Biochemical and Biophysical Research Communications 444 (2014) 406–410

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Kv1.3 potassium channel-blocking toxin Ctri9577, novel gating modifier of Kv4.3 potassium channel from the scorpion toxin family

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ARTICLE INFO

Article history:
Received 15 January 2014
Available online 25 January 2014

Keywords:
Scorpion toxin
Gating modifier
Channel blocker

ABSTRACT

Scorpion toxin Ctri9577, as a potent Kv1.3 channel blocker, is a new member of the α-KTx15 subfamily which are a group of blockers for Kv4.x potassium channels. However, the pharmacological function of Ctri9577 for Kv4.x channels remains unknown. Scorpion toxin Ctri9577 was found to effectively inhibit Kv4.3 channel currents with IC_{50} value of 1.34 ± 0.03 μM. Different from the mechanism of scorpion toxins as the blocker recognizing channel extracellular pore entryways, Ctri9577 was a novel gating modifier affecting voltage dependence of activation, steady-state inactivation, and the recovery process from the inactivation of Kv4.3 channel. However, Ctri9755, as a potent Kv1.3 channel blocker, was found not to affect voltage dependence of activation of Kv1.3 channel. Interestingly, pharmacological experiments indicated that 1 μM Ctri9755 showed less inhibition on Kv4.1 and Kv4.2 channel currents. Similar to the classical gating modifier of spider toxins, Ctri9577 was shown to interact with the linker between the transmembrane S3 and S4 helical domains through the mutagenesis experiments. To the best of our knowledge, Ctri9577 was the first gating modifier of potassium channels among scorpion toxin family, and the first scorpion toxin as both gating modifier and blocker for different potassium channels. These findings further highlighted the structural and functional diversity of scorpion toxins specific for the potassium channels.

1. Introduction

Scorpion toxins specific for K^+ channels (KTx) are a large family of peptide blockers with the diverse structures and functions [1]. Structurally, these toxins typically contain about 30–40 amino acid residues with 3–4 disulfide bridges usually linking an helix and two- or three-stranded β-sheet structures [1–3]; Functionally, scorpion toxins are found to interact with different potassium channels including voltage-gated Kv channels (such as Kv1.1, Kv1.2, Kv1.3 and Kv11.1) [1], small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) [1,4], intermediate conductance Ca^{2+}-activated K^+ channels (IK_{Ca}) [1,5] and big conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) [1,6]. More interestingly, some scorpion toxins sometimes recognize different pharmacological targets with the same binding modes. For example, charybdotoxin can bind Kv1.3, IK_{Ca} and BK_{Ca} channels, and maurotoxin can associate with Kv1.2 and IK_{Ca} channels through interacting with the channel pore regions [1,5,7]. Due to the structural diversity of both scorpion toxins and potassium channels, the potential function of toxins affecting more different potassium channels would be intensely explored nowadays.

At present, scorpion toxins from the α-KTx15 subfamily (such as Aa1, BmTX3, AmmTX3, etc.) were found to be able to block transient or A-type K^+ currents in the cultured neurons, and these currents have been shown to be mediated by Kv4.x potassium channels [8]. Pharmacological experiments further indicated that scorpion toxin BmTX3 was able to effectively block Kv4.1 channel currents and less effective on Kv4.2 and Kv4.3 channels [9]. Toxin AmmTX3 was recently found to effectively block the currents of Kv4.2 and Kv4.3 channels in the presence of the auxiliary subunits DPP6 and DPP10 [10]. Besides the blockade activity of scorpion toxin Aa1 towards Kv4 channels [11], it was also found to block *Shaker* K^+ channel with an IC_{50} value of 4.5 μM [12], which suggested scorpion toxins in the α-KTx15 subfamily would be able to target different types of potassium channels.

