Structural Differences of Bacterial and Mammalian K⁺ Channels*

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Using a peptide toxin, kalio toxin (KTX), we gained new insight into the topology of the pore region of a voltage-gated potassium channel, mKv1.1. In order to find new interactions between mKv1.1 and KTX, we investigated the pH dependence of KTX block which was stronger at pH₇.4 compared with pH₆.2. Using site-directed mutagenesis on the channel and the toxin, we found that protonation of His₃₄₄ in KTX caused the pH dependence of KTX block. Glu³⁵⁶ and Glu³⁵⁸ in mKv1.1, which interact with His³⁴₄ in KTX, were calculated to be 4 and 7 Å away from His³⁴₄/KTX, respectively. Docking of KTX into a homology model of mKv1.1 based on the KcsA crystal structure using this and other known interactions as constraints showed structural differences between mKv1.1 and KcsA within the turret (amino acids 348–357). To satisfy our data, we would have to modify the KcsA crystal structure for the mKv1.1 channel orienting Glu³⁵⁶ 7 Å and Glu³⁵⁸ 4 Å more toward the center of the pore compared with KcsA. This would place Glu³⁵⁰ 15 Å and Glu³⁵⁸ 11 Å away from the center of the pore instead of the distances for the equivalent KcsA residues with 22 Å for Gly³⁴₃ and 15 Å for Gly³⁴₁, respectively. Bacterial and mammalian potassium channels may have structural differences regarding the turret of the outer pore vestibule. This topological difference between both channel types may have substantial influence on structure-guided development of new drugs for mammalian potassium channels by rational drug design.

Voltage-gated potassium channels guide fundamental biological processes such as electrical signaling, osmotic balance and signal transduction (1). Peptide toxins from scorpions, snakes, and sea anemones, which inhibit ion conduction through potassium channels by binding within the outer pore region, have been used to characterize physiological significance, localization, and structural aspects of these channels (2). Charybdotoxin (CTX) has been used to characterize the region of potassium channels bearing both the CTX receptor and the ion conduction pathway and to demonstrate the tetrameric stoichiometry of the channel (3, 4). Estimations of the topology of the external vestibule of various potassium channels were successful because the structure of the peptide toxins, which was structurally defined by NMR studies, reports the complementary shape of the binding partner (5, 6). Structural features of different channel types were announced by specific binding properties of peptide toxins (7). Our knowledge about structural characteristics of potassium channels was confirmed and extended through data from the bacterial Streptomyces KcsA channel based on crystallographic studies (8). Therefore, up to the availability of x-ray data for each potassium channel, further application of peptide toxins as molecular calipers to investigate the architecture of structurally non-defined targets appears to be reasonable.

Several toxins display a blocking affinity dependent on the extracellular pH (pHₒ) but until now only a decreased block at acid pHₒ was known; both with peptide toxins (9–11) and with other blockers working on potassium channels like TEA (12) but also working on sodium channels like tetrodotoxin and saxitoxin (13). The reduced CTX affinity of the F425H mutant Shaker channel and of the wt Kv1.3 channel (9, 11) as well as the lowered TEA affinity of mKv1.1 channels (12) at low pHₒ was caused by protonation of histidine residues within the outer vestibule of the respective channel proteins. Affinity of tityustoxin to the squid Kv1 channel SqKv1A, which contains a histidine at Shaker position 425, is also reduced at acid pHₒ (14). In contrast, in mKv1.1 and H404T mutant mKv1.3 channels, we observed a better KTX block at low pHₒ. In this paper we present evidence that protonation of a histidine in KTX, which interacts with negatively charged amino acids in the outer pore region of mKv1.1 and H404T mutant mKv1.3 channels, is responsible for the higher KTX affinity of these channels. Using electrostatic compliance we found distances of 4 and 7 Å between Glu³⁵⁸ and Glu³⁵⁶ in mKv1.1 and His³⁴₄ in KTX, respectively. Our data define the position of the turret (amino acids 348–357) in Kv1.1 and also Kv1.3 and imply structural differences between mammalian and bacterial K⁺ channels in that region. These results help refine our picture of the spatial arrangement of amino acid residues in the outer pore region of Kv1.1 and Kv1.3 that might facilitate therapeutic drug design.

MATERIALS AND METHODS

Cells—All experiments were carried out on single cells of a rat basophilic leukemia cell line, RBL cells (15). Cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were maintained in a culture medium of Minimal Essential Medium with Earle's salts supplemented with 1 mM l-glutamine and 10% heat-inactivated fetal calf serum in a humidified, 5% CO₂ incubator at 37 °C. Cells were plated to grow non-confluently onto glass 1 day prior to use.

Solutions—The experiments were done at room temperature (21–25 °C). Cells measured in the whole cell configuration were normally bathed in mammalian Ringer's solution containing, in mM: 160 NaCl, 4.5 KCl, 2 CaCl₂, 1 MgCl₂, and 10 X (with X either Tris, HEPES, MES, or citrate), with an osmolarity of 290–320 mOsm. The pH was adjusted to 5.5, 6.2 (X: citrate); to 6.2, 6.6, 6.8, 7.0 (X: MESI), to 7.0, 7.4, 7.8 (X: HEPES), and to 7.8, 8.2 (X: Tris) with NaOH. No differences in current were seen comparing mammalian Ringer's solution containing either citrate or MES at pH₆.2, either MES or HEPES at pH₇.0, and either

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HEPES or Tris at pH 7.8 as described earlier (12). A simple syringe-driven perfusion system was used to exchange the bath solutions in the recording chamber. The internal pipette solution for the whole-cell recordings contained (in mM): 155 Kf, 2 MgCl₂, 10 HEPES, 10 EGTA, adjusted to pH 7.2 with ROH, with an osmolality of 290–320 mOsm.

