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Characterization of the outer pore region of the apamin-sensitive Ca^{2+}-activated K^+ channel *r*SK2

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Abstract

We have studied the interaction between the SK2 channel and different scorpion toxins in order to find similarity and differences to other K^+ channels. Beside apamin, ScTX is a high affinity blocker of the SK2 channel, whereas CTX is unable to block current through SK2. In order to prove that the ScTX affinity can be explained by the character of the different residues in the outer pore of the SK channels we introduced point mutations that render SK2 K^+ channel SK1 and SK3 like. Directed by the results of the toxin receptor on the *Shaker* K^+ channel, we changed single amino acids of the SK2 K^+ channel that should render it sensitive to other peptide toxins like CTX a blocker of the IK channel, or KTX a blocker of the voltage-dependent channel Kv1.1 and Kv1.3. Amino acids V342G, S344E, and G384D of SK2 were changed to amino acids known from *Shaker* K^+ channel to improve *Shaker* K^+ channel CTX sensitivity. Interestingly SK2 V342G became CTX sensitive with a K_d of 19 nM and was also KTX sensitive $K_d = 97$ nM. SK2 S344E ($K_{d\ CTX} = 105$ nM, $K_{d\ KTX} = 144$ nM) and G348D ($K_{d\ CTX} = 31$ nM, Kd KTX = 89 nM) became also CTX and KTX sensitive with a lower affinity. The mutant channels SK V342G, SK2 S344E and SK2 G348D showed reduced ScTX sensitivity ($K_d = 6$ nM, $K_d = 48$ nM, and $K_d = 12$ nM). Because the exchange of a single residue could create a new high affinity binding site for CTX and KTX we concluded that the outer vestibule around position V342, S344, and G348 of the SK2 K^+ channel pore is very similar to those of voltage-gated K^+ channels such as the *Shaker* K^+ channel, Kv1.1 and Kv1.3 channels and also to the prokaryotic KcsA channel. From mutant cycle analysis of KTX position H34 and SK2 position V342G, S344E, and G348D we could deduce that KTX binds in a similar way to SK2 channel mutant pore than to the Kv1.1 pore.

Keywords: Small-conductance Ca^{2+}-activated K^+ channel (rSK2); KcsA; Apamin; Scyllatoxin; Charybdotoxin; Kaliotoxin; Patch-clamp technique

1. Introduction

Ca^{2+}-activated K^+ channels represent a class of potassium channels that respond to changes in the intracellular Ca^{2+} concentration, and couple Ca^{2+} metabolism/signaling to K^+ flux and membrane excitability. Based on the electrophysiological properties, three classes of Ca^{2+} activated K^+ channels have been characterized and were named after their single channel conductance as K^+ channels with big, intermediate and small conductance (BK, IK and SK).

SK channels have been involved in several significant physiological processes. In the central nervous system they modulate the slow afterhyperpolarization, in the proximal colon apamin blocks neurotensin-induced relaxation, and in denervated muscle and muscle of patients with myotonic dystrophy apamin reduces associated hyperexcitability (Behrens et al., 1994).

The SK channel family has 3 members (SK1, SK2, and SK3), which differ in their apamin sensitivity. Within the SK channel heteromeric channels are functional (Ishii et al., 1997). The prominent apamin sensitive SK channel expressed in the human leukemic Jurkat T cell line corresponds to SK2 (Jäger and Grissner, 1997; Jäger et al., 2000) and is that member of the SK channels subfamily with the highest apamin sensitivity. *h*SK2 transcripts are found in various human tissues, including brain, heart, ear, lung, skeletal muscle, liver, and uterus (Catterall et al., 2002). Besides apamin, an 18-mer peptide component of the bee venom *Apis mellifera* (Köhler et al., 1996), SK2 channels are blocked by

Abbreviations: WT, wild type; ScTX, scyllatoxin; CTX, charybdotoxin; KTX, kaliotoxin.

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the 31-mer scorpion toxin scyllatoxin (ScTX, leiurotoxin I) from *Leiurus quinquestriatus hebraeus* (Castle and Strong, 1986; Chicchi et al., 1988; Auguste et al., 1990), by P05 from *Androctonus mauretanicus* (Zerrouk et al., 1993), BmPO5 from *Buthus martensii Karsch* (Romi-Lebrun et al., 1997), Tamapin from the red scorpion *Mesobuthus tamulus* (Pedarzani et al., 2002) and Tsκ from *Tityus serrulatus* (Blanc et al., 1997). Interestingly these highly specific blockers of the SK2 channel apamin and ScTX lack any sequence homology (Fig. 2A). Therefore we were interested to identify their binding determinants. For apamin the differences in the amino acid sequence in the pore region of the SK channels have been proven to influence the apamin sensitivity (Ischii et al., 1997). We wanted to identify the ScTX binding determinants and compare the SK2 outer pore with other K^+ channel pores. Furthermore we tried to create a charybdotoxin (CTX) binding site on the external SK2 channel pore. The differences to CTX sensitive K^+ channels pores are of interest because CTX has been used extensively to characterize Kv, IK, and BK channel pores and therefore a lot of structural data exist describing these pore structures. CTX is isolated from the scorpion *Leiurus quinquestriatus* and does not block SK channels but Ca^{2+}-activated K^+ channel with intermediate and big conductance (IK, BK) and the voltage-gated potassium channel Kv1.3 (Catterall et al., 2002). CTX inhibits ion conduction through K^+ channels by occluding the pore at the extracellular opening by covering or plugging the external mouth of the channel (Miller, 1995). The positively charged ammonium group of K27 of CTX seems to be projected downwards into the pore mimicing K^+ ions entering the pore (Park and Miller, 1992, a,b). For CTX this functional residue is located on the flat β-sheet covering approximately 200 $Å^2$ (18 × 12 Å). There are several others CTX-like toxins like KTX, NTX, Tsκ, and P05 that have a high degree of sequence identity and similar tertiary fold like CTX and interestingly Tsκ and P05 block the SK2 channel and CTX, KTX, and NTX do not block SK channels.