Recently, a new member of the α-KTx15 subfamily, scorpion toxin Ctri9577, was found effectively inhibited Kv1.3 channel currents with an IC_{50} value of 0.49 ± 0.45 nM while it hardly affected the currents of Kv1.1, Kv1.2, Kv11.1 and SK3 potassium channels [13]. According to the pharmacological features of scorpion toxins in the α-KTx15 subfamily, the pharmacological activity of toxin Ctri9577 was further investigated on Kv4.1, Kv4.2 and Kv4.3 channels in this work. It was found that Ctri9577 could efficiently inhibit Kv4.3 channel currents with an IC_{50} value of 1.34 ± 0.03 μM.

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http://dx.doi.org/10.1016/j.bbrc.2014.01.094

Furthermore, the combined kinetic and mutagenesis experiments indicated that toxin Ctri9577 could affect the gating kinetics of Kv4.3 channel. To the best of our knowledge, toxin Ctri9577 was the first gating modifier among the known scorpion toxins affecting potassium channels. These findings revealed that Kv1.3 channel-blocking toxin Ctri9577 was able to recognize Kv4.3 potassium channels with unique gating modifying mechanism, which further enriched the knowledge of functional and mechanic diversity of scorpion toxins specific for the potassium channels.

2. Materials and methods

2.1. Ctri9577 synthesis

Recombinant Ctri9577 (NCBI entry P0DJO5) was produced in *Escherichia coli* and purified as previously described [13].

2.2. Expression of Kv4 channels

The cDNAs encoding mKv4.1, rKv4.2 and rKv4.3 (Kindly gifted by Dr. Michael Morales, State university of New York at Buffalo, USA) were respectively subcloned into pIRES2-EGFP, pEGFP-N1 and pcDNA3 vectors (Clontech, USA) for coexpression with green fluorescent protein (GFP), to ensure the expression of Kv4.1, Kv4.2 and Kv4.3 channels through visual inspection.

HEK293T cells (CCTCC, China) were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum (Invitrogen) supplemented with ampicillin 100 units/mL and streptomycin 100 μg/mL. Plasmids containing mKv4.1, rKv4.2 and rKv4.3 were respectively transfected into HEK293 cells using TurboFect in vitro Transfection Reagent (Fermentas, Europe), and currents were measured 1–2 days after transfection. DNA sequencing (Sunbiotech, China) was performed to verify all the constructions. Kv4.3 mutant channels with respective sites from the linker of helix S3 and S4, were prepared by performing PCR-based site-directed mutagenesis as previously described [14].

2.3. Electrophysiological recording and data analysis

Currents from HEK293T cells expressing Kv4.3 channels were measured using the whole cell patch clamp technique at room temperature. Current measurements and data acquisition were performed with an EPC 10 patch clamp amplifier (HEKA Elektronik, Germany) controlled by a PULSE software (HEKA Elektronik). For measuring Kv1.3, Kv4.1, Kv4.2 and Kv4.3 currents, the internal pipette solution contained (in mM): 140 KCl, 1 $MgCl_2$, 1 EGTA and 5 HEPES (pH 7.2 with KOH), and the external solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose and 10 HEPES (pH 7.4 with NaOH). The Kv4.1, Kv4.2 and Kv4.3 currents were elicited by depolarizing the voltage steps of 500 ms from the holding potential −80 mV to +30 mV. Ctri9577 peptide was dissolved in external solution containing 0.01% BSA for toxin application in electrophysiological experiments. A multichannel micro perfusion system MPS-2 (INBIO Inc., Wuhan, China) was used to exchange the external recording bath solution.

A one-pulse protocol was used to measure the steady-state activation of Kv1.3 channel. From the holding potential −80 mV, 200-ms voltage steps were applied from −80 mV to 0 mV in 5-mV increments. Steady-state inactivation relationships for the open state of Kv4.3 channel were determined using a two-pulse protocol as described previously, the voltage steps were applied from −100 mV to 100 mV in 10-mV increments [15]. Recovery from open-state inactivation of Kv4.3 channel was measured using a two-pulse protocol: pulses to +50 mV (P1) for 800 ms from a holding potential at −80 mV were followed by a second 200 ms depolarization (P2) to +50 mV with a variable time gap [16,17].

