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Residue Phe266 in S5-S6 loop is not critical for Charybdotoxin binding to Ca^{2+}-activated K^+ (mSlo1) channels[1]

Jing YAO[2], Hui LI[2], Ge-liang GAN, Ying WU, Jiu-ping DING[3]

Institute of Biochemistry and Biophysics, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

Key words

charybdotoxin; large-conductance calcium-activated potassium channelss; Slo1

[1] Project supported by Grants from the National Natural Science Foundation of China (30470449).
[2] These authors contributed equally to this work.
[3] Correspondence to Prof Jiu-ping DING.
Phn/Fax 86-27-8779-2024.
E-mail jpding@mail.hust.edu.cn

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Abstract

Aim: To gain insight into the interaction between the Charybdotoxin (ChTX) and BK channels. **Methods:** Site-directed mutagenesis was used to make two mutants: mSlo1-F266L and mSlo1-F266A. The two mutants were then expressed in *Xenopus* oocytes and their effects were tested on ChTX by electrophysiology experiments. **Results:** We demonstrate an equilibrium dissociation constant K_d= 3.1–4.2 nmol/L for both the mutants mSlo1-F266L and mSlo1-F266A similar to that of the wild-type mSlo1 K_d=3.9 nmol/L. **Conclusion:** The residue Phe266 does not play a crucial role in binding to ChTX, which is opposed to the result arising from the simulation of peptide-channel interaction.

Introduction

Large-conductance Ca^{2+}- and voltage-gated potassium channels termed BK channels are widely distributed in many tissues from pancreas to smooth muscle to brain[1]. BK channels play a crucial role in the control of excitability and secretion. Scorpion toxins such as Charybdotoxin (ChTX), Iberiotoxin (IbTX) and Slotoxin (SloTX) are among the most potent and important tools for studying function and structure of ion channels. Most of the scorpion toxins have a well-conserved three-dimensional structure stabilized by three or four disulfide bridges[2]. They bond with high affinity and specificity to the BK channels and causally to the voltage-gated K^+ channels $K_V1.3$[3,4]. The channels encoded with pore-forming Slo1 α and auxiliary β subunits usually have very different sensitivity to toxins in comparison to the channels encoded with Slo1 α subunits alone. Toxins have been used as tools to recognize the existence of β subunits and to identify the stoichiometry of channels by their sensitivity to toxins[5–9]. The reversibility of toxins such as ChTX and IbTX is usually very poor. This made it difficult to study functions of BK currents, especially in current-clamp experiments. Based on the crystal structure of KcsA channels, a docking model predicts that the residue Lys27 of ChTX inserts into the pore to occlude entranceway of ions and the residue Phe266 is one of the binding sites by π-π stacking with the aromatic residues Trp14 and Tyr36 of peptides[4]. To understand the function of the residue Phe266, we mutated the Phe266 to leucine (Leu) or alanine (Ala) to verify whether it is a binding site between the ChTX peptides and the Slo1 channels. In the present work, we report that both the mutations Slo1-F266L and Slo1-F266A have the equilibrium dissociate constant K_d similar to the one of the wild-type mSlo1 channels, but with a perfect reversible recovery. Our results oppose the idea that the residue Phe266 is a site associated tightly with the ChTX peptide, whereas the reason for irreversibility of mSlo1 is still unknown.