The prokaryotic K^+ channel KcsA from *Streptomyces lividans* has a high similarity with the eukaryotic K^+ channels (Schrempf et al., 1995) and the crystal structure was determined at 3.2 Å (Doyle et al., 1998). The structure revealed a tetrameric complex with a centrally located pore, each with two transmembrane helices flanking a selectivity filter. Despite the fact that KcsA is gated by pH while most of the eukaryotic K^+ channels use voltage or Ca^{2+} it is assumed that the structures conferring the K^+ selectivity are the same T/SXGYD. This has been confirmed by the recent studies from Jiang et al. (2003) who showed that the region of the KvAP channel and the KcsA channel conferring K^+ selectivity are almost superimposable. The top of the selectivity filter with the turret on the extracellular side is not as informative compared to the selectivity filter of the KcsA. For example, the side chains of the amino acid residues of the turret are indeed rather flexible; some could even not be located in the KcsA crystal, like amino acid at position 51 and 71. Even the backbone structure may differ between K^+ channels not only because of different amounts of non-conserved prolines in the turret region (Fig. 1). Therefore peptide toxin studies are still helpful to compare the three-dimensional structure of different potassium channels and have shown differences between the KcsA model and voltage-gated Kv1.1 and Kv1.3 channels in the outer vestibule (Wrisch and Grissmer, 2000). Here, we wanted to see whether SK channels look more like KcsA channels or more like voltage-gated K^+ channels using similar approaches as have been used before.

2. Material and methods

2.1. Cells

TsA201 cells, a HEK293 cell line stable transfected with the SV40 T-antigen (DuBridge et al., 1987) (tsA 201 = formerly called 293/tsA1609neo; Heinzel et al., 1988) were grown in MEM containing 10% fetal bovine serum and 10% heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria), and 2 mM L-Glutamax I (Gibco, Eggenstein, Germany) at 37 °C and a CO_2 atmosphere of 5%.

2.2. Construction of the SK2 expression vector

The *r*SK2 *c* DNA (Köhler et al., 1996) was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen, Groningen, Netherlands) which provides a CMV promotor and an SV40 replication origin. The QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to generate point mutations coding for the amino acid exchanges.

2.3. Transfection

tsA201 cells were transiently transfected by the $CaPO_4$ method (Ausubel et al., 1987). DNA for transfection was prepared using Qiagen anion-exchange columns (Qiagen, Hilden, Germany). The SK2 coding plasmids for transfections were used together with a CD8 coding plasmid. Anti-CD8 antibodies coupled to paramagnetic beads (Dynal, Hamburg, Germany) were used to identify transfected tsA201 cells. After 1–3 days cells were used for whole-cell current measurements.

2.4. Electrophysiology

Experiments were carried out using the whole-cell mode of the patch-clamp technique as described earlier (Hamill et al., 1981). SK2 channels were activated by whole-cell