Results are mostly shown as mean ± S.E., n being the number of individual experiments. Current amplitudes were measured using Pulse as acquisition software while Data was analyzed with Sigmaplot 9 (SPSS Inc. USA). Concentration–response relationships were fitted according to modified Hill equation: $I_{toxin}/I_{control} = 1/1 + ([toxin]/IC_{50})$, where I is the peak current and [toxin] is the concentration of toxin. Concentrations of half maximal effect (IC_{50}) were the parameters that need to be fitted. A fourth power Boltzmann function ($f(V) = 1/\{1 + \exp[-(V - V_{1/2})/k]\}^4$) was used to fit the data to calculate the steady-state activation relationship, where $V_{1/2}$ and k are the half-activation potential and the slope factor, respectively. Steady-state inactivation relationships were fitted by the Boltzmann function $f(V) = 1/\{1 + \exp[(V - V_{1/2})/k]\}$, where $V_{1/2}$ and k are the half-inactivation potential and the slope factor, respectively. The kinetics of recovery from open-state inactivation were determined by fitting recovery of the normalized peak current by a single exponential function, $f_{rec}(t) = A * \exp(-t/\tau) + C$, where A is the initial amplitude, t is the time for recovery, τ is the time constant and C is the baseline.

3. Results

3.1. Ctri9577, a gating modifier of Kv4.3 potassium channel

Scorpion toxin Ctri9577 is a new member in the α-KTx15 subfamily, and it was found to effectively inhibit Kv1.3 potassium channel currents with IC_{50} value of 0.49 nM [13]. According to the pharmacological features of scorpion toxins from the α-KTx15 subfamily blocking Kv4.x channel currents [8,10], the effect of Ctri9577 on Kv4.3 channel was investigated in this work.

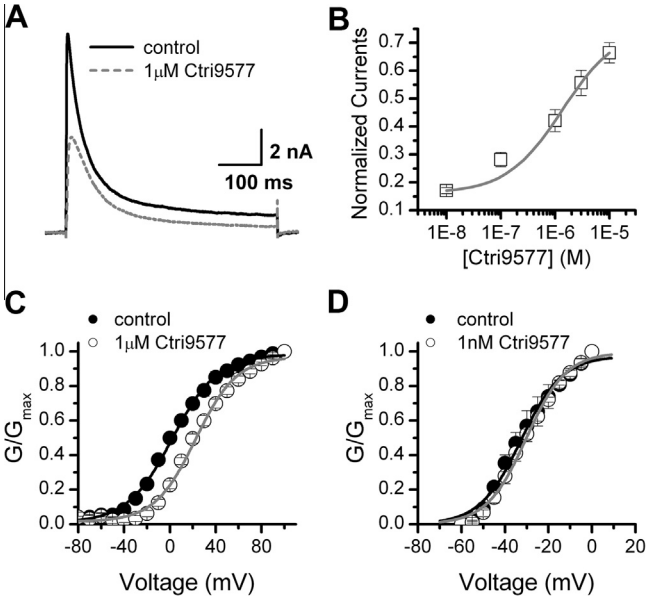

Fig. 1. Inhibitory effects of Ctri9577 on Kv4.3 potassium channel. (A) Inhibiting influence of 1 μM Ctri9577 on Kv4.3 channel at +30 mV. (B) Average normalized current inhibition by various concentrations of Ctri9577 for Kv4.3 channels. Hill equation fitting gives an IC_{50} value of 1.34 ± 0.03 μM (n = 5). (C) The activation G–V curves of Kv4.3 channel before and after applying 1 μM Ctri9577. Solid lines indicate Boltzmann fitting before (black) and after (grey) applying toxin. $V_{1/2}$ values are 2.2 ± 1.0 mV and 22.4 ± 1.6 mV, k values are 19.6 ± 1.0 and 17.9 ± 1.5 respectively (n = 6). (D) The activation G–V curves of Kv1.3 channel before and after applying 1 nM Ctri9577. Solid lines indicate Boltzmann fitting before (black) and after (grey) applying toxin. $V_{1/2}$ values are −30.2 ± 1.0 mV and −32.6 ± 1.3 mV, k values are 9.1 ± 0.7 and 9.5 ± 1.0 respectively (n = 3). Data represent the mean ± S.E. of at least three experiments.