Materials and methods

Site-directed mutagenesis The QuikChange protocol (Stratagene) was used to produce two point mutations mSlo1-F266L and mSlo1-F266A. With the wild-type mSlo1 as a template and a pair of complementary mutagenesis primers, the reactions were performed by polymerase chain reaction (PCR). The primers for the mutants mSlo1-F266L and mSlo1-F266A

are 5'-CAGGGGACCCATGGGAAAATCTTCAAAACAA-CCAGGCACTTAC-3'/5'-GTAAGTGCCTGGTTGTTTTGAA-GATTTTCCCATGGGTCCCCTG-3' and 5'-CAGGGGACCC-ATGGGAAAATGCTCAAAACAACCAGGCACTTACG-3'/ 5'-CGTAAGTGCCTGGTTGTTTTGAGCATTTTCCCATGG-GTCCCCTG-3', respectively. Then the enzyme DpnI was used to cut the PCR reaction mixture to digest the template of the wild-type mSlo1. Finally, the PCR products were transformed into competent bacterial cells to amplify the mutated plasmids of mSlo1. All mutant constructs were verified by sequence analysis.

Expression in *Xenopus* oocytes After DNA was linearized with MluI, SP6 RNA polymerase (Roche) was used to synthesize cRNA for oocyte injection. Methods of expression in Stage V–VI *Xenopus* oocytes have been described previously[5]. Oocytes were defolliculated by treatment with 2 mg/mL collagenase I (Sigma-Aldrich Corp, St Louis, MO, USA) in zero-calcium ND-96 solution. Between 2 and 24 h after defolliculation, 1–2 ng of a (mSlo1) cRNA were injected into *Xenopus* oocytes using a Drummond Nanoject II (Drummond Scientific Co, USA). After injection, oocytes were then incubated in ND-96 solution at 18 ºC. Currents were recorded 2–7 d after cRNA injection. The ND-96 solution (pH 7.5) contained (in mmol/L): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 2.5 Na pyruvate, and 10 H^+-HEPES, supplemented with 100 IU/mL penicillin and 100 mg/mL streptomycin (only for incubation).

Solutions Oocytes were bathed in the ND-96 solution. For all the experiments, currents were recorded in outside-out patches. The intracellular recording solution was (in mmol/L): 160 $MeSO_3K$, 10 H^+-HEPES, and 2 $MgCl_2$, adjusted to pH 7.0 with $MeSO_3H$. Pipettes were filled with a solution containing (in mmol/L): 160 $MeSO_3K$, 10 H^+-HEPES, and 5 HEDTA with added Ca^{2+} to make 10 mol/L free Ca^{2+}, as defined by the EGTAETC program (E McCleskey, Vollum Institute), with pH adjusted to 7.0. The solutions for 20 mmol/L Tetraethylammonium chloride (TEA) and 100 nmol/L Charybdotoxin (ChTX) were made by adding the 20 mmol/L TEA and the 100 nmol/L ChTX into the intracellular solution, respectively. All of the chemicals were obtained from Sigma.

Electrophysiology Recording pipettes were used to have a resistance of 2–6 MΩ while filled with internal solution. An outside-out patch was obtained by excising from oocytes. Currents were recorded with the EPC-9 patch-clamp amplifier and PULSE software (HEKA Electronics, Germany). Data were typically collected with a sampling frequency of 20 kHz. Macroscopic records were filtered at 2.9 kHz during digitization.

During experiments, the control, drug and recovery solutions were locally perfused onto the patches via a perfusing pipette with seven solution channels. All experiments were performed at room temperature (22–25 ºC).

Data analysis Data were analyzed with IGOR (Wavemetrics, Lake Oswego, OR, USA), Clampfit (Axon Instruments, Inc USA), and SigmaPlot (SPSS Inc USA) softwares. Unless stated otherwise, the data are presented as mean±SEM, significance was tested by Student's *t*-test, and differences in the mean values were considered significant at $P \leq 0.05$.

The onset and recovery (offset) from blockade by ChTX were fit with the first-order blocking reaction, in which the time constants of onset and offset were given by $\tau_{on}=1/[f\times(\text{drug})+b]$ and $\tau_{off}=1/b$, where f is the forward drug blocking rate in $M^{-1}s^{-1}$, b is the drug dissociation rate in s^{-1}.