Kaliotoxin was purchased from Bachem Biochemica GmbH (Heidelberg, Germany) or made by recombinant methods (see below). Because of different batches of KTX, KTX from only one charge was used in each experiment. CTX was obtained from Bachem Biochemica GmbH (Heidelberg, Germany). Noxioxotin (NTX) was a generous gift from Dr. Stephen Brady (Merck Institute, West Point, PA). The Lys⁴⁰⁴→Thr (H404T) KTX (K27R, K27Thr, Lys⁴⁴I, K27I) were generous gifts from Dr. K. George Chandy (University of California, Irvine, CA). KTX was kindly provided by Dr. Martin-Eauclaire (Mar- seille, France). KTX mutants H34A, H34K, R31A/H34A, and K32A were generated recombinantly with friendly help of Dr. H. G. Knaus and Maria Trieb at the Department of Biochemical Pharmacology (University of Innsbruck, Innsbruck, Austria) (17).

The lyophilized peptides were stored at −70 °C. Stock solutions of 10–100 μM were made with mammalian Ringer’s solution containing 0.1% bovine serum albumin. The final dilutions were prepared shortly before the experiment.

Electrophysiology—Experiments were carried out using the whole-cell recording mode of the patch-clamp technique as described before (14). Pipettes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, United Kingdom) in three stages, coated with Sylgard (Dow Corning, Seneffe, Belgium), and fire-polished to resistances measured in the bath of 2.5–4 MΩ. Membrane currents were recorded with an EPC-9 clamp amplifier (HEKA Elektronik, Lambrecht, Germany) interfaced to a Macintosh computer running acquisition and analysis software (Pulse and PulseFit). Capacitative and leak currents were subtracted using the P/10 procedure. Series resistance compensation (>90%) was employed if the current exceeded 1 nA. The holding potential in all experiments was −80 mV.

Expression—pBsta plasmids containing the entire coding sequence of the mKv1.1 wild type (wt) gene, and the pSP64T plasmids containing the sequences for the H404T mutant mKv1.3 channel (18) (a generous gift from Dr. K. George Chandy) were linearized with PsI and EcoRI, respectively, and in vitro transcribed with the T7 (mKv1.1) and SP6 (mKsA H404T) Cap-Scribe system (Roche Molecular Biochemicals). The resulting cRNA was phenol/chloroform-purified and could be stored at −75 °C for several months.

Injection—The cRNA was diluted with a fluorescent FITC dye (0.5% FITC-dextran in 100 mM KCl) to a final concentration of 1 μg/ml RBL cells. The cRNA was injected with the cRNA/FITC solution filled in glass capillaries (Femtotips®) using an Eppendorf microinjection system (Mi- cromanipulator 5171 and Transjector 5246). In the visualized cells, specific currents could be measured 3–6 h after injection.

Mutagenesis—All mutants of mKv1.1, mKv1.3, and KTX were generated by using the QuikChange™ site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). Mutations were confirmed by se- quencing plasmid DNA with the Cy5-AutoRead kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Thermodynamic Mutant Cycle Analysis—Thermodynamic mutant cycles assist in studying of coupling energies between pairs of amino acid in a protein-protein complex. The dimensionless Ω value, which indicates the interaction strength of a given channel-toxin pair, was calculated as shown before (9, 19). The change in coupling energy, ∆G, for the channel-toxin pairs was calculated using the formula ∆G = kT ln Ω. The distances between this pair of residues was estimated based on the studies of Schreiber and Fersht (20) and assuming that the free energy of binding correlates linearly with the free energy of the interaction between the terminal amino group of the central Lys27 in KTX and the Cα atoms of Tyr⁴⁴I in the GYGD motive (21). The distances were measured as described earlier (7).

RESULTS

To initially characterize the effect of extracellularly applied KTX on current through mKv1.1 channels, we measured whole cell currents in response to depolarizing steps from −80 to +40 mV in the absence and presence of KTX (Fig. 1A, left). 60 nM KTX (Bachem) added to the external mammalian Ringer solution resulted in a peak current reduction of about 50%. To quantify the KTX affinity of mKv1.1, we measured, in additional experiments, the effect of different KTX concentrations on current through mKv1.1 channels and plotted the normalized peak currents against the applied KTX concentration (Fig. 1B, open triangles). A Hill equation was fitted to the data with a Hill coefficient of 1, indicating a 1:1 stoichiometry suggesting as described before (7).

The protein backbone of the mKv1.1 homology model was identical to the Kvα1 backbone. Docking of KTX into the mKv1.1 homology model was performed using the interactions between mKv1.1 and KTX as described before (7, 21). Additionally, the well characterized interaction between the terminal amino group of the central Lys27 in KTX and the Cα atoms of Tyr⁴⁴I in the GYGD motive was exploited (6, 21). The docking configuration and the distances between interacting amino acids were analyzed using RasMol 2.7.1.