Table 1
Effects of toxin Ctri9577 on peak *I–V*, G/G_{max}, steady-state inactivation in Kv4.3, Kv4.3(L275A), Kv4.3(V276A), Kv4.3(N280A), Kv4.3(E281A), Kv4.3(V288A), and Kv4.3(L275A/V276A).

	n	Peak *I–V*	G/G_{max}		SSI	
			$V_{1/2}$, mV	*k*	$V_{1/2}$, mV	*k*
Kv4.3 Control	6	1.00	2.2 ± 1.0	19.6 ± 1.0	−37.1 ± 1.4	7.7 ± 0.5
Kv4.3 + 1 μM 9577	6	0.57 ± 0.06†	22.4 ± 1.6†	17.9 ± 1.5	−18.8 ± 1.9*	9.3 ± 0.7
Kv4.3(L275A) Control	4	1.00	1.3 ± 1.9	19.3 ± 2.2	−39.5 ± 2.0	6.6 ± 0.4
Kv4.3(L275A) + 1 μM 9577	4	0.72 ± 0.01†	19.5 ± 2.2†	16.5 ± 2.0	−21.7 ± 0.6*	8.2 ± 0.5
Kv4.3(V276A) Control	4	1.00	−6.3 ± 2.6	25.3 ± 2.7	−34.3 ± 0.7	5.8 ± 0.2
Kv4.3(V276A) + 1 μM 9577	4	0.70 ± 0.01†	21.2 ± 1.7*	20.9 ± 1.7	−19.3 ± 1.8†	5.0 ± 0.1
Kv4.3(N280A) Control	8	1.00	5.3 ± 1.2	15.2 ± 0.3	−28.6 ± 0.9	8.4 ± 0.4
Kv4.3(N280) + 1 μM 9577	8	0.74 ± 0.03†	2.3 ± 2.3	16.6 ± 0.5	−28.4 ± 2.3	8.1 ± 0.3
Kv4.3(E281A) Control	5	1.00	4.3 ± 3.6	27.1 ± 3.5	−28.0 ± 1.2	7.7 ± 0.4
Kv4.3(E281A) + 1 μM 9577	5	0.59 ± 0.01†	24.4 ± 2.0†	18.7 ± 1.9*	−7.0 ± 0.8†	9.7 ± 1.6
Kv4.3(V288A) Control	4	1.00	−2.8 ± 2.5	33.1 ± 2.9	−43.9 ± 2.8	8.0 ± 0.6
Kv4.3(V288A) + 1 μM 9577	4	0.72 ± 0.02†	3.9 ± 1.8	21.5 ± 1.8	−35.1 ± 1.8	9.0 ± 0.8
Kv4.3(L275A/V276A) Control	5	1.00	N.D.	N.D.	N.D.	N.D.
Kv4.3(L275A/V276A) + 1 μM 9577	5	0.84 ± 0.07*	N.D.	N.D.	N.D.	N.D.

Values are means ± S.E.; *n* = no. of cells. Peak *I–V*, peak current–voltage relationship; G/G_{max}, normalized conductance; SSI, steady-state inactivation; $V_{1/2}$, half-activation and half-inactivation potentials; *k*, slope factor; N.D., not determined.
* $p < 0.05$ vs. control.
† $p < 0.01$ vs. control.