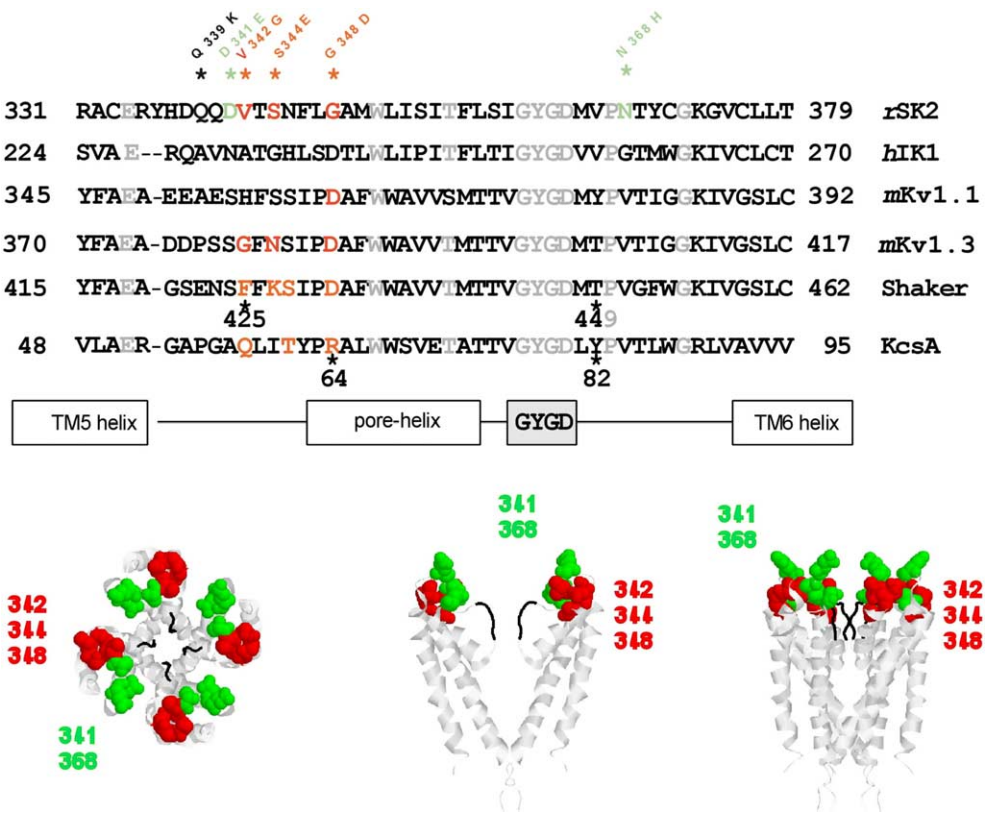

Fig. 1. Amino acid sequence alignment of the pore region of some K^+ channels. The T/SXGYGD motive and conserved amino acids among potassium channel are indicated by gray letters. Top and side views of KcsA channel (Protein Data Bank: 1BL8) Amino acid positions conferring CTX/ KTX sensitivity on SK2 channels are highlighted in red, major binding determinates for ScTX are highlighted in green.

dialysis with a pipette solution (in mM: 135 K-aspartate, 2 $MgCl_2$, 10 HEPES, 8.7 $CaCl_2$, 10 EGTA, pH 7.2) containing 1 μM free Ca^{2+}. $[Ca^{2+}]_i$ was calculated assuming a K_D for EGTA and Ca^{2+} at pH 7.2 of 10^{-7} M (Portzehl et al., 1964). Microelectrodes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK), coated with Sylgard (Dow Corning Corp., Midland, MI), and fire polished to resistances of 2–5 MΩ. Ramp currents were elicited by 400 ms voltage ramps from −160 to 40 mV every 20 s before and after application of a K^+ solution. The bath solution contained (in mM): 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and either 10 MES, 10 HEPES or 10 Tris, adjusted to pH 6.0, 7.4 or 9.0 with NaOH (290–320 mOsm) or 164.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 MES, 10 HEPES or 10 Tris, adjusted to either pH 6.0, 7.4, or 9.0 with NaOH (290–320 mOsm). External solutions were changed by a syringe-driven perfusion system. Membrane currents were recorded with an EPC-9 patch clamp amplifier (HEKA elektronik, Lambrecht, Germany), filtered at 2.9 kHz, and digitized. The holding potential in all experiments was −80 mV. Stimulation and recording was controlled by a Macintosh computer with appropriate software (Pulse and PulseFit, HEKA elektronik, Lambrecht, Germany). Series resistance compensation (80%) was used, if the current exceeded 2 nA. All figures were corrected for capacitative currents using the auto C_{fast} and C_{slow} function of Pulse (HEKA elektronik, Lambrecht, Germany). Voltage ramps from −160 to +40 were elicited in the presence of different toxin concentrations (I_{toxin}) and were divided by the current without toxin. Current measured at −160 mV was used to determine the K_D values ($K_D = conc._{toxin} * I_{toxin}/(I_{max} - I_{toxin})$. Data are given as mean ± SEM from at least three independent experiments. Analysis was also performed using IGOR (Wavemetrics, Lake Oswego, OR, USA) software.

2.5. Toxins

Peptide toxin block was measured in the presence of 0.1% bovine serum albumin. Apamin and CTX were purchased from Latoxan (Rosans, France) and were applied by a separate tubing system; ScTX was purchased from Bachem (Heidelberg, Germany). KTX mutants H34A, H34K, R31A/H34A, were generated recombinantly (Wrisch and Grissmer, 2000). All other chemicals were from Sigma (Taufirchen, Germany). The apamin NMR data were kindly provided by Dr David E. Wemmer (University of California, Berkeley, CA, USA). The molecular structures of the toxins and the KcsA channel were viewed with Rasmol 2.7.

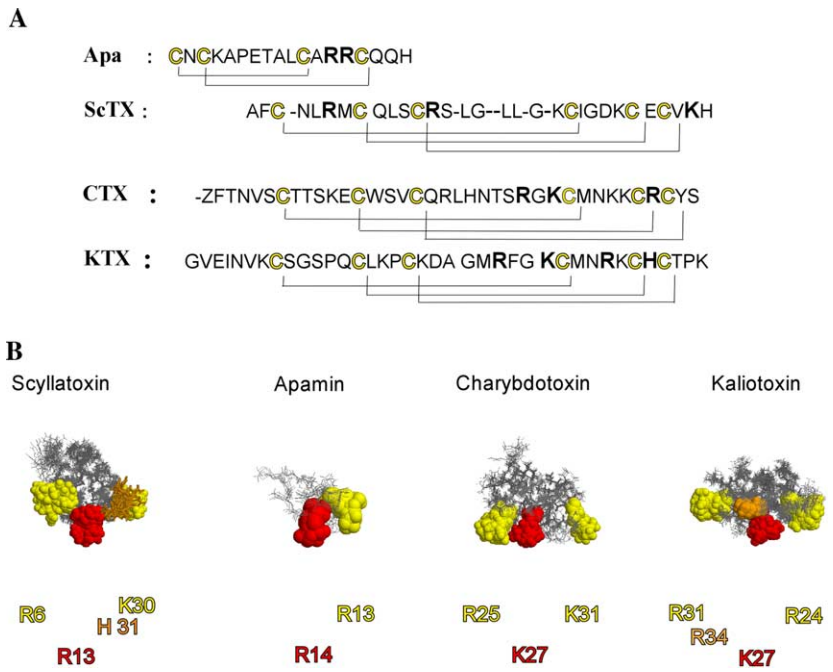

Fig. 2. Comparison of peptide structures. *A*: Sequence alignment of apamin, ScTX, CTX, and KTX. Cysteins are highlighted in yellow disulfide bridges are indicated by bars. B: The toxins are shown as wire frames including all chains of the NMR structure. Important amino acids known to modulate affinity on SK2 channel are shown as solid CPKs in color. Apamin coordinates were friendly provided by Dr D. Wemmer (University of California, Berkeley, CA, USA). All other coordinates came from the Protein Data Bank (ScTX: 1SCY; CTX: 2CRD; and KTX: 2KTX).