During application of drug (for $t_0<t\leq t_1$),

$$I(t)=(I_0-I_{SS})\times\exp(-t/\tau_{on})+I_{SS} \quad (1)$$

During recovery (for $t>t_1$), $I(t)=I_0-(I_0-I_r)\times\exp(-(t-t_1)/t_{off})$ (2)

Where I_0 is the mean control current amplitude, I_{SS} is $I_0\times b/(f\times[\text{drug}]+b)$ and indicated a steady-state level of current during blockade by a given drug concentration, Ir is the empirically determined current that is unblocked at the end of the drug application period, $t=0$ at the time of the drug application, and $t1$ is the time of drug washout. The equilibrium dissociation constant K_d was defined by $K_d=b/[f\times(\text{drug})]$[10].

Results

Large-conductance Ca^{2+}- and voltage-gated K^+ channels (BK channels) encoded by mammalian mSlo1 genes are abundantly distributed in the nervous system. It regulates excitability in response to intracellular Ca^{2+} and membrane potentials. BK channels likely share similar pore structural determinants and sensitivities to toxins with voltage-dependent K^+ channels (K_V channels)[4]. Some peptidyl scorpion toxins such as Charybdotoxin (ChTX) not only block $K_V1.3$ as well as a mutation F425H of shaker channels, but also block the BK currents encoded by both the Slo1 a subunits and the b subunits but with a higher EC_{50}[3,11,12]. In Figure 1, a conserved residue Phe266 (mSlo1) labeled with the symbol ▼ (the upper panel) is supposed to interact with the residues that are highlighted in the lower panel with the same symbol[4,13].

The blocking behaviors of those toxins commonly show poor reversibility, therefore making it difficult to study functions of BK channels, especially in current-clamp experiments[5–7]. For mSlo1 channels (Figure 2A), an approximate 20% irreversible component remains after a 3 min recovery period, compared to the unblocking currents. Fits of the

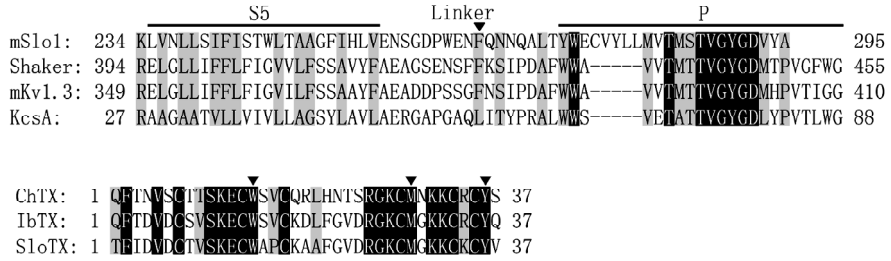

Figure 1. The multi-sequence alignments of some of the K^+ channels and of the scorpion toxin peptides. The multi-sequence alignment within the S5-S6 loop region of mSlo1 and other three K_V channels is shown at the top panel. Residues in which the nature of the side chain is preserved (more than 50% similarity) are marked in grey and homologous residues are marked in black. The symbol ▼ labels the residue F266 of mSlo1 channels and refers to the amino acid that we mutated into leucine (Leu) or alanine (Ala) for this study. The multi-sequence alignment of the scorpion peptides ChTX, IbTX and SloTX is shown at the bottom panel. The symbols ▼ highlight the residues binding with the F266 of the mSlo1 channels with π-π interaction.