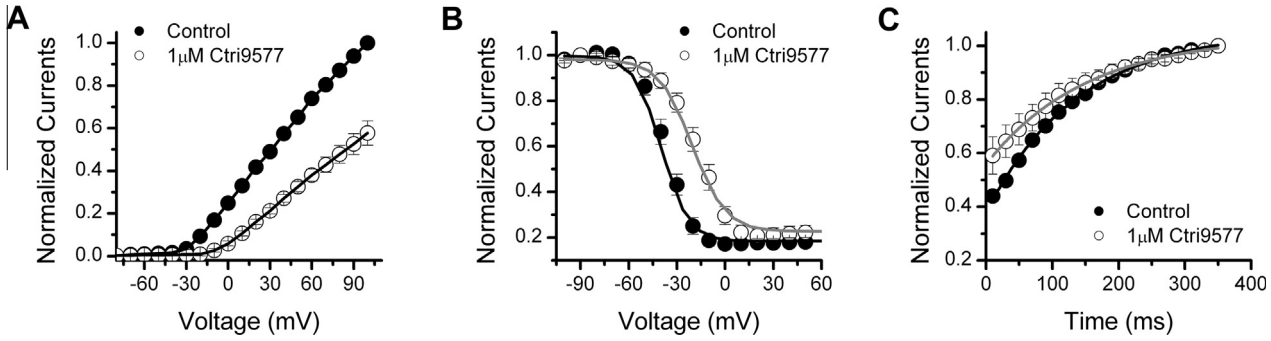

Fig. 2. The influence of Ctri9577 on Kv4.3 channel gating kinetics. (A) Current–voltage relationship curves of Kv4.3 channel before and after applying 1 μM Ctri9577. (B) Steady-state inactivation curves of Kv4.3 channel before and after applying 1 μM Ctri9577. Solid lines indicate Boltzmann fitting before (black) and after (grey) applying toxin. $V_{1/2}$ values are −37.1 ± 1.4 mV and −18.8 ± 1.9 mV, *k* values are 7.7 ± 0.5 and 9.3 ± 0.7 respectively. (C) Recovery curves from open-state inactivation of Kv4.3 channel before and after applying 1 μM Ctri9577. Solid lines indicate single exponential fitting before (black) and after (grey) applying toxin. Recovery from open-state inactivation time constants are 178.4 ± 7.7 ms and 213.5 ± 23.3 ms. Data represent the mean ± S.E. of at least three experiments.

As shown in Fig. 1A, 1 μM Ctri9577 was able to inhibit 43 ± 6% of Kv4.3 channel currents ($n = 6$). Dose–response analyses provided IC_{50} value for Ctri9577-induced current inhibition of 1.34 ± 0.03 μM (Fig. 1B, $n = 5$). To characterize whether Ctri9577 was a blocker same as other members in the α-KTx15 subfamily [8,10], the activation *G–V* curves of Kv4.3 potassium channel were also measured in the absence and presence of Ctri9577. As shown in Fig. 1C, the *G–V* curve of Kv4.3 potassium channel had a significant shift in the presence of 1 μM Ctri9577, and the $V_{1/2}$ values changed from 2.2 ± 1.0 mV in the absence of Ctri9577 to 22.4 ± 1.6 mV in the presence of Ctri9577 (Fig. 1C and Table 1). However, 1 nM Ctri9577 did not significantly change the *G–V* curve of Kv1.3 potassium channel, and the $V_{1/2}$ values were −30.2 ± 1.0 mV and −32.6 ± 1.3 mV in the absence and presence of Ctri9577, respectively (Fig. 1D). These differential activation *G–V* curves demonstrated that Ctri9577 was a gating modifier of Kv4.3 potassium channel.