2.6. Mutant cycle analysis

With the thermodynamic mutant cycles analysis one can calculate the coupling energies between pairs of amino acids in a protein-protein complex. The dimensionless Ω value, which describes the interaction strength of a given channel-toxin pair, was determined as described before (Aiyar et al., 1995; Hildago and MacKinnon, 1995). The change in coupling energy, $\Delta\Delta G$ for the channel-toxin pairs was calculated using the formula $\Delta\Delta G = kT \ln \Omega$ as described earlier (Rauer et al., 2000). The distances between a pair of residues was estimated based on the studies of Schreiber and Fersht (1995); Hidalgo and MacKinnon (1995), assuming that $\Delta\Delta G$ values >0.5 kcal mol^{-1} corresponds to a distance of <5 Å between the residues.

2.7. Electrostatic compliance measurements

For the estimation of the distances between the charges at KTX and the SK channel pore an electrostatic compliance calculation was used in order to determine the distances (Stocker and Miller, 1994; Aiyar et al., 1995; Bretschneider et al., 1999). Protonation of H34 in KTX creates a higher effective KTX concentration at the receptor site of the channel compared with the bulk concentration. Calculation of the potential ψ causing the higher effective blocker concentration and the appropriate distances between the interacting residues was performed as described by Bretschneider et al. (1999).

3. Results

In order to prove the assumption that the different apamin sensitivities among SK channels are conferred by the differences in their outer pore regions amino acid position 339, 341 and 368 of SK2 were changed to their SK1 and SK3 counterparts (see Fig. 1). SK1 has a lysine at the SK2 339 equivalent position and a glutamate at the SK2 341 equivalent position, while SK3 has a histidine at the SK2 368 equivalent position. Furthermore we wanted to examine if apamin and scyllatoxin may have convergent features, although apamin has no obvious sequence homology to scyllatoxin or other peptide toxins, which interact with K^+ channels (Fig. 2). To characterize the effect of extracellular applied toxins on current through SK2 channels, we applied ramp currents from -160 to $+40$ mV for 400 ms in the presence and absence of different toxin concentrations. Fig. 3 shows in the upper row current through SK2 wt channels in the absence and presence of 0.3 nM ScTX. This concentration of ScTX was able to reduce the slope of the ramp current through SK2 wt channels by almost 50%

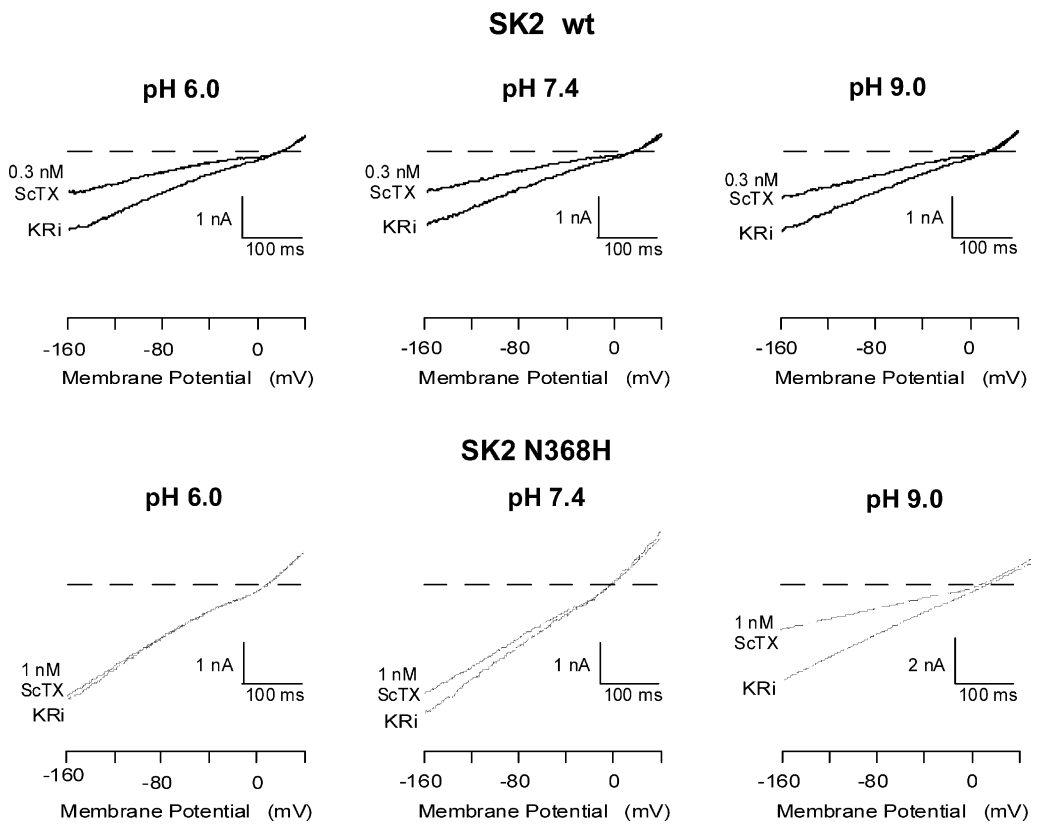

Fig. 3. pH_o effect on current through SK2 wt (upper row) and SK2 N368H (lower row) mutant channels expressed in tsA201 cells. SK2 channels were activated by whole-cell dialysis with a pipette solution containing 1 μM free Ca^{2+}. Traces were obtained after full activation of the current in K^+ Ringer in the absence and presence of 0.3 nM ScTX for the wt and 1 nM ScTX for the SK2 N368H mutant channel at pH 6.0, pH 7.4 and pH 9.0.