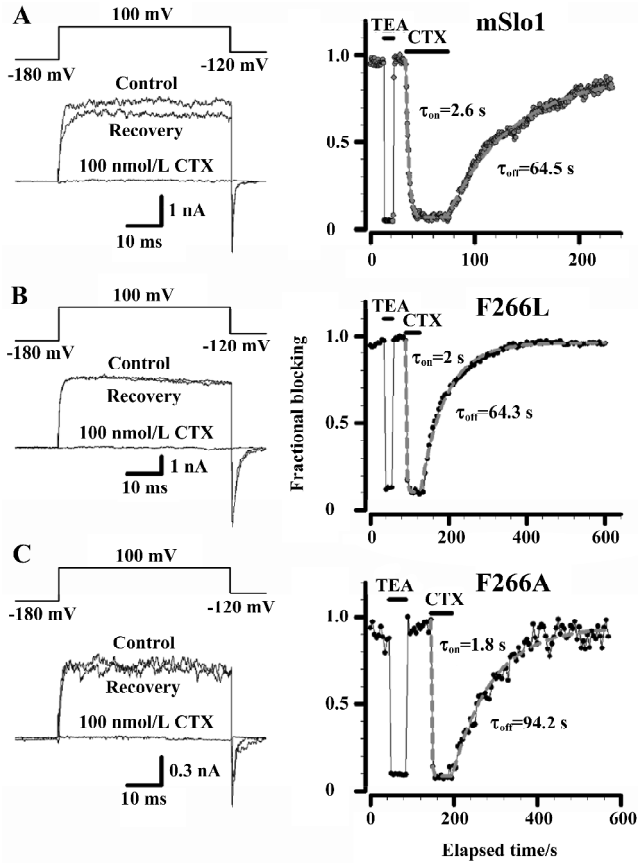

Figure 2. Inhibitory characteristics of the scorpion toxin Charybdotoxin (ChTX) on the currents of BK channels composed of mSlo1 alone or its mutations. (A) Traces show the BK currents recording from an outside-out patch from a *Xenopus* oocyte injected with cRNA encoding mSlo1 a subunits. Channels were activated by a 50 ms voltage step to +100 mV, after a prepulse to -180 mV, in the symmetrical 160 K^+ saline in absence or in presence of 20 mmol/L TEA or 100 nmol/L ChTX, with intracellular 10 μmol/L Ca^{2+}. A voltage protocol is shown at the top panel. BK currents encoded with mSlo1 were remarkably reduced by 100 nmol/L ChTX. All the traces shown on the left were subtracted from the current in the presence of 20 mmol/L TEA. Normalized values of the steady-state currents from the outside-out patches shown on the right are plotted as a function of elapsed time. The currents were repeatedly activated, by a voltage step from -180 mV to +100 mV with a time interval 0.5 s, before, during, and after the application of 100 nmol/L ChTX or 20 mmol/L TEA. In some cases, we also chose 3 s or 5 s as the time interval of the repeated pulses. The dash lines in gray are fits of Equation (1) and Equation (2) respectively. The patch was perfused with 20 mmol/L TEA (the upper) and 100 nmol/L ChTX (the lower) indicated by the horizontal bars. Fits to the onset and offset time courses of 100 nmol/L ChTX give τ_{on}=2.6 s and τ_{off}=64.5 s, respectively. (B) and (C) They are exactly the same as shown in A, except Figure 2B is for mSlo1-F266L and Figure 2C is for mSlo1-F266A. In 2B, the onset and offset time constants for mSlo1-F266L are τ_{on}=2 s and τ_{off}=64.3 s, respectively. In C, the onset and offset time constants for mSlo1-F266A are τ_{on}=1.8 s and τ_{off}=94.2 s, respectively.

onset and offset time give τ_{on}=2.6 s and τ_{off}=64.5 s on graph, with a mean τ_{on}=2.8±0.2 s and τ_{off}=73±14 s. The mean K_d calculated from the fitted time constants gives 3.9±0.5 nmol/L (n=3). To gain insight into the molecular determinants of peptide-channel complex such as ChTX-mSlo1, Yao *et al* (2005) has reported that the aromatic residues Phe266 and Tyr294 of mSlo1 may stabilize binding of ChTX by π-π stacking with the aromatic residues Trp14 and Tyr36 of peptides[13].

