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EXPERIMENTAL PAPERS

$K_V1.2$-Selective Peptide with High Affinity

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Abstract—The isoform of voltage-gated potassium channels $K_V1.2$ is of interest because mutations in its gene are associated with various diseases, such as ataxia and epilepsy. Selective ligands are needed to study the function of $K_V1.2$ in health and disease. In our work, we obtained such a ligand based on the known scorpion peptide toxin, charybdotoxin (ChTx, α-KTx1.1) from the venom of *Leiurus hebraeus*, by introducing a single amino acid substitution M29I into its structure. ChTx_M29I peptide was produced in a bacterial expression system. Its pharmacological characterization was carried out in *Xenopus laevis* frog oocytes expressing a panel of human K_V1 channels. We found that, compared to the parent toxin, ChTx_M29I peptide showed lower affinity for $K_V1.1$, 1.3, and 1.6 channels, while its activity against $K_V1.2$ increased manifold. We attribute this effect to the interaction of the peptide with a specific channel residue (V381 in $K_V1.2$). If there is a relatively small residue at this position, then an advantageous contact is formed that increases the affinity. ChTx_M29I peptide studied by us presents one of the highest affinity (with a half-maximal inhibitory concentration $IC_{50} \approx 6$ pM) and selectivity among $K_V1.2$ ligands (affinity for other isoforms is lower by 680 times or more).

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Voltage-gated potassium channels (K_V) are transmembrane proteins and contain four major α-subunits [1]. Each α-subunit consists of a cytoplasmic T1 domain and six transmembrane helices (S1–S6). The S1–S4 helices form the voltage-sensing domain, in which it is the S4 helix that operates as the voltage sensor. The amino acid residues K and R, which are not typical for the intramembrane space, are found in its structure. In response to changes in the transmembrane potential, they cause movement of the entire helix, which ultimately regulates the opening and closing of the channel. The S5 and S6 helices of all four α-subunits form the pore domain, in which the selectivity filter contains the conserved TVGYG sequence that interacts with K^+ ions.

Forty genes encoding K_V α-subunits have been found in the human genome, making this group

Table 1. Sequences of primers used to obtain the full-length gene encoding ChTx_M29I

Name	Sequence 5'–3'
F1	AGCTGCGGTACC**GACGACGACGACCGT**CAGTTTACCAATGTGAGCTGC
F2	GAGCGTTTGCCAGCGTCTGCATAACACCTCTCGCGGCAAGTGTATTAATA
R1	ACTTGAGGATCCTTAAGAATAACAACGGCATTTCTTATTAATACACTTGC
R2	CGCTGGCAAACGCTCCAACATTCTTTAGACGTGGTGCAGCTCACATTGGT

Restriction sites are underlined, and the enteropeptidase hydrolysis site is highlighted in bold.

the largest among ion channels. In particular, channels of the K_V1 subfamily are widely represented in the mammalian brain, with K_V1.1, 1.2, 1.4, and 1.6 being particularly common [2]. These channel isoforms can form both homo- and heteromers, which affects their characteristics [3, 4]. Homotetrameric channels are mostly available to study due to the limitations of experimental systems, in which it is difficult to control the expression of heteromeric channels.

The K_V1.2 channel isoform is of particular interest because, firstly, it is relatively uniformly expressed in the central nervous system among other K_V1 isoforms, and secondly, it can form both functional homomeric and heteromeric channels with other K_V1 [3]. Several diseases, such as ataxia and epilepsy, are associated with mutations of this isoform [5, 6]. Selective and high-affinity ligands are needed to study K_V1.2 function in normal and pathological conditions.