independent of the applied pH_o. From these reductions we calculated K_D values for the ScTX block of current through SK2 wt channels of 0.3 ± 0.03 nM ($n = 5$). At pH_o 7.4 SK2 Q339K behaved like wild type ($K_D = 0.6 \pm 0.04$ nM, $n = 4$, data not shown), SK2 N368H (SK3-like) showed reduced ScTX sensitivity (see Fig. 3 lower row, middle; $K_D = 3 \pm 0.9$ nM, $n = 5$) and SK2 D341E (SK1-like) was no longer ScTX sensitive ($K_D = 128 \pm 37$ nM, $n = 5$, data not shown). The current reduction by ScTX was fully reversible upon washout. Block of SK2 N368H channel was strongly pH_o sensitive (Fig. 3, lower row). SK2 N368H, based on the crystal structure of Doyle et al. (1998) is positioned at the outer entrance of the channel pore. We tested the possibility that protonation of N368H might repel ScTX which has positively charged residues in its binding surface. We tested this possibility by examining the pH_o-dependence of toxin block. The determination of the ScTX affinity of SK2 N368H at pH_o 9.0 showed a K_D of 1.9 ± 0.5 nM, $n = 4$ while at pH_o 6.0 a K_D of 31.1 ± 8 nM, $n = 6$ was determined. Raising pH_o from 6.0 to 9.0 increased the ScTX affinity about 10 -fold. For the WT channel SK2 that has a non-chargeable asparagine at position 368 no pH_o-dependence of block was found. Indicating that the introduced histidine at position 368 is most likely the only titratable residue in the channel pore. It is probable that the toxin is electrostatically repelled by the protonated N368H of the mutant channel. Like the ScTX block, the extracellular TEA^+ block is pH_o dependent in SK2 N368H (data not shown).

Several examples are known for amino acid substitutions that increase the sensitivity for certain peptide toxins. For example introducing a glycine at position F425 could increase *Shaker* affinity for CTX (Goldstein et al., 1994) while substituting three amino acids of the KcsA in the pore (Q58A, T61S, R64D) an although weak agitoxin2 (AgTX2) binding site could be generated with a $K_D = 620$ nM (MacKinnon et al., 1998) and also a CTX binding site (Heginbotham et al., 1999). Under the circumstances that we could generate a CTX binding site on SK2 channels the *Shaker* and maybe also the KcsA data should serve as a template to model the SK2 pore structure. Therefore amino acids V342, S344 and G384 of SK2 were changed to amino acids known from *Shaker* K^+ channel to improve *Shaker* K^+ channel CTX sensitivity. While SK2 wt was not blocked by

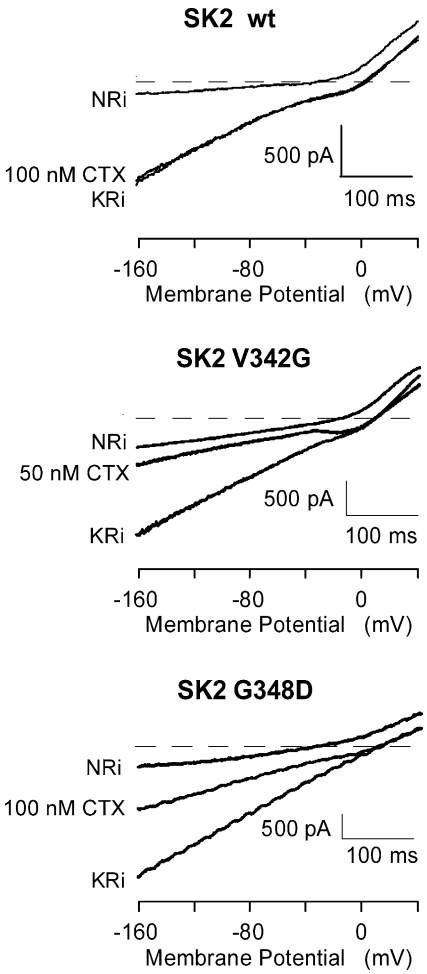

Fig. 4. Effect of CTX on current through SK2 wt, SK2 V342G and SK2 G348D channels expressed in tsA201 cells. SK2 channels were activated by whole-cell dialysis with a pipette solution containing 1 μM free Ca^{2+}. Ramp currents were elicited by 400 ms voltage-ramps from −160 to 40 mV and were obtained after full activation of the current in K^+ Ringer in the absence and presence of 50 or 100 nM CTX. The block by CTX was completely reversible upon washout.

100 nM CTX (Fig. 4, top), the SK2 V342G became CTX sensitive (Fig. 4, middle) with a K_D of 19 nM and was also KTX sensitive (K_D = 97 nM). SK2 S344E (K_D for CTX was 105 nM, K_D for KTX was 144 nM) and G348D (Fig. 4, bottom) (K_D for CTX was 31 nM, K_D for KTX was 89 nM) became also CTX and KTX sensitive, however, with a lower affinity. In addition, the mutant channels SKV342G, SK2 S344E, and G348D showed reduced ScTX sensitivity (K_D = 6 nM; K_D = 48 nM; K_D = 12 nM).