In Figure 2B, we show that the mutation mSlo1-F266L has τ_{on}=2.4±0.5 s, τ_{off}=77.6±8.9 s and a mean K_d=3.1±0.5 nmol/L (n=8). In Figure 2C, we find that the mutation mSlo1-F266A has τ_{on}=2.6±1.2 s, τ_{off}=73.6±20 s and mean K_d=4.2±1.2 nmol/L (n=4). However, both the mSlo1-F266L and the mSlo1-F266A mutations show a nearly completed recovery from inhibition by 100 nmol/L ChTX in most of cases (eg n=8/9 for mSlo1-F266L).

There are three irreversible data of wild-type mSlo1 channels and eight reversible data of F266L channels shown as labeled in Figure 3, respectively. Applying the recovery saline for 400 to 800 s, we only found a 70%–80% recovery arising from mSlo1 and nearly a 100% recovery from the mSlo1-F266L. The irreversible recovery occurs sometimes in many blocking experiments of the BK-type channels by applying toxins such as ChTX or IbTX, even though it was never brought to an important place before[5,9]. Another interesting phenomenon is that the successive recovery level after the first application of toxins is always "reversible"[9]. The repeated recovery experiments (Figure 4) show that there is an irreversible component arising from blocked mSlo1 channels that are only apparent after the first application of ChTX (Figure 4A). In contrast, the irreversible component is never observed in both the mSlo1-F266L and mSlo1-F266A cases (Figure 4B, 4C).

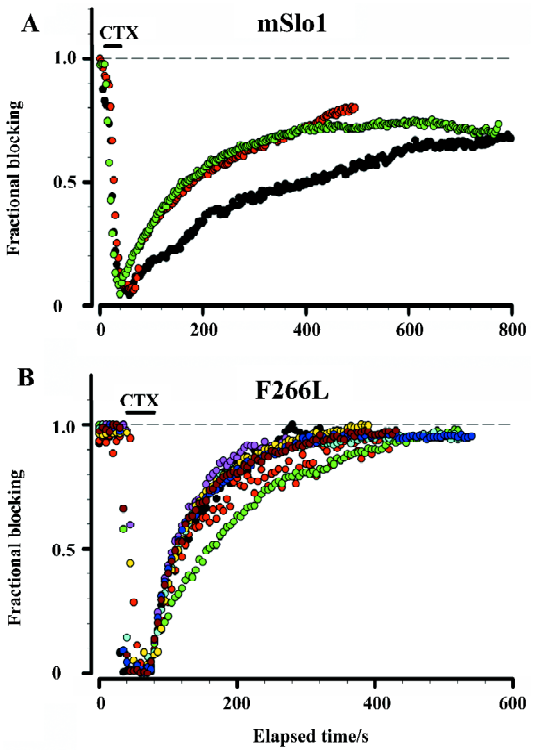

Figure 3. Irreversible inhibition of ChTX on the BK currents encoded with the wild-type mSlo1. (A) The time course of the mSlo1 channels blocked by 100 nmol/L ChTX shows that currents were partially recovered from washout for about 800 s (n=3). For a better comparison with a variety of different blocking time courses, we shifted the blocking time courses artificially by reducing the points of data around the steady-state minimum. Here the horizontal bar just represents the period applying the 100 nmo/L ChTX symbolically. (B) The currents of mSlo1-F266L were almost recovered completely in less than 600 s after removing of 100 nmol/L ChTX (n=8).