One of the rich sources of K_V ligands is scorpion venom. Many peptide K_V blockers (KTx) have been isolated from the venom of various scorpion species; according to the Kalium database, there are about 200 of them [7]. According to the amino acid sequence, they are divided into several families: α-, β-, γ-, δ-, ε-, κ-, and λ-KTx. The α-KTx family is the largest and most studied; it includes peptides containing ~20–40 residues that assume the cysteine-stabilized α-helix/β-sheet fold (CSα/β). They are also characterized by the so-called "functional dyad" of K and Y residues, where K physically blocks the channel pore and Y interacts with its outer vestibule [8].

Charybdotoxin (ChTx, α-KTx1.1) is a classic toxin from the venom of the scorpion *Leiurus hebraeus* that has been studied on many channels. It shows high affinity and selectivity to K_V1.3 channels as well as the calcium-activated potassium channels K_{Ca}1.1 and K_{Ca}3.1 [9, 10]. A number of derivatives of ChTx were obtained in Chris Miller's laboratory during the studies of its interaction of with various potassium channels. A peptide with an M29I substitution caught our attention [11]. It was shown that if the T449F replacement was introduced in the K_V of *Drosophila* Shaker, the affinity of ChTx_M29I dropped by a factor of 1650 compared with that of wild-type ChTx. In human channels, various residues including large aromatic residues are found in an analogous position. Therefore, we decided to test how this mutation would affect the affinity of the toxin with respect to human channels.

MATERIALS AND METHODS

Recombinant protein production. ChTx_M29I was obtained according to the standard protocol that we used in previous studies [12]. The peptide was produced in a bacterial expression system as a fusion protein containing: the carrier protein thioredoxin (Trx) [13], the hydrolysis site of human enteropeptidase light chain, and a His-tag for protein purification by affinity chromatography.

Cloning of the target gene. The DNA sequence encoding ChTx_M29I was obtained by two-step PCR using synthetic oligonucleotides. In the first step, four primers were used to obtain a full-length copy of the gene (Table 1). In the second step, the reaction mixture from the first step was used as a matrix for amplification with primers F1 and R1. The resulting DNA was cloned into the expression vector pET-32b (Novagen) at the KpnI and BamHI sites.

Expression and purification of the fusion protein. The expression strain *Escherichia coli* SHuffle T7

Express (New England Biolabs) was transformed with the vector carrying the ChTx_M29I gene; cell biomass growth in LB medium took place at 37°C until reaching the mid-exponential phase. Expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a concentration of 0.4 mM. The cell biomass was then cultured at room temperature for 16 h. The cells were then disrupted by ultrasonication, and the cell lysate was applied to an HisPur Cobalt Resin column (Thermo Fisher Scientific). The hybrid protein was purified according to the resin manufacturer's protocol.

Purification of the target peptide. The purified chimeric protein was dissolved in 50 mM Tris-HCl (pH 8.0) to a concentration of 1 mg/mL. Enzymatic hydrolysis was performed using human enteropeptidase light chain (1 U of enzyme per 1 mg of protein) at 37°C for 16 h [14]. The resulting hydrolysate was separated by reversed-phase high-performance liquid chromatography (RP-HPLC) in a linear gradient of acetonitrile concentration (0–60% for 60 min) on a Jupiter C_5 column (4.6 × 250 mm, Phenomenex). Detection was based on the optical absorbance of the eluate, and the compound of interest was determined by comparing the calculated and experimentally obtained masses determined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.

Mass spectrometry. An Ultraflex TOF-TOF spectrometer (Bruker Daltonik) was used to measure the molecular masses as described previously [15]. 2,5-Dihydroxybenzoic acid (Sigma-Aldrich) was used as a matrix. The measurements were performed in the reflectron mode with a mass-accuracy error of no more than 100 ppm. Mass spectra were analyzed using Data Analysis 4.3 and Data AnalysisViewer 4.3 software (Bruker).