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FIG. 1. Effect of extracellularly applied KTX on currents through mKv1.1 and H404T mutant mKv1.3 channels. A. pH-dependent KTX block of mKv1.1 channels. Currents were elicited by 200 ms depolarizing voltage steps from a holding potential of −80 mV to +40 mV every 20 s at pH 7.4 and 6.2, respectively. Currents were recorded before (control) and after external application of 60 nM KTX. The applied KTX was purchased from Bachem. B. dose-response curve for KTX to block peak current through mKv1.1 channels at pH 7.4 (●) and pH 6.2 (○). C. Data points, peak currents elicited as in A (top) in the presence of different KTX concentrations (I/KTX) were divided by the peak currents without KTX (Icontrol). Ratios I/KTX/Icontrol of at least four independent experiments are shown as mean ± standard deviation (error bars are only shown if they exceed the size of the symbol) and plotted against the indicated KTX concentration. The lines through the points were fitted to a modified Hill equation I/Icontrol = 1/(1 + ([KTX/Kd])) with a Hill coefficient of 1 revealing a Kd of 62 nM at pH 7.4 and of 19 nM at pH 6.2, respectively.
that one KTX molecule is sufficient to block one mKv1.1 channel. The fit gave a dissociation constant $K_d$ at pH$_o$ 7.4 of 62 nM ($n = 25$), which is in agreement with earlier reports (22). The current reduction by KTX was fully reversible upon washout of KTX even after wash-out for more than 1 h (data not shown). Application of 60 nM KTX (Bachem) to the channels with a non-protonatable glycine instead of a histidine at position 379 of the recombinant KTX from Bachem (Table I) probably because of higher purity of the recombinant KTX. To avoid possible problems with rundown versus block, we tested KTX on H404T mutant mKv1.3 channels. The H404T/mKv1.3 channel also contains no protonatable histidine in the outer pore region (Fig. 2A). Instead of the histidine, it has a glycine at the corresponding position 380; the histidine residue of the wt mKv1.3 channel at position 404 (equivalent to position 379 of mKv1.1) is changed to a threonine in this mutant of the channel (Fig. 2A). We did identical experiments with KTX from Bachem for H404T mutant mKv1.3 channels as we did for mKv1.1 channels. KTX displayed an increased affinity to mKv1.3 channels at low pH$_o$ 6.2 (open squares) versus previously published data (24) reported that the KTX block of calcium-depen-
dent potassium channels (MaxiK channels) and of voltage-dependent potassium channels is voltage-independent at physiological pH$_o$ but there were no studies testing a possible voltage dependence of KTX block at acid concentrations. Therefore, lowering pH could effect the pH dependence of KTX even after wash-out for more than 1 h (data not shown).

### Table I

| Channel Structure | $K_d$ at pH$_o$ 7.4 | $K_d$ at pH$_o$ 6.2 |
|-------------------|---------------------|---------------------|
| Kv1.1             |                     |                     |
| KTX$^a$           | 62                  | 19                  |
| KTX$^b$           | 10.6 ± 2            | 3.3 ± 0.5           |
| D381N Kv1.1       |                     |                     |
| KTX$^a$           | 272 ± 67            | 82 ± 12             |
| E353S Kv1.1       |                     |                     |
| KTX$^a$           | 94 ± 33             | 40 ± 12             |
| H404T Kv1.3       |                     |                     |
| KTX$^a$           | 0.24                | 0.10                |
| KTX$^b$           | 0.059 ± 0.006       | 0.025 ± 0.004       |
| CTX               | 0.25                | 0.25                |
| NTX               | 5.4                 | 7.7                 |
| KTX$^a$           | 0.15 ± 0.015        | 0.17 ± 0.025        |
| KTX-H34A          | 0.097 ± 0.008       | 0.096 ± 0.019       |
| KTX-H34K          | 0.017 ± 0.003       | 0.016 ± 0.006       |
| KTX-K32A          | 0.98 ± 0.1          | 1.07 ± 0.1          |
| KTX-K27Dap        | 0.24 ± 0.04         | 0.11 ± 0.03         |
| KTX-K27Dab        | 5.4 ± 0.3           | 1.4 ± 0.04          |
| KTX-K27ThLys     | 5.6 ± 0.3           | 3.2 ± 0.3           |
| KTX-K27Nle        | 0.19 ± 0.02         | 0.08 ± 0.01         |
| KTX-K27A          | 42                  | 16                  |
| D375A/D376A/H404T Kv1.3 | 79 ± 4               | 33.7 ± 2.5           |
| KTX$^a$           | 0.111 ± 0.01        | 0.102 ± 0.01        |
| D375A/H404T Kv1.3 |                     |                     |
| KTX$^a$           | 0.059 ± 0.006       | 0.056 ± 0.005       |
| D376A/H404T Kv1.3 |                     |                     |
| KTX$^b$           | 0.094 ± 0.002       | 0.040 ± 0.008       |
| S378E/H404T Kv1.3 |                     |                     |
| KTX$^b$           | 0.057 ± 0.003       | 0.016 ± 0.002       |
| P377A/S378E/H404T Kv1.3 | 0.064 ± 0.006      | 0.019 ± 0.002       |

$^a$ KTX from Bachem.  
$^b$ Recombinant KTX.

In order to find out whether voltage-dependent binding of KTX could effect the pH$_o$ dependence of KTX block, we investigated voltage dependence of KTX block on current through H404T mutant mKv1.3 channels (Fig. 3). Crest et al. (23) and Mourre et al. (24) reported that the KTX block of calcium-dependent potassium channels with large conductance (MaxiK channels) and of voltage-dependent potassium channels is voltage-independent at physiological pH$_o$ but there were no studies testing a possible voltage dependence of KTX block at acid concentrations. Therefore, lowering pH could effect the pH$_o$ dependence of KTX even after wash-out for more than 1 h (data not shown). To avoid possible problems with rundown versus block, we tested KTX on H404T mutant mKv1.3 channels. The H404T/mKv1.3 channel also contains no protonatable histidine in the outer pore region (Fig. 2A). Instead of the histidine, it has a glycine at the corresponding position 380; the histidine residue of the wt mKv1.3 channel at position 404 (equivalent to position 379 of mKv1.1) is changed to a threonine in this mutant of the channel (Fig. 2A). We did identical experiments with KTX from Bachem for H404T mutant mKv1.3 channels as we did for mKv1.1 channels. KTX displayed an increased affinity to H404T mutant mKv1.3 channels at low pH$_o$ despite the absence of protonatable histidines in the outer pore region. The dose-response curve for pH$_o$ 6.2 was shifted toward lower concentrations compared with pH$_o$ 7.4 (Fig. 1C) and revealed $K_d$ values of 0.24 nM ($n = 28$) and 0.10 nM ($n = 26$) for pH$_o$ 7.4 and 6.2, respectively.
for the higher affinity at low pH that protonation of an amino acid in KTX might be responsible
channels lacking histidine residues in the pore region indicated
ment of m currents (see also Fig. 1).