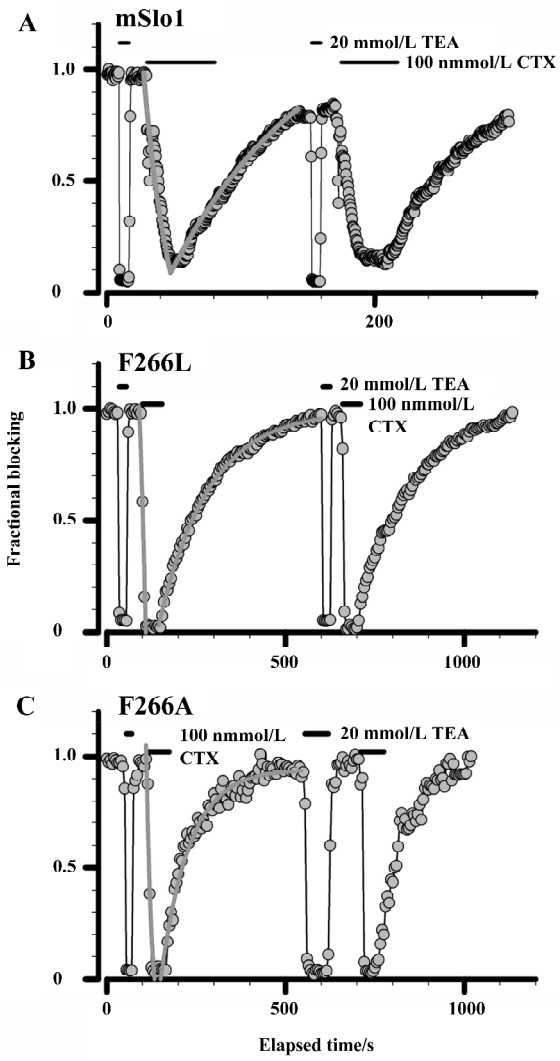

Figure 4. The recovery characteristics of the BK channels blocked by ChTX during consecutive recoveries. (A) The consecutive recoveries returning to the last recovered level occurred after the first irreversible blockade on the wild-type mSlo1 channels by 100 nmol/L ChTX. (B) and (C) The levels of the consecutive recoveries of both the mutation mSlo1-F266L and mSlo1-F266A as indicated were approaching the previous levels while applying with 100 nmol/L ChTX. The horizontal bars represent the durations applying with the 20 mmol/L TEA (the upper) and the 100 nmol/L ChTX (the lower).

Discussion

To date, the scorpion toxins have been used as a tool for exploring the structure and function of the ion-channel proteins. It is important to know how it can associate with channel proteins and what function it exerts on the channels. Simulation of peptide-channel complex interaction reveals that the residues Phe266 and Tyr294 in mSlo1 channels may bind to the aromatic residues Trp14 and Tyr36 of the scor-

pion toxin peptides by π-π stacking[4,13]. The mutant Y294V proves insensitive to both TEA and CTX (n=8, data not shown), which means that Y294 is the binding site of CTX. However, the permanently lost component in BK channels by ChTX or IbTX has never been paid enough attention before. We often selected the results of the subsequent application of toxins so that we could examine the nature of the peptide-channel complex. In this study, we were attempting to gain an insight into the interaction mechanism of ChTX-mSlo1 complex by mutations of the residue Phe266.

Based on experiment results from both the mutants mSlo1-F266L and mSlo1-F266A in this study, we did not find any significant difference on the equilibrium dissociation constant Kd, as well as time constants τ_{on} and τ_{off}. Therefore, the results in this study suggest that the residue Phe266 does not clearly show its ability for stabilizing binding to the peptide ChTX by π-π stacking as the predication given by simulation of peptide-channel complex[4]. The only difference we found in this study is that the mutants eliminate irreversible components. It is unclear why the mSlo1 currents continue to contain an irreversible component during the first application of toxin. A hypothesis is that the BK channels encoded with mSlo1 might have a rundown in currents during a long recovering period. Consequently, we performed experiments to test the stability of mSlo1 currents and found that the steady-state currents of mSlo1 (n=4/4 patches) only showed less than the 10% rundown in -20 min (Unpublished data). Finally, we cannot completely exclude the possibility that the wild-type mSlo1 may need an extra long time to recover its permanent part, or that the residue Phe266 itself might have a role in removing the irreversible component. However, more precisely designed experiments should be undertaken to verify this idea in the future.

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