mM Tris-HCl pH 7.4, 0.1% bovine serum albumin and different concentrations of unlabeled peptide. Incubations were performed at room temperature for ca 20 h. Non-specific binding was determined in the presence of 100 nM NxTX-S14W.

by one residue each at the N- and C-termini has the same backbone length as IbTX and ChTX. Fig. 5 shows that NxTX and NxTX- Δ Thr1- Δ Asn39 displace bound [125 I]HgTX-1-A19Y/Y37F from membranes containing $K_V1.3$ channels with IC_{50} values of 0.6 and 8 nM, respectively. In contrast, the N31G mutant of NxTX- Δ Thr1- Δ Asn39 has no effect on bound [125 I]HgTX-1-A19Y/Y37F at 300 nM. This finding is also consistent with previous studies where mutation of this 'Asn 30' in ChTX and agitoxin-2 (AgTX-2 or α -KTx 3.2) weakened binding to the *Shaker* K_V channel by 840- [22] and 700-fold [26], respectively, compared to the wild-type peptides. Together these findings suggest that 'Asn 30' in the α -KTx beta turn is critical for its high affinity interaction with K_V channels.

Our finding that replacing the smaller Gly in IbTX with the larger Asn restores a high affinity interaction with K_V channels is remarkable, and it suggests that steric interactions are not responsible for the effects. The most reasonable interpretation of this result is that Asn 30 forms a critical hydrogen bond with a H-bond acceptor on the K_V channel and that a similar H-bond is not critical for interaction of α -KTx peptides with the maxi-K channel. Indeed, recent models of ChTX bound to the $K_V1.3$ outer vestibule reveal the presence of an important H-bond between Asn30 in ChTX and Asp

Table 1
Effects of ChTX, IbTX-S10A and mutants on binding to maxi-K and $K_V1.3$ channels

Peptide	K_i (nM) maxi-K	K_i (nM) $K_V1.3$
ChTX	0.007 ± 0.002	0.034 ± 0.006
IbTX-S10A	0.13 ± 0.02	no effect 100 nM
IbTX-S10A-D4N	0.02 ± 0.0002	no effect 1,000 nM
IbTX-S10A-D4N-D6N	0.007 ± 0.0008	no effect 300 nM
IbTX-S10A-D24N	0.15 ± 0.037	no effect 300 nM
IbTX-S10A-D4N-D6N-D19N-D24N	0.027 ± 0.006	no effect 300 nM
IbTX-S10A-G30N	0.37 ± 0.1	43 ± 25

K_i values were determined from competition experiments with either [125 I]HgTX-1-A19Y/Y37F binding to $K_V1.3$ channels or [125 I]IbTX-D19Y/Y36F binding to maxi-K channels as described in Figs. 3 and 4, respectively.

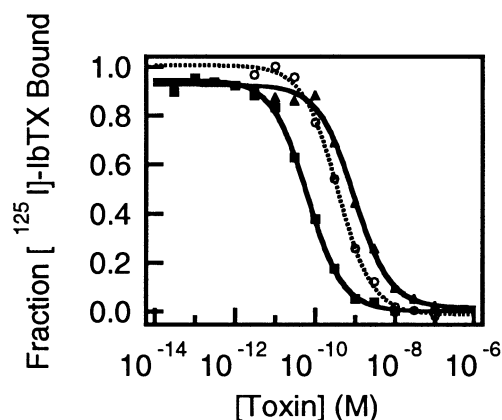


Fig. 4. In IbTX, the G30N mutation exerts little effect on its interaction with maxi-K channels. Specific binding of [125 I]IbTX-D19Y/Y36F to HEK membranes expressing maxi-K α subunits is plotted as a function of increasing concentrations of IbTX-S10A (open circles), IbTX-S10A-G30N (filled triangles) and IbTX-S10A-D4N-D6N-D19N-D24N (filled squares). Membranes were incubated in a medium containing 5 pM [125 I]IbTX-D19Y/Y36F, 10 mM NaCl, 20 mM Tris-HCl pH 7.4, 0.1% bovine serum albumin and different concentrations of unlabeled peptide. Incubations were carried out at room temperature for ca 20 h. Non-specific binding was determined in the presence of 100 nM IbTX.

381 in the channel [27]. For the maxi-K channel model, the analogous H-bond between Asn30 in the peptide and Glu276 in the channel cannot be formed [27]. Together, these results suggest that IbTX specificity for the maxi-K vs. $K_v1.3$ channel results, in part, from the absence of a critical H-bond between Asp381 in the channel and Gly 30 in IbTX.

Our findings clearly show that Asn 30 in the α -KTx peptides is critical for their interaction with K_v channels. However, the interaction of IbTX-S10A-G30N with the $K_v1.3$ channel is still ~ 1000 -fold weaker compared to ChTX. Since the S10A mutation weakens binding to K_v channels by no more than 10-fold [22], it is likely that other residues in domains I, III and V of IbTX contribute to its specificity. Studies with synthetic ChTX-IbTX chimera also suggest that the major determinants for IbTX specificity lie in the C-terminal half of IbTX [10]. Approaches to understand the molecular

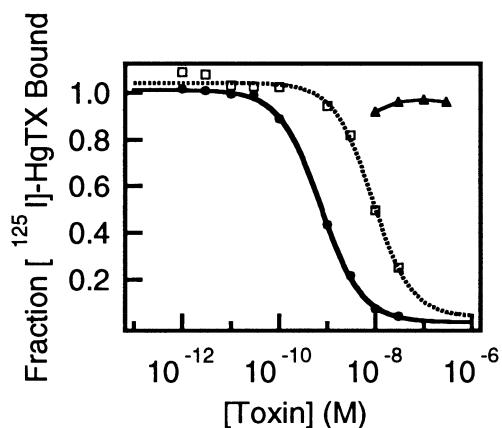


Fig. 5. 'Asn30' in the NxTX beta turn is critical for its interaction with the $K_v1.3$ channel. Specific binding of [125 I]HgTX-1-A19Y/Y37F to HEK membranes transfected with $K_v1.3$ channels is plotted as a function of increasing concentrations of NxTX (filled circles), NxTX- Δ Thr1- Δ Asn39 (open squares), NxTX-N31G- Δ Thr1- Δ Asn39 (filled triangles). Incubations were as described in Fig. 3.

basis for α -KTx specificity are likely to reveal novel features of the maxi-K and $K_v1.3$ outer vestibules. Moreover, the critical role of Asn30 in defining α -KTx specificity will be instrumental in the rational design of specific α -KTx blockers and potassium channel inhibitors.

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