CTX blocked the current through H404T/mKv1.3 with equal strength at pH 7.4 as well as at pH 6.2, which can be seen by the identical Kd values shown in Table I for pH 7.4 (0.25 nM; n = 28) and pH 6.2 (0.25 nM; n = 22). In addition, H404T mutant mKv1.3 channels showed a slight but opposite pH dependence of NTX block with Kd values of 5.4 nM (n = 24) and 7.7 nM (n = 20) at pH 7.4 and 6.2, respectively (Table I). We therefore concluded that the increase in affinity by lowering pH is specific for KTX since CTX and NTX did not show this behavior.

By comparing the amino acid sequence of the toxins, we found that KTX possesses a unique histidine at position 34 not present in CTX and NTX (Fig. 2B). In order to find out whether His34 might be the protonatable amino acid in KTX, we used KTX3, which has an Asp at position 34 whose protonation should not vary between pH 7.4 and 6.2. KTX3 exhibited nearly the same affinity to H404T/mKv1.3 channels at pH 7.4 and 6.2 with Kd values of 150 ± 15 pm (n = 6) and 170 ± 25 pm (n = 6) at pH 7.4 and 6.2, respectively (Table I). The loss of the pH-dependent KTX effect in KTX3 indicated that His34 of KTX might be responsible for this effect because of the lack of a histidine residue at position 34 in KTX3.

Additional experiments with KTX-Lys27 mutants on H404T mutant mKv1.3 channels were made to exclude Lys27 of KTX as the protonatable amino acid of KTX. The KTX mutants K27Dap, K27Dab, and K27ThrLys contained non-naturally positively charged lysine analogs of varying side chain lengths (Dap, 2.5 Å from Cα; Dab, 3.8 Å; ThrLys, 7.7 Å; natural lysine, 6.3 Å), whereas in K27A and K27Ne the neutral alanine and the non-neutral natural norleucine replaced lysine (6, 9). If Lys27 was somehow involved in the pH dependence of KTX block, we would expect a pH-dependent block with the noncharged amino acids at position 27 and perhaps an altered pH dependence with the shorter and longer non-natural lysine analogs Dap, Dab, and ThrLys. Table I shows that all KTX-Lys27 mutants bound in a pH-dependent manner to H404T mutant mKv1.3 channels with a higher affinity at low pH, revealing that Lys27 is not responsible for pH dependence of KTX block.

To further substantiate that protonation of His34 in KTX is responsible for the pH dependence of KTX block on current through mKv1.1 and H404T mutant mKv1.3 channels, we made mutants of this peptide toxin by recombinant methods (17). His34 of KTX seemed to be a good candidate for the protonatable amino acid in KTX, as indicated by the investigations with H404T/mKv1.1 channels and CTX, NTX, and KTX3. Therefore, we investigated the KTX mutants H34A and H34K. In addition, we also replaced Arg31 and Lys32 in KTX with neutral amino acids to examine whether these two positions in KTX were also involved since earlier work indicated that these two positions participate in important interactions (9, 27). We therefore tested the pH dependence of block of all four KTX mutants with H404T mutant mKv1.3 channels (Table I). The KTX mutant K32A still had a weaker blocking effect at pH 7.4 compared with pH 6.2 by a factor of about 2.1 on H404T mutant mKv1.3 channels, suggesting that Lys32 in KTX did not cause the pH dependence of block (Table I). In contrast, KTX-H34A blocked H404T mutant mKv1.3 with the same potency at pH 7.4 and 6.2, respectively (Table I). We therefore concluded that the increase in affinity by lowering pH is specific for KTX since CTX and NTX did not show this behavior.

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By a set of 200-ms voltage steps from a holding potential of −80 mV to voltages between −60 and +100 mV in 20-mV increments every 20 s, in the absence (top) and the presence (bottom) of 50 μM recombinantly produced KTX at pH_7.4. B, corresponding conductances for the currents shown in A are plotted against the applied membrane potential, in the absence (□) and in the presence (●) of KTX. The lines through the points were fitted by a Boltzmann equation: $g_{K,max} = \frac{g_{K,max}}{1 + \exp([E_{1/2} - E]/k)}$ with values for $g_{K,max}$ of 24 and 16 nS without and with KTX, respectively. The determined values for $k$ (6 mV) and $E_{1/2}$ (−24 mV) were confirmed by additional experiments. C, currents elicited as described in A at pH_6.2. D, corresponding conductance-voltage relations for the currents shown in C, in the absence (□) and in the presence (●) of KTX. The Boltzmann fit was made as described in B with values for $g_{K,max}$ of 25 and 10 nS without and with KTX, respectively. Values for $k$ (6 mV) and $E_{1/2}$ (−10 mV) were also confirmed by additional experiments.

**FIG. 3.** Voltage dependence of KTX block on current through H404T mutant mKv1.3 channels at pH_7.4 and 6.2. A, currents elicited by a set of 200-ms voltage steps from a holding potential of −80 mV to voltages between −60 and +100 mV in 20-mV increments every 20 s, in the absence (top) and the presence (bottom) of 50 μM H404T mutant (A). Corresponding conductances for the currents shown in A are plotted as a function of applied membrane potential, in the absence (□) and in the presence (●) of KTX. The lines through the points were fitted by a Boltzmann equation: $g_{K,max} = \frac{g_{K,max}}{1 + \exp([E_{1/2} - E]/k)}$ with values for $g_{K,max}$ of 24 and 16 nS without and with KTX, respectively. The determined values for $k$ (6 mV) and $E_{1/2}$ (−24 mV) were confirmed by additional experiments. C, currents elicited as described in A at pH_6.2. D, corresponding conductance-voltage relations for the currents shown in C, in the absence (□) and in the presence (●) of KTX. The Boltzmann fit was made as described in B with values for $g_{K,max}$ of 25 and 10 nS without and with KTX, respectively. Values for $k$ (6 mV) and $E_{1/2}$ (−10 mV) were also confirmed by additional experiments.