For SK2 V342G having a KTX affinity (K_D = 97 nM) and SK2 G348D (K_D = 89 nM) we tested if the KTX docking configuration in the external vestibule is similar for SK and Kv channels. Changing pH_o from 6.0 to 8.2 improved the KTX affinity of SK2 V342G by a factor of 4. For SK2 G348D we found the opposite effect by a rise in pH from 6.0 to 8.2 we found a decrease affinity by a factor of 9 (Fig. 5). To find out whether protonation of a histidine at position 34 of KTX is responsible for the pH_o dependent KTX block, like it was shown for mKv1.1 (Wrisch and Grissmer, 2000), we determined the affinity of the KTX analogs KTX H34A, H34R and R31A/H34A (Fig. 5). As expected, KTX H34K blocked SK2 V342G with a 9 fold higher affinity compared to KTX H34A. A mutant-cycle analysis revealed a $\Delta\Delta G$ value of 1.54 kcal/mol, which indicates a proximity of <5 Å. In addition we could show that KTX R31 interacts with position 342 of SK2 in the V342G mutant channel. A mutant-cycle analysis revealed a $\Delta\Delta G$ value of 0.62 kcal/mol, which also indicates a proximity of <5 Å identical to the interaction point that was found for KTX R31 and mKv1.1 (H355) or Kv1.3 (G380) (Aiyar et al., 1995). Furthermore we could show that KTX H34 interacts with position 348 of SK2 in the G348D mutant channel ($\Delta\Delta G = 0.76$ kcal/mol). No coupling was found for KTX R31 and position 348 of SK2 in the G348D mutant channel ($\Delta\Delta G = 0.41$ kcal/mol). From these data we assume that KTX does block SK2 V342G/SK2 G348D with a similar docking conformation like for mKv1.1 or mKv1.3.

To calculate the distances between His 34 in KTX and position 342, and 348 in SK2, we performed electrostatic compliance. Because it was not possible to determine the number of interacting channel subunits without investigating heterotetrameric channels, we calculated the potentials for one, two and four interacting channel subunits. We estimated distances of 3.7; 5.8, and 8.3 Å between His 34/KTX and one, two, or four interacting V342GSK2 and distances of 3.3, 5.4; and 7.6 Å between H34/KTX and one, two, or four interacting G348D/SK2. The electrostatic compliance calculation confirmed the distance calculation by mutant cycle analysis and suggested the interaction of His 34/KTX and only one channel subunit.

4. Discussion

Like for other K^+ channels a variety of pharmacological tools including peptide toxins and potent synthetic small molecules have been used to characterize SK channels. In contrast to other K^+ channels blocking toxins, apamin and ScTX have been proven to be a high affinity blocker exclusively for SK channels, exhibiting no activity on voltage-gated, inward rectifier or other classes of Ca^{2+}-activated K^+ channels. Therefore we wanted to characterize the ScTX sensitivity as tool to characterize the SK channel pore.

Despite the absence of extensive amino acid sequence or structural homology, ScTX competes with apamin for the same, or overlapping binding sites on the outer vestibule of SK channels (Fig. 2A) (Auguste et al., 1992). Two other

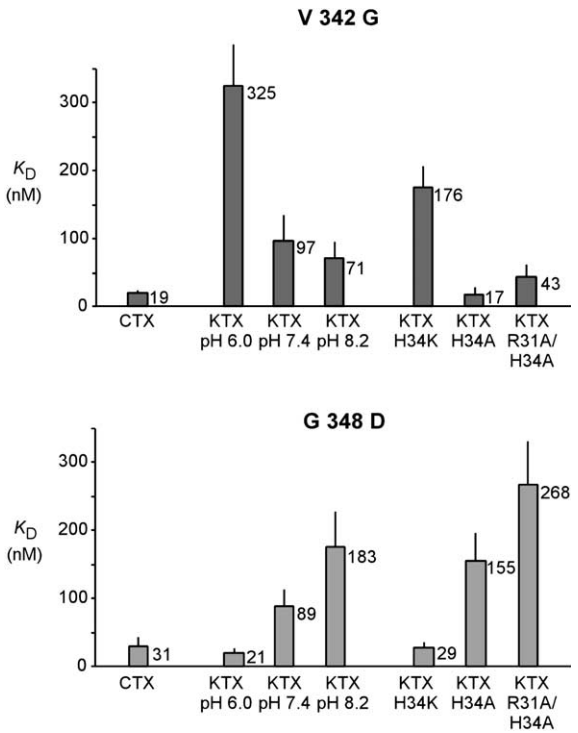

Fig. 5. Influence of single amino acid substitutions in the pore region of SK2 channel on KTX and mutant KTX binding. The bar diagram shows the affinity of the peptide toxins to the individual channel as quantified through the K_D values. Data represent mean ± SEM ($n = 3 - 8$).

scorpion venom toxins, P05 from *Androctonus mauretanicus mauretanicus* and BmP05 from *Buthus martensi* which exhibit high sequence similarity to ScTX, have been found to compete with ^{125}I-apamin binding to synaptosomes, suggesting that they too interact with the SK channel pore. Tsκ from *Tityus serulatus* and maurotoxin from *Scorpio maurus* also inhibit ^{125}I-apamin binding to synaptosomes.