3.2. Effects of Ctri9577 on gating kinetics of Kv4.3 channel

Next, the effects of Ctri9577 on gating kinetics of Kv4.3 potassium channel were further characterized in this work. The current–voltage relationship of Kv4.3 channel was first evaluated in the presence of 1 μM Ctri9577. As shown in Fig. 2A, there is stronger inhibition of Kv4.3 channel currents at more negative voltage. During the steady-state inactivation process, a significant difference was observed for Kv4.3 channel in the absence and presence of 1 μM Ctri9577, $V_{1/2}$ changed from −37.1 ± 1.4 mV to −18.8 ± 1.9 mV (Fig. 2B and Table 1). In addition, the effect of Ctri9577 on the recovery from open-state inactivation of Kv4.3 channel was also examined. The recovery from open-state inactivation was fitted by a single exponential function. As shown in Fig. 2C, the recovery from open-state inactivation was slower after application of 1 μM Ctri9577, and however the time constant for recovery from open-state inactivation was increased from 178.4 ± 7.7 ms to 213.5 ± 23.3 ms ($n = 5$). These results revealed that toxin Ctri9577 could remarkably affect the gating kinetic features of Kv4.3 channel.

3.3. Ctri9577 with less inhibition activities on Kv4.1 and Kv4.2 channels

Based on the pharmacological properties of Ctri9577 on Kv4.3 channel, the first gating modifier from the scorpion toxins (Fig. 1), we further investigated its effects on other two members of Kv4 subfamily in this work. As shown in Fig. 3A, Ctri9577 could inhibit about 14 ± 5% of Kv4.1 channel currents at a concentration of 1 μM. As for Kv4.2 channel, its currents were also less inhibited

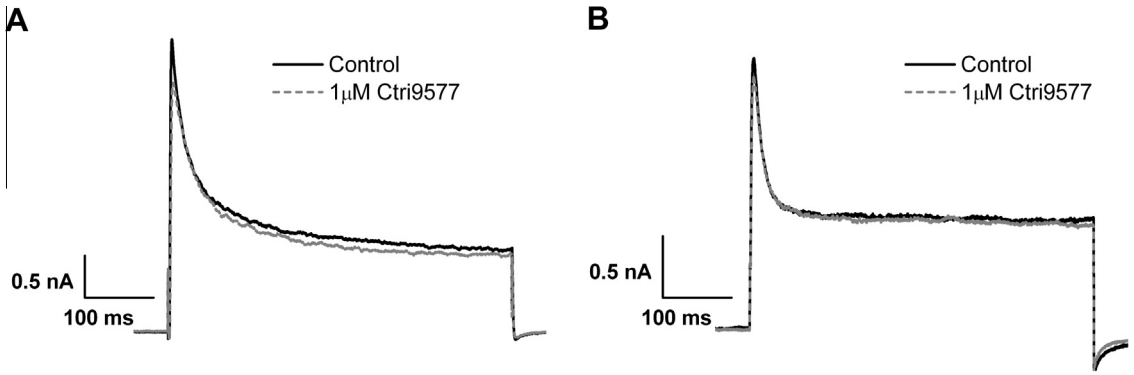

Fig. 3. Inhibition of Kv4.1 and Kv4.2 channel currents by Ctri9577. (A) Inhibiting effect of 1 μM Ctri9577 on Kv4.1 channel at +30 mV. (B) Inhibiting effect of 1 μM Ctri9577 on Kv4.2 channel at +30 mV.

