



Block of maurotoxin and charybdotoxin on human intermediate-conductance calcium-activated potassium channels (hIKCa1)

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Abstract

Using human intermediate-conductance calcium-activated potassium (hIKCa1) channels as a model we aimed to characterize structural differences between maurotoxin (MTX) and charybdotoxin (CTX) and to gain new insights into the molecular determinants that define the interaction of these pore-blocking peptides with hIKCa1 channel. We report here that the block of MTX, but not of CTX on current through hIKCa1 channels is pH₀ dependent. The replacement of histidine 236 from hIKCa1 channel with a smaller amino acid, cysteine, did not change MTX binding affinity, however, partially affected the pH₀ dependency of its block at low pH₀. In contrast, CTX binding affinity to the hIKCa1_H236C channel mutant was increased suggesting that His236 might play a role in the binding of CTX, but has only a weak influence in the binding of MTX to hIKCa1 channels.

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1. Introduction

Scorpion venoms constitute a rich source of peptidyl inhibitors of K⁺ channels. Previous studies have shown that these toxins usually bind to the extracellular side of the channel and block it by occluding the channel pore. Knowledge of the three-dimensional structure of these peptides, combined with site-directed mutagenesis has allowed the identification of those toxin residues critical for binding to the channel pore, and has provided a picture of the interaction surface with K⁺ channels (Hidalgo and MacKinnon, 1995; Aiyar et al., 1996; Rauer et al., 2000). To gain insight into the molecular determinants that define

the specificity of interaction of pore-blocking peptides, such as charybdotoxin (CTX), and maurotoxin (MTX), we focussed on their interaction with hIKCa1 channels. The intermediate-conductance, calcium-activated potassium channel, IKCa1 (Ishii et al., 1997; Jensen et al., 1998), is a well-recognized therapeutic target present in erythrocytes, human T-lymphocytes, and colon (Grissmer et al., 1993; Ishii et al., 1997; Logsdon et al., 1997; Vanderpe et al., 1998; Jensen et al., 1998; Neylon et al., 1999; Ghanshani et al., 2000; Chandry et al., 2001). More recent studies associate its up-regulation with cell proliferation and suggest the fact that hIKCa1 might be used as a marker of physiological and pathological cell proliferation (Vanderpe et al., 1998; Khanna et al., 1999; Pena and Rane, 1999; Wulff et al., 2000; Rane, 2000; Kohler et al., 2000; Elliot and Higgins, 2003).

To further investigate the interaction of different toxins with hIKCa1 channels, we have chosen MTX and CTX, two scorpion toxins belonging to the α KTX-toxins family

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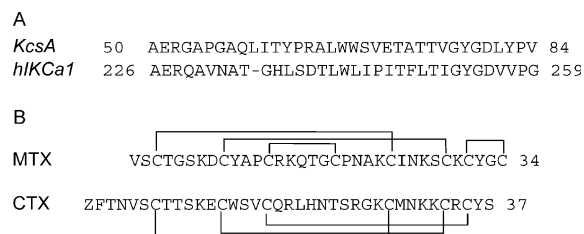


Fig. 1. Alignment of channels and toxins. (A) Amino acid alignment of *KcsA*, and *hKCa1* channel sequences in the putative pore region; (B) alignment of the amino acid sequences of MTX and CTX.

(Tytgat et al., 1999). Even if they are far related they both block *hKCa1* channels with high affinity (Castle et al., 2003; Rauer et al., 2000). MTX is a 34 amino acid peptide toxin cross-linked by four disulfide bridges that have an atypical organisation pattern among scorpion toxins (C1–C5, C2–C6, C3–C4, and C7–C8, instead of C1–C4, C2–C5, C3–C6 for three-disulfide bridged toxins and C1–C5, C2–C6, C3–C7, and C4–C8 for four-disulfide bridged toxins; Fig. 1B) isolated from the venom of the Tunisian chactoid scorpion *Scorpio maurus palmatus* (Kharrat et al., 1996, 1997; Castle et al., 2003). In comparison with MTX, CTX is a slightly bigger peptide toxin of 37 amino acids, cross-linked with three disulfide bridges (C1–C4, C2–C5, C3–C6; Lambert et al., 1990; Fig. 1B). Despite their different sizes and disulfide bridge organisations, MTX and CTX have the classical three-dimensional structure of peptide scorpion toxins constituted by one α -helix and two or three β -sheets (Fig. 2).