pH_7.4 and 6.2 (Fig. 4A (top) and Table I). Additional replacement of Arg_31 with alanine in the H34A/R31A mutant KTX exhibited no further effect (Table I). Replacement of His_34 in KTX by lysine led to the same effect as substitution by alanine concerning the pH-independent block but exhibited a higher affinity to H404T mutant than H34A and wt KTX (Fig. 4A (bottom) and Table I). Therefore H34A, H34K, and R31A/H34A mutants of KTX blocked currents through H404T mutant mKv1.3 channels in a pH-independent manner, whereas KTX-H34A block is influenced by pH. These results suggest that only the histidine residue at position 34 of KTX is the protonatable amino acid of KTX that causes pH-dependence of block while Arg_31 and Lys_32 of KTX do not seem to play a role in this effect.

If protonation of His_34 in KTX is the reason for the better block of mKv1.1 and H404T mutant mKv1.3 channels with lowering pH_7.4 A and H34K mutants of KTX should (a) have a pH-independent block as shown before (Fig. 4A and Table I) and (b) represent the fully protonated (H34K) and the fully unprotonated (H34A) form of wt KTX responsible for the pH-dependence of block. Fig. 4B shows the titration of wt KTX and the mutants H34K and H34A from pH_5.5 to pH_7.8 examined on H404T mutant mKv1.3 channels. KTX-H34K exhibited the same K_d of ~16 pM in the investigated pH_range, and KTX-H34A had identical K_d values of ~100 pM at different pH values between pH_5.5 and 7.4. Linear regressions through the data points of both KTX mutants had slopes of zero, indicating that these KTX mutants cannot be titrated in the pH_range used. This also suggests that histidine at position 34 of KTX is protonatable. The affinity of wt KTX to H404T mutant mKv1.3 channels decreased with increasing pH. The fit through the data points using the Hill equation gave a K_d(min) of 18 pM at pH_5.5 and a K_d(max) of 60 pM at pH_7.8 and a K_d of 6.5, which represents the pH of the half-maximum protonation. The Hill coefficient of 1.7 suggests that more than one negatively charged amino acids of the channel protein could sense His_34 of KTX. The K_d(min) of 18 pM wt KTX at pH_5.5 is the expected value with good agreement with the K_d of KTX-H34K, which stands for the fully protonated form of wt KTX. In contrast, KTX-H34A, which represents the unprotonated wt KTX, had a lower affinity to H404T/mKv1.3 channels with 100 pM compared with K_d(max) of wt KTX with 60 pM at pH_7.8. The reason for this difference might be a non-ionic interaction between the unprotonated His_34 of wt KTX and an amino acid of the channel that is abolished by the substitution with alanine. A more conservative replacement with glutamine or asparagine might solve this question (28, 29). Since the data confirmed our predictions apart from this difference between K_d(max) of wt KTX and K_d of KTX-H34A, we concluded that indeed protonation of His_34 in KTX caused the pH-dependence of block.

To determine which amino acid(s) of the channel protein interacted with the protonatable His_34 of KTX, we tested some mutants of the mKv1.1 and the H404T/mKv1.3 channels, respectively. Six negatively charged residues in the mKv1.1 pore region could be candidates for the interaction with His_34 in KTX: Glu_348, Glu_350, Glu_351, Glu_343, Asp_361, and Asp_377 (Fig. 2A). The mKv1.3 channel contains all of these negatively charged amino acids except one at position 378, which corresponds to position 353 of mKv1.1. We did not investigate the Asp_377 of mKv1.1 (Asp_402 in mKv1.3) of the GYG motif, since we had found that the shorter lysine analogs Dap and Dab at position 27 of KTX that were shown to interact with Asp_402 in mKv1.3 (6) still blocked in a pH-dependent manner (Table I). In addition, from the KcsA crystal structure data, we concluded that the negative charges at positions 348–353 of mKv1.1 would be the best candidates for the interacting amino acids. We expected that replacing negatively charged amino acids in

**Mammalian K^+ Channel Structure**

39349

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**Table I.**

| pH | K_d(min) | K_d(max) |
|----|----------|----------|
| 5.5 | 18 pM    | 60 pM    |
| 7.8 | 6.5      |          |
the channel that interact with His34 with neutral residues would diminish or abolish pH-dependent KTX block, depending on whether the negatively charged residue alone or together with others could sense His34 in KTX. As a starting point, we investigated Glu350 of mKv1.1 as an interacting residue because of the dissimilar amino acid at the equivalent position of H404T/mKv1.3 channels, which might explain the different ratios (Kd, pH 7.4/Kd, pH 6.2) of both channels. The E353S mutant mKv1.1 channel showed still an improvement of KTX affinity with lowering pH, with Kd values of 94 nM and 40 nM (n = 5) for pH 7.4 and 6.2, respectively (Table I). Nevertheless, the ratio of (Kd, pH 7.4/Kd, pH 6.2) was reduced to 2.4, the same ratio as for H404T mutant mKv1.3 channels. Therefore, we concluded that (a) the lack of a negative charge at position 378 in mKv1.3 (equivalent to 353 in mKv1.1) reduced the (Kd, pH 7.4/Kd, pH 6.2) ratio, and (b) that Glu350 in mKv1.1 is one of the negatively charged residues in mKv1.1, which interacts with the protonatable amino acid in KTX. This result was confirmed by the introduction of a negatively charged amino acid at mKv1.3 position 378 (P377A/S378E/H404T and S378E/H404T mutant mKv1.3 channels) exhibiting the stronger improvement of KTX block with lowering pH, like mKv1.1 channels (Table I). Therefore, protonation of His355 in mKv1.1 (12) seemed not to cause the stronger pH effect of mKv1.1 compared with H404T/mKv1.3.