4.1. Apamin and ScTX

As in the case for apamin, positively charged residues appear to be essential for biological activity of scorpion venom toxins. Indeed, it has been suggested that much of the biological activity of apamin, P05 and BmP05 reside in a common (R/K)RCQ amino acid sequence motif (Sabatier et al., 1993, Sabatier et al., 1994, Shakkottai et al., 2001). A similar sequence, RMCQ is found in ScTX. Interestingly, a synthetic form of ScTX in which the methionine 7 has been replaced by an arginine has been found to be ten times more potent than the native toxin (Sabatier et al., 1994). A conserved arginine residue at position 13 and the terminal histidine has also been proposed to contribute to the blocking activity of scyllatoxin and related toxins (Auguste et al., 1992). Chemical modifications of ScTX have shown that two arginines in the sequence, Arg6 and Arg13, are essential for binding to the SK (Auguste et al., 1992).

4.2. ScTX binding determinants

The three subtypes of small conductance Ca^{2+}-activated K^+ channels differ in their apamin sensitivity (Catterall et al., 2002). By producing amino acid substitutions by site directed mutagenesis, we identified determinants of the scyllatoxin (ScTX) block. SK2 channel positions D341E mimicking SK1 and N368H mimicking SK3 showed a drastically reduced ScTX sensitivity. Therefore the SK1-like and SK3-like substitutions in the SK2 channel can explain the different ScTX sensitivity among SK channels.

4.3. ScTX N368H

A change in pH from pH 9.0 to 6.0 diminished the N368H mutant SK2 channel sensitivity to block by ScTX 10-fold. We conclude that lowering pH_o increases protonation of N368H of each homomer of SK2 channels thereby repelling ScTX electrostatically. Because the ScTX affinity of WT SK2 channel is not pH_o sensitive it is unlikely that His31/ScTX is the candidate for the protonation effect. One could speculate that R6 and K30 (Fig. 2B) do find space in the rim between two monomers with R13 facing into the pore. This assumption is based on the data of the SK2 N368K channel, which shows a drastically reduced ScTX affinity like the SK2 N368H channel at pH 6.0. The only two negative charges of ScTX do face away from the pore in this model and may account for lowered affinity of the mutant channel which posses a new or a larger side chain with a negative charge, like in the mutant SK2 channels D341E, S344E, and G348D. Shakkottai et al. (2001) changed the character and size of scyllatoxin residues at position 6 and 7 by introducing a non-natural amino acid di-aminobutanoic acid (Dab) at position 7, which is located in direct neighborhood of Arg 6. In a model these residues are positioned right opposite to His 31, the residue we tested upon acidification. To analyze this modified toxin Shakkottai et al. (2001) replaced His 521 in hSK3 for an Asn the corresponding residues of SK2 channels. They could increase the ScTX R7Dab (Lei-Dab 7) sensitivity of hSK3 H521N compared to wild type SK3 channel more than 100-fold and deduced from mutant cycle studies that the residues H521 in the channel and R6 in the toxin lay in close proximity. These data make it likely that Arg6 or Arg7 do sense the protonation of the SK2 N368H channel pore.

4.4. CTX binding site

In 1998 the first X-ray data of a K^+ channel from *Streptomyces lividans* were published (Doyle et al., 1998). Although the amino acids of the K^+ channel selectivity filter are highly conserved, as has been confirmed by yet another crystal structure of a voltage dependent bacterial K^+

channel, KvAP from *Aeropyrum pernix* (Jiang et al., 2003), the residues lining the entryway are quite variable. One big advantage of peptide toxins is that their affinity to the external pore is determined by their 3-D surface, which is much larger as other blockers like TEA or the plant alkaloid *d*-tubocurarine (dTC). The characteristic fold of the toxins is held rigidly by two to four disulfide bridges, which made them useful for mutant cycle analysis. We tried to prove the concept that if SK, IK, and Kv channels are similar in there surface structure they should behave similarly on substitutions in that region. The amino acid sequence of SK channels exhibit about 40% identity with the intermediate conductance, calcium-activated potassium channel, IKCa1, which is selectively blocked by charybdotoxin (CTX) and about 35% identity with the *Shaker*-like K^+ channel. In the past studies employing site-directed mutagenesis of the KcsA, *Shaker*, Kv1.1, Kv1.3 and IK K^+ channel have mapped the scorpion toxin binding site to regions corresponding to the extracellular entryway of the K^+ channel. From *Shaker* K^+ channel and IK channel it is known that at position V342 (equivalent to *Shaker* position 425) a small side chain and at S344 and G348 equivalent positions negative charges are favorable to confer CTX sensitivity to these channels (Fig. 1) (Goldstein et al., 1994). The authors suggested that some of the 2000-fold increase in CTX sensitivity of F425G channels is due to the ability of the toxin to move deeper into the channel vestibule owing to the elimination of the steric interaction of toxin threonines 8 and 9 and the phenol ring on the phenylalanines at channel position 425. This reduction in size enables the toxin to enter the pore and creates a CTX binding site. Therefore we wanted to test if a reduction of side chain lengths of the valine side chain at position V342 make the ScTX sensitive SK2 channel CTX-sensitive. We found that we created new high affinity binding sites for CTX and KTX in SK2 V342G, SK2 S344E and SK2 G348D proteins. Like for the KcsA (Q58A) and *Shaker* (F425G) channels a small uncharged amino acid at SK2 position 342 is necessary for creating a high affinity CTX binding site ($K_d = 19$ nM) as well as negative charges at *Shaker* 427 and 431 equivalent positions (Fig. 1). Comparing the CTX sensitivity of the IK channel with a $K_D = 5$ nM and Kv1.3 ($K_D = 2 - 3$ nM) with the V342G mutant SK2 channel ($K_D = 19$ nM) we generated a very good binding surface for CTX. The affinity of KTX of V342G SK2 mutant channel was in the range of mKv1.1 KTX affinity with about 62 nM. On the other hand the ScTX affinity of the V342G mutant channel was drastically reduced by the substitution of valine for glycine. Interestingly currents through V342G, S344E, and G348D mutant channel were not AgTX2 sensitive (data not shown). These results demonstrate that the overall structures of the ScTX receptor sites of the SK2 and the *Shaker* K^+ channel are very similar in this region of the outer pore and that different toxin surfaces are able to plug one 'pore-type'. This conclusion justifies the use of energetic data borrowed from the *Shaker* and also from the KcsA K^+ channel studies to assist in the docking of peptide toxins onto the SK2 channel structure. No estimations could be drawn for the S5 near pore loop of SK2 channels because we did not examine this part.