Electrophysiology. The experiments were performed according to the protocols published earlier [12]. Human voltage-gated potassium channels $K_V1.1$ (GenBank ID: NM000217), 1.2 (NM004974), 1.3 (NM002232), and 1.6 (NM002235) were expressed in the oocytes of the frog *Xenopus laevis*. For this purpose, mRNAs encoding the channels were obtained using the mMESSAGE mMACHINE T7 kit (Thermo Fisher Scientific). The obtained mRNAs were then injected into the oocytes using a microinjector (Drummond Scientific).

Currents through the oocyte membrane were recorded at room temperature using the two-electrode voltage clamp method. Data were obtained using a GeneClamp 500 amplifier and Clampex 9 software (Molecular Devices). The holding potential was set at –90 mV, channel opening was induced by membrane depolarization to 0 mV for 500 ms, then the potential was maintained at –50 mV for another 500 ms and returned to the holding potential value.

To build the dose-response curve, the studied peptide was serially diluted and added to the oocyte chamber, where the final studied concentration was reached. The data obtained were analyzed using the Hill equation:

$$y = \frac{100}{1 + \left(\frac{IC_{50}}{C_{peptide}}\right)^h},$$

where y is the current inhibition in %, $C_{peptide}$ is the concentration of the peptide under study, IC_{50} is the half-maximum inhibitory concentration, and h is the Hill coefficient.

All data were obtained in at least three independent experiments ($n \geq 3$). Results were processed using Origin software (OriginLab Corporation).

RESULTS

Obtaining the recombinant peptide ChTx_M29I. One of the main ways to obtain peptides with amino acid substitutions is to use bacterial expression systems. In order to make the peptide we want in bacteria, we need to obtain the genetic construct encoding it. We cloned the gene encoding ChTx_M29I into the expression vector pET-32b at the KpnI and BamHI restriction sites. We transformed the *E. coli* SHuffle T7 Express strain, which is designed to express disulfide-rich proteins [16], with the obtained construct. The target peptide was produced as a fusion protein with Trx, which was purified by immobilized metal ion affinity chromatography. The fusion protein was then subjected to enzymatic hydrolysis at the human enterokinase site, and the hydrolysate was separated by RP-HPLC (Fig. 1). The peak on the chromatogram corre-

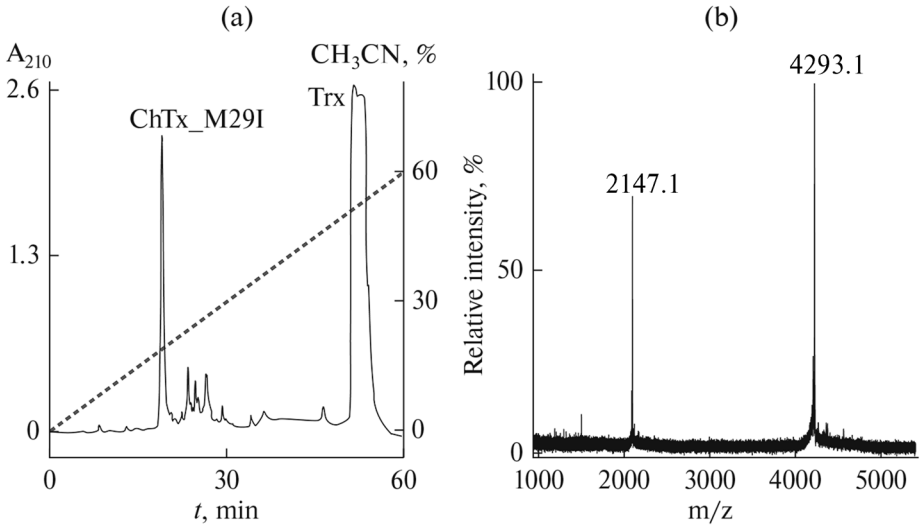

Fig. 1. (a) Chromatographic separation of products of the fusion protein hydrolysis by enteropeptidase. (b) Spectrum of purified ChTx_M29I obtained by MALDI mass spectrometry in the reflectron mode, showing the corresponding monoisotopic masses. The calculated monoisotopic mass $[M+H]^+$ is 4293 Da, and the experimentally measured mass is also 4293 Da.