As additional candidate for an interacting amino acid of the channel, we investigated the D361N mutant mKv1.1 channel lacking the negative charge at position 361 (equivalent to position 386 in mKv1.3). As can be seen from Table I, despite the deficiency in this negative charge, KTX block is still pH-dependent and exhibited the same (Kd, pH 7.4/Kd, pH 6.2) ratio as wt KTX. Therefore we concluded that Asp376 in mKv1.1 (Asp386 in mKv1.3) did not interact with His34 in KTX.

Furthermore, we investigated whether the negative charges in mKv1.1 position 350 and 351 (corresponding to positions 375 and 376 in mKv1.3) sense the protonatable His34 in KTX. These studies were performed with three mutants of the H404T/mKv1.3 channel since equivalent mKv1.1 mutants could not be expressed in RBL cells. Substitution of both negatively charged aspartate residues with neutral alanines in the D375A/D376A/H404T mutant mKv1.3 channel diminished the pH dependence of KTX block (Table I). Since we could not detect any statistical significant difference between the Kd values at pH 7.4 and 6.2 by using Student’s t test (p < 0.05), removal of the negative charges at position 375 and 376 in mKv1.3 abolished the pH dependence of KTX block. To test whether both or only one of these glutamates could interact with His34 in KTX, we replaced only one of the negative charged residues by a neutral alanine. The D376A/H404T mutant mKv1.3 channels showed the same pH-dependent KTX block as H404T/mKv1.3 channels with an unchanged (Kd, pH 7.4/Kd, pH 6.2) ratio of 2.4 (Table I). In contrast, KTX displayed the same affinity to D375A/H404T mutant mKv1.3 channels at pH 7.4 and 6.2 with an identical (Kd, pH 7.4/Kd, pH 6.2) ratio to D375A/D376A/H404T mutant mKv1.3 channels. Again there was no statistical significant difference between the Kd values at pH 7.4 and 6.2 analyzed with Student’s t test with p < 0.05; accordingly, we concluded that only Asp375 interacts with the protonatable His34 in KTX. From this result we could also exclude Glu373 in mKv1.1 and the equivalent Glu348 in mKv1.1 to interact with His34 in KTX.

The knowledge about the effective KTX concentration enabled us to determine the potential ψ, which caused the higher effective KTX concentration at low pH. ψ for wt KTX was −30.4 mV, for E353S/mKv1.1 1 − 22 mV, and for the calculated mutant E350A/mKv1.1 − 8.6 mV. These values for ψ were correct if only one Glu350 and Glu356 with His34/KTX. E353S mutant mKv1.1 channels exhibited the exclusive interaction between Glu350/mKv1.1 and His34/KTX with an effective KTX concentration of 235 nM at pH 6.2 in contrast to the bulk concentration of 100 nm. The sole interaction between Glu353/mKv1.1 and His34/KTX was calculated by dividing the Kd factor for mKv1.1 by the same factor for E353S/mKv1.1, indicating that protonation of His34/KTX would raise KTX concentration 1.4 times due to the interaction with Glu353 in mKv1.1.

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Å between His\textsuperscript{34}/KTX and one, or four interacting Glu\textsuperscript{350}/mKv1.1 and distances of 6.5–7, 9.5, or 12.5 to 14 Å between His\textsuperscript{34}/KTX and one, two, or four interacting Glu\textsuperscript{350}/mKv1.1.

Corresponding calculations were performed for interacting residues in H404T/mKv1.3 and His\textsuperscript{34} in KTX. We estimated the same distances for His\textsuperscript{34}/KTX and Asp\textsuperscript{375}/mKv1.3 as for His\textsuperscript{34}/KTX and Glu\textsuperscript{350}/mKv1.1. The distance between His\textsuperscript{34}/KTX and S378E/mKv1.3 also seemed to be the same as for His\textsuperscript{34}/KTX and Glu\textsuperscript{350}/mKv1.1.

Furthermore, we calculated the distance between the only interacting residue in H404T/mKv1.3 channels, Asp\textsuperscript{375} and His\textsuperscript{34}/KTX by electrostatic compliance using KTX\textsubscript{3} and KTX\textsubscript{3}H34K. By application of the known charge difference of two between Asp\textsuperscript{35} in KTX\textsubscript{3} and Lys\textsuperscript{34} in H34K (Fig. 2) and the \(K_d\) values (Table I), we calculated a local potential of \(-55.9\) mV. Therefore, we found distances between Asp\textsuperscript{375}/mKv1.3 and His\textsuperscript{34}/KTX of 4–4.5 Å for the interaction with one channel subunit, of 6.5–7 Å for the interaction with two subunits, and of 9 Å for the interaction with all four channel subunits, respectively. The agreement of this electrostatic compliance calculation using the known charge difference between H34K and KTX\textsubscript{3} at position 34 with the electrostatic compliance using the degree of protonation at His\textsuperscript{34}/KTX enabled us to confirm the above determined \(pK_a\) of His\textsuperscript{34}/KTX.

Estimation of the distance between Asp\textsuperscript{375}/mKv1.3 and His\textsuperscript{34}/KTX was also carried out by mutant cycle analysis. The KTX block of the channel mutants D375A/H404T and D375A/H404T was tested at pH 7.4 and 6.2 (Table I) using the different degrees of protonation at KTX position His\textsuperscript{34} instead of mutations at KTX position 34. The change in coupling energy \(\Delta G\) of 0.5 kcal mol\(^{-1}\) indicated a distance of maximally 5 Å between Asp\textsuperscript{375} of mKv1.3 and His\textsuperscript{34} of KTX (7, 20). The mutant cycle analysis confirmed the distance calculation by electrostatic compliance and suggested the interaction of His\textsuperscript{34}/KTX with Asp\textsuperscript{375} in only one channel subunit.