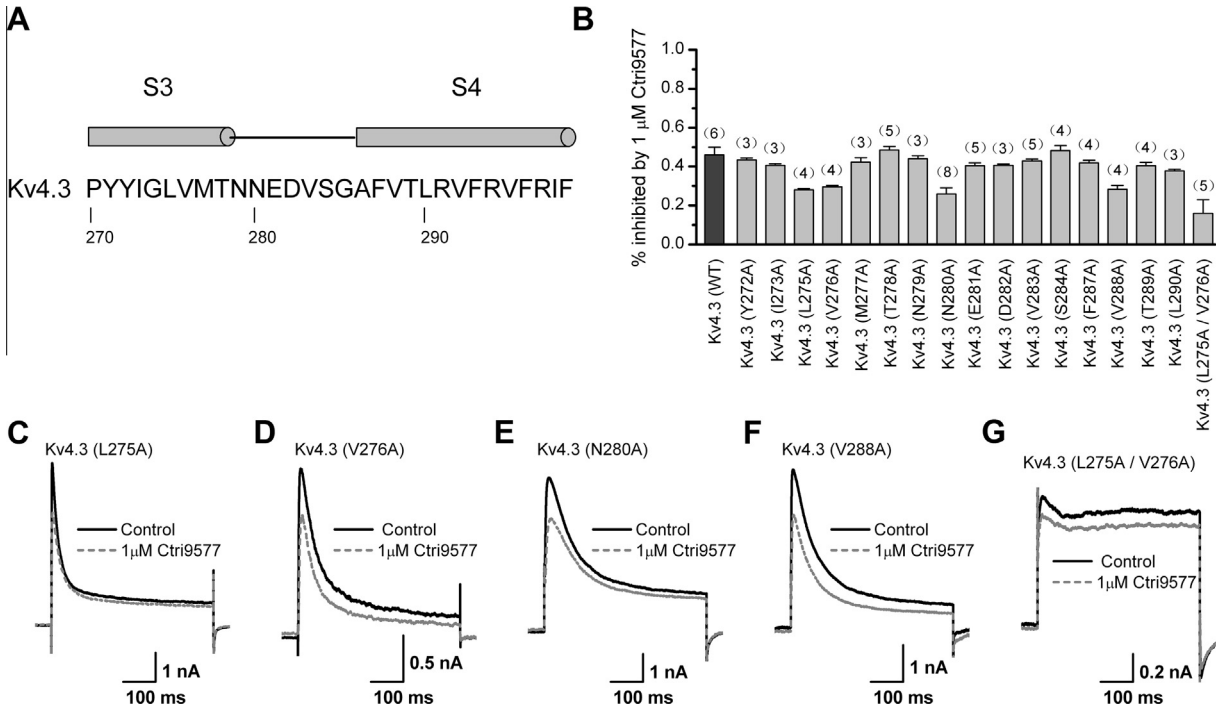

Fig. 4. Inhibition of Kv4.3 mutant channels by Ctri9577. (A) Sequence of S3–S4 linker region in Kv4.3 channel. (B) Average inhibition of wild-type and mutant Kv4.3 channel currents by 1 μM Ctri9577. Data represent the mean ± S.E. of at least three experiments ($p < 0.05$). (C–G) Representative current traces of Kv4.3 channel mutants showing decreased inhibition by 1 μM Ctri9577 than wild type Kv4.3 channel.

by toxin Ctri9577 (Fig. 3B). These data indicated that Ctri9577 was a relatively selective gating modifier for Kv4.3 channel.

3.4. The binding sites of Ctri9577 on Kv4.3 channel

Scorpion toxin Ctri9577 as a gating modifier of Kv4.3 channel, it would likely interact with the linker of transmembrane helix S3 and S4 in Kv4.3 channel, which was same as that of spider toxin Heteropoda venatoria Toxin 2 (HpTX2) as a gating modifier modulating Kv4.3 channel [14,18]. In order to test this inference, we explored the binding sites of toxin Ctri9577 in channel S3–S4 linker by the alanine-scanning strategy. In comparison with the inhibition capability of wild-type toxin Ctri9577, four residues L275, V276, N280, V288 were found more important for toxin Ctri9577 binding since 1 μM Ctri9577 caused 28 ± 1%, 30 ± 1%, 26 ± 3%, and 28 ± 2% decrease of potassium currents for Kv4.3(L275A), Kv4.3(V276A), Kv4.3(N280A) and Kv4.3(V288A), channels, respectively (Fig. 4 and Table 1). Based on the functional effects of L275 and V276 residues, a double mutant Kv4.3(L275A/V276A) channel was further constructed. As shown in Fig. 4, 1 μM Ctri9577 caused 16 ± 7% decrease of potassium currents for Kv4.3(L275A/V276A) channel, whose effect was more significant than those of both Kv4.3(L275A) and Kv4.3(V276A) channels. Besides the effects of Kv4.3 channel residues on toxin affinity, we also investigated the changes of Kv4.3 channel kinetics with and without Ctri9577. For two Kv4.3(L275A) and Kv4.3(V276A) with lower toxin sensitivity, and Kv4.3(E281A) channel with the higher toxin sensitivity, the $V_{1/2}$ values from the activation and steady-state inactivation were found to have a significant shift in the presence of toxin Ctri9577 (Table 1). Meanwhile, the gating kinetics of Kv4.3(N280A) and Kv4.3(V288A) channels with lower toxin sensitivity were less influenced by Ctri9577 (Table 1). In addition, currents elicited from Kv4.3(L275A/V276A) channel were much small so that its kinetics could not be determined. In summary, these data demonstrated that four L275, V276, N280 and V288 residues were important for the interaction between Ctri9577