Table 2. Pharmacological characterization of charybdotoxin (ChTx) and its mutant ChTx_M29I

Peptide	Sequence	K_V1.1	K_V1.2	K_V1.3	K_V1.6
ChTx	ZFTNVSCTTSKECWSVCQRLHNTSRGKCMNKKCRCYS	1500	9	0.19	22
ChTx_M29I	QFTNVSCTTSKECWSVCQRLHNTSRGKCINKKCRCYS	2000/3.1[1]	0.006	4.1	2000/9.6

[1] X/Y, where X—peptide concentration, Y—percent of inhibition. Cysteine residues are underlined, and the position of the substitution is double underlined. Z indicates a pyroglutamic acid residue. The values of K_d (for ChTx) [10, 17] and IC_{50} (for ChTx_M29I) are indicated in nM.

sponding to the ChTx_M29I peptide was determined by MALDI mass spectrometry. The yield of the target peptide was 4 mg from 1 liter of medium.

Electrophysiological study of the obtained peptide. We characterized the ChTx_M29I peptide pharmacologically using the two-electrode voltage clamp technique on a panel of K_V1 channels. Compared with ChTx, it presents much lower affinity for K_V1.1 and 1.6 channels: at a concentration of 2 μM, it blocks these channels by 3.1 ± 2.7% and 9.6 ± 0.6%, respectively (Fig. 2, Table 2). Among K_Vs, ChTx exhibited selectivity toward the K_V1.3 channel (Table 2), whereas the resulting mutant was 1500 times more active toward the K_V1.2 channel (IC_{50} = 6 ± 0.4 pM) and 20 times less active against K_V1.3 (IC_{50} = 4.1 ± 0.8 nM). Thus, the ratio between the IC_{50} values with respect to the K_V1.2 and 1.3 channels is 680-fold, making ChTx_M29I one of the most selective K_V1.2 ligands.

Previously, Miller et al. suggested that there is an interaction between the ChTx toxin residue M29 and the Shaker channel residue T449 (Table 3). This leads to a high-affinity complex ($K_d \approx 0.063$ nM) [11]. Introducing substitutions into the toxin (M29I) and channel (T449F) produced a less stable complex ($K_d \approx 1100$ nM). So, the introduction of a large aromatic residue to the 449 position of the channel leads to an extreme deterioration in the binding of the ChTx_M29I derivative. In the case of human K_V1 isoforms, the deterioration of binding of this derivative to the K_V1.1 and 1.6 channels is not unexpected because the corresponding position contains a large aromatic residue (Y379 and Y429, respectively; Table 3).

In the case of K_V1.3 we also observe a decrease in affinity, which can similarly be attributed to the presence of a large residue (H451) in this position.