To refine the docking conformation of mKv1.1 with KTX, we confirmed the known interacting pair of Gly\textsuperscript{386}/Kv1.3 and Arg\textsuperscript{31}/KTX for the mKv1.1 channel (9). We investigated wt and H355K mutant mKv1.1 channels with the KTX mutants H34A versus R31A/H34A. wt mKv1.1 exhibited an \(-10\) times higher affinity to H34A compared with R31A/H34A with \(K_d\) values of 38 ± 4 nm and 410 ± 40 nm, respectively. In contrast, R31A/H34A blocked current through H355K mutant mKv1.1 channels only 2 times more weakly than the H34A mutant KTX with \(K_d\) values of 1120 ± 180 nm and 620 ± 80 nm for R31A/H34A and R34A block, respectively. Application of mutant cycle analysis revealed a \(\Delta G\) value of 1.05 kcal mol\(^{-1}\), which indicates a proximity of ≤5 Å between the interacting residues His\textsuperscript{355} in mKv1.1 and Arg\textsuperscript{31} in KTX similar to the Kv1.3-KTX complex.

Using a homology model of the mKv1.1 channel based on the known crystal structure of the Kv\textsubscript{Ca} channel (8), we visualized the docking configuration of mKv1.1 and KTX. The docking of KTX into the mKv1.1 homology model is shown in Fig. 5. KTX is shown in green, mKv1.1 in gray. The interacting amino acids of channel and toxin (Arg\textsuperscript{31} and His\textsuperscript{34} of KTX and Gly\textsuperscript{350}, Glu\textsuperscript{353}, and His\textsuperscript{355} of mKv1.1) are drawn as Corey-Pauling-Koltun surfaces, the remaining channel as schematic, the remaining toxin as ribbon. His\textsuperscript{34} of KTX interacts with Gly\textsuperscript{350} and Glu\textsuperscript{353} of one channel subunit as indicated by the mutant cycle analysis between Asp\textsuperscript{375}/mKv1.3 and His\textsuperscript{34}/KTX. The large distances between two adjacent or opposite channel subunits would not yield the interaction strength measured by electrostatic compliance and mutant cycle analyses. The docking of KTX into the mKv1.1 homology model based on the Kv\textsubscript{Ca} structure was performed using the interactions between channel and toxin described here and, additionally, the interaction between Tyr\textsuperscript{377}/mKv1.1 and Lys\textsuperscript{377}/KTX (6). The docking exhibited distances of 11 Å between Glu\textsuperscript{350}/mKv1.1 and His\textsuperscript{34}/KTX, of 11 Å between Glu\textsuperscript{350}/mKv1.1 and His\textsuperscript{34}/KTX, and of 5 Å between Glu\textsuperscript{350}/mKv1.1 and Arg\textsuperscript{31}/KTX. Therefore, the docking distance between Glu\textsuperscript{350}/mKv1.1 and His\textsuperscript{34}/KTX was 7 Å and between Glu\textsuperscript{350}/mKv1.1 and His\textsuperscript{34}/KTX 4–4.5 Å larger compared with the experimentally determined distances. The docking distance of 5 Å between His\textsuperscript{355}/mKv1.1 and Arg\textsuperscript{31}/KTX was identical to the distance calculated by mutant cycle analysis according to the experimental results. The docking suggested distances of 22 Å between Glu\textsuperscript{350}/mKv1.1 and of 15 Å between Glu\textsuperscript{350}/mKv1.1 and the center of the pore. In contrast, the experimentally derived results indicated distances of 15 Å between Glu\textsuperscript{350} and of 10.5–11 Å between Glu\textsuperscript{350} in mKv1.1 and the pore axis. The mKv1.1 homology model has the same amino acid sequence compared with the mKv1.1 clone, but the structure of the protein backbone was identical to Kv\textsubscript{Ca}. Therefore, deviations between the experimentally determined distances and the docking distances might indicate a different spatial arrangement of the turret in mKv1.1 and Kv\textsubscript{Ca} (amino acids 348–357 in mKv1.1). Guiding the turret at position 3507 Å and at position 3534–4.5 Å more toward the center of the pore as simulated by the yellow line in Fig. 5 would match experimentally determined and docking distances. To satisfy our data, we must modify the Kv\textsubscript{Ca} crystal structure for mKv1.1, which suggests structural differences between mKv1.1 and Kv\textsubscript{Ca} in the outer pore region. Since mKv1.3 exhibited the same interacting positions and interaction strengths in the turret like mKv1.1, the mKv1.3 structure might differ from Kv\textsubscript{Ca} as well.
mKv1.1 and mKv1.3 and maybe other mammalian voltage-gated potassium channels seem to be structurally different from KcsA in the turret.

**DISCUSSION**

In this report we demonstrate that the pH dependence of KTX block on current through mKv1.1 and H404T mutant mKv1.3 channels is caused by protonation of the histidine at KTX position 34. We could define two interacting positions of the channel proteins: 350/353 in mKv1.1 and 375/378 in H404T mutant mKv1.3 channels, respectively. Furthermore, we could confirm an equivalent interaction between Arg31 in KTX and His355 in mKv1.1 known for Kv1.3 and KTX (9). Comparing experimentally derived and docking distances between interacting residues of mKv1.1 and KTX indicated that the turret of mKv1.1 is oriented more toward the center of the pore compared with KcsA whose structure was used as template for the mKv1.1 homology model. In summary, we present new pairs of interacting residues between KTX and the potassium channels mKv1.1 and mKv1.3 to increase the knowledge about the outer pore region of these channels, which might be advantageous in designing novel drugs of higher affinity and specificity.