and Kv4.3 channel, and the channel S3–S4 linker was responsible for the toxin binding.

4. Discussion

Scorpion toxins specific for K^+ channels are a large family of peptides blockers which recognize channel pore region by plugging the sidechain of the conserved basic residue into the selectivity filter [5,6,19–22]. To the best our knowledge, no scorpion toxin as the gating modifier for K^+ channels has been identified so far.

In this work, scorpion toxin Ctri9577, a new member of α-KTx15 subfamily interacting with Kv4.x potassium channels [8–11], was found to be a novel gating modifier instead of the classical channel pore blocker for Kv4.3 potassium channel. Ctri9577 was found to effectively inhibit Kv4.3 potassium channel currents with IC_{50} value of 1.34 ± 0.03 μM (Fig. 1A and B), and this inhibition also affected the gating kinetics of Kv4.3 channel (Figs. 1C and 2). However, the blocking process of Ctri9577 is not voltage dependence for Kv1.3 potassium channel (Fig. 1D), which means that toxin Ctri9577 effectively blocked the currents of Kv1.3 channels at nanomolar concentration level through the interaction between toxin and the pore region of Kv1.3 channel [13]. Different from the interaction mode between Ctri9577 and Kv1.3 channel, the key functional residues in the S3–S4 linker region in Kv4.3 channel were revealed for toxin Ctri9577 binding (Fig. 4). These data indicated that Ctri9577 would like use different molecular surfaces to respectively recognize Kv1.3 and Kv4.3 channels, and become the first scorpion toxin modulating the different potassium channels with two distinct mechanisms so far.

As for other scorpion toxins from the α-KTx15 subfamily, they were the classical channel blockers without changing the gating kinetics of Kv4.x channels [8,10]. Such different functions between Ctri9577 and other members in the α-KTx15 subfamily were likely determined by their different structures. The sequence alignment indicated that Ctri9577 displayed lower than 50% identity with other scorpion toxins from the α-KTx15 subfamily [13], such as AmmTX3 [8,10], Aa1 [23], BmTX3 [24] and Discrepin [25]. At present, there is no clear structure–function relationship to be elucidated for scorpion toxins in the α-KTx15 subfamily. Therefore, it is essential to resolve the spatial structure of Ctri9577, and further elucidate the effects of the differential residues on toxin functions as the channel gating modifier or the pore blocker near the future.

In conclusion, the pharmacological function of scorpion toxin Ctri9577 was found to be a novel gating modifier of Kv4.3 potassium channel with IC_{50} value of 1.34 ± 0.03 μM. Mutagenesis experiments further indicated that Ctri9577, like the classical gating modifier of spider toxins, interacted with the linker between the transmembrane S3 and S4 helical domains. To the best of our knowledge, scorpion toxin Ctri9577 was a both novel gating modifier and pore blocker for different potassium channels, whose differential mechanisms would be an interesting question among the scorpion toxins specific for potassium channels in the future.

Acknowledgment

This work was supported by grants from the National Basic Research Program of China (2010CB529800), National High Technology Research and Development Program of China (2012AA020304), and National Natural Sciences Foundation of China (number 31170789). New Century Excellent Talents in Wuhan University by the Ministry of Education of China (No. NCET-10-0651), and Wuhan City Science and Technology Foundation of China (No. 2013070204020046).

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