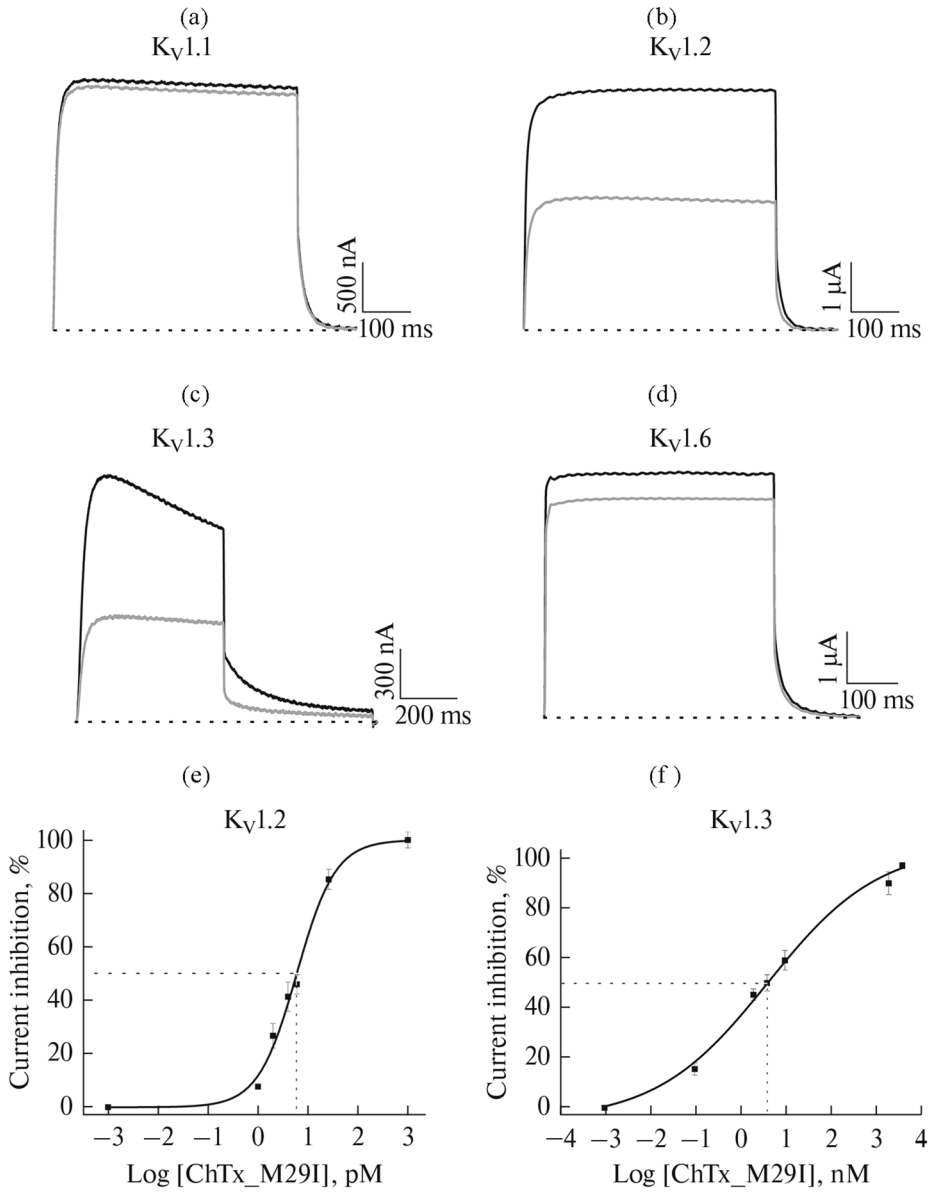

Fig. 2. Pharmacological characterization of ChTx_M29I. (a–d) Recordings of currents through the oocyte membrane in control (black curves) and in the presence of the peptide (2 μM for $K_V1.1$ and 1.6, 6 pM for $K_V1.2$ and 4 nM for $K_V1.3$; gray curves). (e, f) Dose-response curves for channels $K_V1.2$ and 1.3, the dotted lines show IC_{50} values. The Hill coefficients (h) are 1.1 ± 0.1 and 0.35 ± 0.02, respectively.

In turn, the $K_V1.2$ channel has a relatively small hydrophobic residue (V381) in this position; van der Waals interactions with it seem to explain the high affinity of ChTx_M29I to this channel. To interpret the observed effects, we can consider the already known structure of the ChTx complex with the $K_V1.2/2.1$ chimera [18]. We assume that ChTx_M29I is located in the vestibule of the channel pore in the same way as ChTx itself. In Fig. 3, we can see that the residue M29 of the toxin and V381 of the channel are close together in space. Accordingly, if the channel has a large aromatic residue in this position, it leads to steric hindrance and decreased affinity.