4.5. Differences between ScTX and CTX

CTX possesses the complete motif common to all scorpion toxins; this motif being stabilized in particular by a cluster of hydrophobic side chains while CTX interacts with its receptor by the β-sheet surface (Park and Miller, 1992a). ScTX is shorter, the β-sheet, and the connection between the helix and the sheet is different from that of charybdotoxin. The situation is rather different for ScTX (Auguste et al., 1992), which blocks the SK channels. Chemical modifications of Arg 6, Arg 13, and His 31 abolished both the binding on rat brain calcium activated K^+ channels and the toxin capacity to contract guinea pig *taenia coli*, while simultaneous modification of Lys 20, Lys 25, and Lys 30 modifies the activity of the toxin on *taenia coil* but does not alter the binding on rat brain channels. From this, Auguste et al., conclude that ScTX interacts with its receptor by the helix side of the toxin and that the β-sheet charged surface is important for a second step following toxin binding. Even there are major differences in the two-dimensional structure ScTX and CTX show an astonishing similarity in there three-dimensional structure focusing on the residues R6, R13, and K30 of ScTX and R25, K27, and K31 of CTX (see Fig. 2B). A pH effect of ScTX binding on wt SK2 channel was not observed probably because H31 of ScTX is very close to Lys 30 of ScTX, which is bigger in size and positively charged and might also exert an influence on the pKa value of ScTX H31.

4.6. KTX binding site

There are several others CTX-like toxins like KTX or NTX that have a high degree of sequence identity and similar tertiary fold like CTX. KTX R31 and H34 are suitable position to examine the orientation of KTX binding to the SK2 pore because V342G and G348E mutant SK2 channel showed a pH dependence of binding. Further KTX mutants were already well characterized on Kv1.1 channel in our lab (Wrisch and Grissmer, 2000). H34 of KTX interacts with only one subunit of the channel tetramer at position 342 and 348 because the short distances determined by mutant cycle analysis exclude the interaction of H34 with amino acids 342 and 348 of opposite subunits. H34 of KTX has a strong influence on SK2 channel position 342 and a shows interaction with position 348. From energy coupling we conclude that H34 is 3.7 Å away from position 342 and 3.3 Å away from position 348. From mutant cycle analysis data we can deduce that R31/KTX is less than 5 Å away from position 342 and not in contact with position 348, therefore R31 should face into the rim between two monomers like for Kv1.1 and Kv1.3 channels. The strong

interaction of position 342 with H34 also might explain why the valine to glycine substitution was so important to create a KTX binding site.

4.7. Mutant cycle analysis on position SK2 V342G

KTX R31A does bind better compared to wt and KTX wt does decrease affinity upon protonation in low pH buffer. This result is more complicated to explain. The only positively charged residues in the SK2 pore R335, H337 K373 are all very far away from the center of the pore. R31 could dive too deep into the rim between two monomers getting repelled by K373. Explaining the fact that KTX H34A does bind with a higher affinity. The same could be true for H34 staying in conflict with K373 of another monomer of the tetramer. However, this effect may also be caused by steric changes as well.

4.8. Mutant cycle analysis on position SK2 S344E

The relative low KTX affinity can be explained by the position of SK2 S344E. It is located more outward compared to the positions 342 and 348 and therefore pH effects were not observed. SK2 position 344 is probably too far away from the H34 in KTX to sense the protonation of the KTX molecule.

4.9. Mutant cycle analysis on position SK2 G348D

The negative charged residue at position SK2 G348D that creates a KTX binding site on the SK2 pore should bind KTX in its protonated form better than unprotonated and that was exactly what we found. Further the KTX H34A mutant showed a lower affinity compared to wt KTX and KTX H34K a higher affinity. Therefore we can deduce that position 348 stays in close contact to KTX H34 in the bound state and the interaction is ionic in nature. H34 of KTX lies right opposite of KTX R24 (Fig. 2B). For R24 it is known that it interacts with the SK2 G348 homologue of Kv1.3 D386 of another channel monomer on the other side of the vestibule (Aiyar et al., 1995). Therefore the flanking positive charges R24 and R31/34 do find space between two monomers of the channel pore similar as we expected it for the ScTX binding to the SK2 wt pore (compare Fig. 2B).

5. Conclusion

In summary, we characterized the major binding determinants for ScTX. We generated CTX and KTX sensitive mutant SK2 pores and could verify that KTX binds to the V342G or G348E mutant SK2 channel pore similar to KTX binding to the Kv1.1 pore. Therefore we conclude that with the existing data on toxin-receptor sites of voltage-gated K^+ channels and of the KcsA channel, these channel structures are good templates for models of the SK channel pore.

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