This is the first report of a peptide toxin binding to potassium channels whose affinity to the channel’s receptor was increased in low pH. Deutsch et al. (10) reported about an enhanced CTX binding to the voltage-gated K+ channel in human T lymphocytes with increasing pH. The reduced CTX and KTX block of wt Kv1.3 in acid pH was shown to be a consequence of protonation of His314 in wt Kv1.3 channels, which repelled the positively charged toxins (9). Replacing His314 in Kv1.3 with non-protonatable threonine caused a pH-independent CTX block and an increased KTX block corresponding to our results (9). The diminished CTX block of F425H mutant Shaker channels at low pH was also a result of protonation of F425H of this Shaker mutant (11). Diminished sensitivity of tityustoxin to the squid potassium channel SgKv1A was also attributed to the protonation of a histidine at position 351 (equivalent to Shaker position 425) of the channel (14).

Other blocking agents like TEA also exhibited a pH dependence of block. Diminished TEA block on current through mKv1.1 channels was caused by protonation of His355 in mKv1.1, which induced an electrostatic repulsion of the positively charged TEA (12). Weaker sensitivity of saxitoxin and tetrodotoxin to block voltage-gated sodium channel at low pH, was also discussed to be a result of protonation of the receptor (1, 13), but there is also evidence that the decreased block with increasing [H+] or increasing concentration of di- or trivalent cations is caused by neutralization of negative surface charges lowering the saxitoxin and tetrodotoxin concentration at its receptor site (30).

The reduced blocking strength of the above toxins was caused by protonation of the receptor or by changing the surface potentials. None of these examples revealed altered affinity because of protonation of the blocker itself. Therefore, our observations regarding the pH dependence of KTX block are different in two respects. First, the KTX block of mKv1.1 and H404T mutant mKv1.3 channels is increased with decreasing pH. Second, this higher affinity is the result of protonation of an amino acid in the peptide toxin, whereas protonation of the KTX receptor seemed not to play a role in causing this effect.

The pH independence of CTX and KTX block on current through H404T/mKv1.3 indicated that protonation of His314/KTX could cause the pH dependence of KTX block. Our hypothesis was proven by the pH-independent KTX-H34A and KTX-H34K block. Titration of wt and mutant KTX revealed that H34K behaved like the fully protonated wt KTX. On the other hand, H34A, which should behave like fully unprotonated wt KTX, certainly blocked H404T/mKv1.3 in a pH-independent manner, however, with a lower affinity compared with wt KTX at pH 7.8 with a degree of protonation less than 5%. The difference in affinity between wt KTX at high pH and H34A might be caused by an interaction between His355/KTX and residues of the channel via the nitrogen atoms, which is destroyed by the substitution with alanine. More conservative exchanges of His355 with glutamine or asparagine might have the same blocking strength as unprotonated wt KTX, as shown for several histidine interactions in other proteins (28, 29).

Titration of wt KTX also indicated a pHK of 6.5 for His354/KTX. Gairi et al. (27) determined the pHK of His354 in unbound KTX by NMR investigations with a value of about 5.2. These two observations seem to be in contrast; however, we investigated the pHK of KTX that was interacting with H404T/mKv1.3 channels and not while unbound in solution. The stronger interaction of protonated His354/KTX with negatively charged residues of the H404T channel might facilitate the protonation of His354 and therefore raise its pHK. Similar observations with lowered pHK values (20) and elevated pHK values (31) due to unfavorable and advantageous interactions with the receptor or ligand have been reported previously.

Replacing negatively charged residues in the outer pore vestibule of mKv1.1 and H404T/mKv1.3 or vice versa revealed Glu350 and Glu353 in mKv1.1 and Asp357 in H404T to sense protonation of His354 in KTX. Since mKv1.1 and mKv1.3 exhibited the same interacting positions and the same strength of interaction, we could transfer results from one channel to the other. Using electrostatic compliance we were able to calculate the distances between the interacting residues of the KTX and both channels (5, 9). Application of two mutant cycles refined the docking configuration of KTX and suggested that His354/KTX interacted with only one subunit of the channel.

We used these experimental data for a docking of KTX into a mKv1.1 homology model based on the KcsA coordinates (8). The average sequence identity of about 30% between KcsA and mKv1.1 is sufficient to generate homology models using the KcsA structure as template (32). The docking of KTX into the mKv1.1 homology model built on the basis of the KcsA structure exhibited a distance of about 5 Å between His355/mKv1.1 and Arg31/KTX, which agrees with the experimentally derived distance. The pore diameter of about 30 Å at position 355/mKv1.1 corresponds to the equivalent positions in Kv1.3 and Shaker channels (9, 19). In contrast, the docking distances at mKv1.1 positions 350 and 353 within the turret disagreed from the experimentally obtained distances, suggesting shorter pore diameter at these positions for mKv1.1 compared with KcsA. To get an agreement between the experimental and the docking data, we would have to modulate the KcsA structure within the turret (amino acids 348–357) guiding Glu350 7 Å and Glu353 4–4.5 Å more toward the center of the pore. Therefore, the shape of the mKv1.1 turret seemed to be distinct from KcsA, particularly in the region near the S5 segment but not in the region close to the pore helix. The spatial arrangement of the turret in mKv1.3 and mKv1.1 seems to be very similar since we found identical interacting positions and the same distances between the interacting positions. Evidence for topological differences between KcsA and voltage-gated potassium channels within the turret is also indicated by distinct toxin affinities of both channel types (33, 34). Only the replacement of the complete outer pore region of KcsA with the equivalent region of a mammalian voltage-gated potassium channel could create a similar toxin affinity in KcsA (35).

In conclusion, using KTX as a molecular caliper, we were able to characterize topological features within the turret of the voltage-gated potassium channels mKv1.1 and mKv1.3. The
turret of both channels seems to be structurally different compared with the bacterial KcsA channel. Since the receptor for many potassium channel modulators is located in the outer pore region, this information could aid the rational drug design and therefore accelerate the generation of new and improved drugs working on potassium channel.

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