A toxin from the venom of the scorpion *Mesobuthus eupeus* named MeKTx11-1 (α-KTx1.16) and showing high affinity to $K_V1.2$ ($IC_{50} \approx 0.2$ nM) was previously obtained and characterized in our laboratory [19]. However, this toxin is inferior in both affinity and selectivity to the

Table 3. Comparison of amino acid sequences of the pore region of Shaker and K_V1 channels

Shaker	416	FAEAGSENSFFKSIPDAFWWAVVTMTTVGYGDM**T**PVGVW	454
hK_V1.1	346	FAEAEEAESHFSSIPDAFWWAVVSMTTVGYGDM**Y**PVTIG	384
hK_V1.2	348	FAEADERESQFPSIPDAFWWAVVSMTTVGYGDM**V**PTTIG	386
hK_V1.3	418	FAEADDPTSGFSSIPDAFWWAVVTMTTVGYGDM**H**PVTIG	456
hK_V1.6	396	FAEADDDDSLFPSIPDAFWWAVVTMTTVGYGDM**Y**PMTVG	434

Differing residues are underlined, and the key residues presumably interacting with the M/I29 residue of toxin are shown in bold.

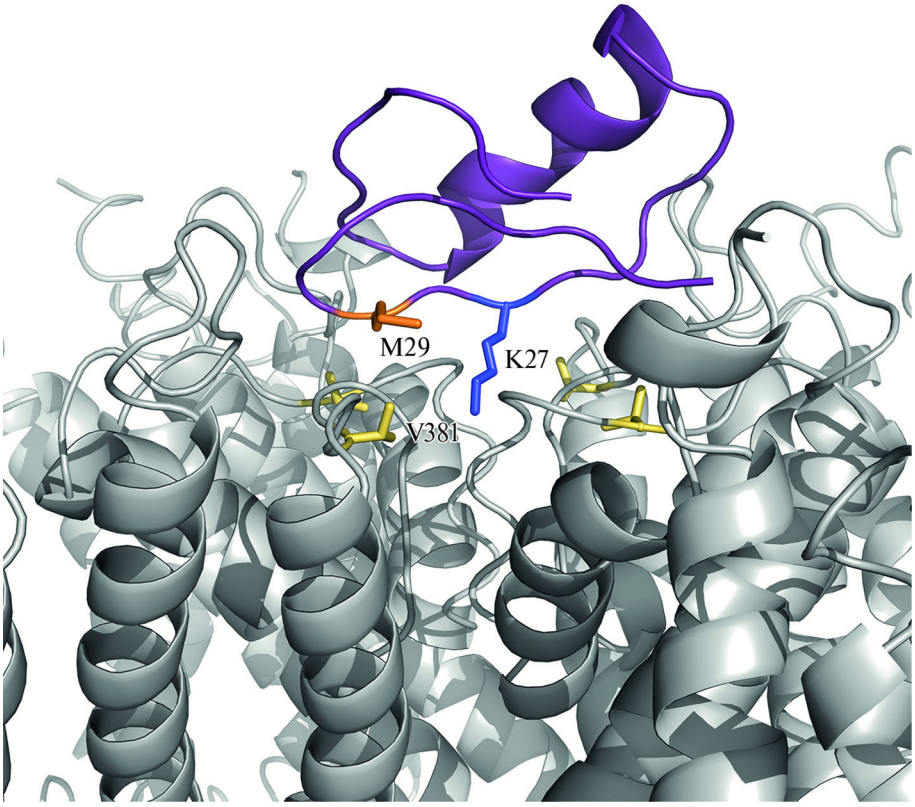

Fig. 3. Structure of the ChTx complex with the K_V1.2/2.1 channel. The channel is shown in gray, and residue V381 is highlighted in yellow; the toxin is shown in purple, K27 in blue, and M29 in orange.

ChTx_M29I peptide discussed in this article. MeKTx11-1 has a methionine residue in its structure at position 29, which suggests that a more selective ligand could be obtained by making the same substitution M29I.

In order to compare our results with the literature, a list of selective ligands of the K_V1.2 channel was compiled using the Kalium database (Table 4). Most of them are scorpion toxins, but several toxins of sea anemones, cone snails, and snakes can also be noted. The general trend observed is that most of the ligands are not highly selective. In addition to MeKTx11-1, urotoxin [20] and mesomartoxin (MMTX) [21], which have comparable selectivity to ChTx_M29I, but whose affinity is significantly lower, are worth mentioning. Finally, Pi-4 from the venom of the scorpion *Pandinus imperator* [22] has similar affinity and even exceeds ChTx_M29I in selectivity, but the activity of this peptide has not been

Table 4. List of known polypeptide ligands, selective to $K_V1.2$

	$K_V1.1$	$K_V1.2$	$K_V1.3$	$K_V1.6$	Reference
ChTx_M29I	2000/3[1]	0.006[2]	4.1	2000/10	This work
MeKTx11-1	2110	0.19	67	8900	[19]
MeKTx11-3	130	3.1	78	910	[19]
Css20	>10[3]	1.3	7.2	[4]	[23]
Toxin II.10.4	>10	3.6	~72		[24]
Toxin II 10.5	>10	0.3	8.3		[24]
Toxin II.12.5	>10	0.7	26.2		[24]
Toxin II.12.8	4.8	2.9	>10		[24]
TsTX-K-alpha	1000/85	0.2	1000/85	1000/94	[25, 26]
Tst26	>10	1.9	10.7		[27]
Pi-1	>5000	0.44	9.7		[28, 29]
Maurotoxin	45	0.8	180		[30]
Pi-4	>10000	0.008	>10000		[22]
Urotoxin	253	0.16	91		[20]
OdK1	>400	183	>400		[31]
Kbot1	145	2.5	15		[32]
CoTx1	24400	27	5300		[33]
OsK-2	>250	97	>250		[34]
Ts15	500/10	196	508	500/20	[35]
MMTX	>50000	15.6	12500		[21]
Bcs3a	405	0.03	74	1.31	[36]
Bcs4a	3000/54	173	1007	2246	[36]
RIIIJ	~4000	33	~10000	~8000	[37]
RIIIK	>10000	280	>10000	5000/10	[38, 39]
α-DTX	9.4	0.38	>100	9	[40–42]
DTX-I	3.1	0.13	4533	10/26	[43–45]

[1] X/Y, where X is the concentration of peptide, nM, and Y is the percent of inhibition. [2] IC_{50}, nM. [3] Ligand is not active in concentration more than the indicated value in nM. [4] No data available.

studied against $K_V1.6$.

Mutations in the $K_V1.2$ channel gene can lead to epileptic encephalopathy [6]. These are mainly loss-of-function mutations, in which the channel loses its ability to open in response to a depolarizing stimulus. However, pathogenic $K_V1.2$ gain-of-function mutations are known to cause a shift in the threshold potential of channel activation into the negative direction. In such a case, selective blockers could just find application as a pharmacological agent correcting the function of the mutant channel.

CONCLUSIONS

In this work, we demonstrated that a single amino acid substitution can lead to a significant change in the affinity and selectivity of a potassium channel ligand using the well-studied charybdotoxin (ChTx) as an example. The M29I substitution led to a decrease in the affinity of the toxin with respect to $K_V1.1$, 1.3, and 1.6 channels, and we observed an increase in affinity of more than 1500-fold ($IC_{50} \approx 6$ pM) with respect to the $K_V1.2$ channel. The resulting peptide ended up

being a high-affinity and high-selectivity ligand of the K_V1.2 channel.

AUTHORS' CONTRIBUTION

A.M.G. and A.A.V. planned the study. A.M.G. performed biochemical experiments and obtained recombinant peptides. S.P. and E.L.P.-J. performed electrophysiological experiments. A.A.V. supervised the biochemical experiments. J.T. supervised the electrophysiological experiments. A.M.G. and A.A.V. wrote the article.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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