In order to design selective peptide blockers for *hKCa1* channel we must elucidate, as precisely as possible, all the topographies by which known blockers bind to the external pore region of this channel. All the scorpion toxins that block K^+ channels have a functional amino acid dyad formed by a positively charged amino acid (usually a Lys) that is protruding into the channel pore, and a positively charged or aromatic amino acid that is stabilising toxin-channel interaction (Lys, Arg, or Tyr; Hidalgo

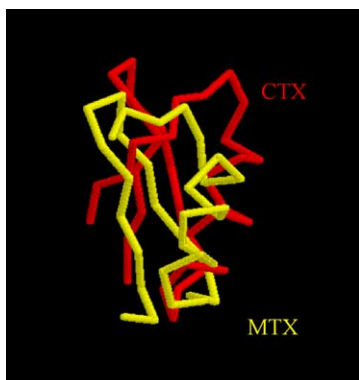


Fig. 2. Superimposed backbone structures of MTX (yellow) and CTX (red).

and MacKinnon, 1995; Aiyar et al., 1996; Rauer et al., 2000; Avdonin et al., 2000; Castle et al., 2003). The other interactions that peptide toxins establish with the channels are weaker, but important for toxin binding affinity and specificity.

Here, we report the importance in toxin binding of a histidine situated far from the pore region of *hKCa1* channel that is influencing CTX, but not MTX binding to the *hKCa1* channel pore.

2. Methods

Cells. The tsA cell line was maintained in MEM with Earle's salts supplemented with Glutamax-I (Gibco, UK), and 10% heat-inactivated FCS. Cells were kept in a humidified, 10% CO_2 incubator at 37 °C.

Solutions. All experiments were carried out at room temperature (21–25 °C). Cells were measured in normal mammalian Ringer's solution containing (in mM): 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 X (with X either MES, HEPES, or Tris), with an osmolarity of 290–320 mOsm. The pH_0 was adjusted to 6.2 (X: MES), 7.4 (X: HEPES), 8.3, or 9.1 (X: Tris) with NaOH. A simple syringe-driven perfusion system was used to exchange the bath solution in the recording chamber. The internal pipette solution used for measuring Ca^{2+} -activated potassium currents contained (in mM): 135 K-aspartate, 8.7 $CaCl_2$, 2 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.2 (with KOH), with an osmolarity of 290–320 mOsm (free $[Ca^{2+}]_i = 10^{-6}$ M).

Toxins. CTX was obtained from Bachem Biochemica GmbH (Heidelberg, Germany). MTX was obtained by the solid-phase method (Merrifield, 1986) and the homogeneity and identity of the synthesized toxin were verified as described previously (Kharrat et al., 1996). All lyophilized peptides were kept at –20 °C, and the final dilutions were prepared before the measurements in normal Ringer's solution containing 0.1% bovine serum albumin.

Electrophysiology. All the experiments were carried out using the whole-cell recording mode of the patch-clamp technique (Hamill et al., 1981). Currents through *hKCa1* channels were elicited with 400 ms voltage ramps from –120 to 0 mV, every 30 s. Electrodes were pulled from glass capillaries (Science Products, Germany) in three stages, and fire-polished to resistances measured in the bath of 2.5–5 M Ω . Membrane currents were measured with an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) interfaced to a Macintosh computer running acquisition and analysis software Pulse and PulseFit (HEKA Elektronik, Lambrecht, Germany). The holding potential in all experiments was –80 mV. Data analysis was performed in IgorPro 3.1 (WaveMetrics, Oregon, USA), and K_D values were deduced by fitting a modified Hill equation ($g_{toxin}/g_{control} = 1/[1 + ([toxin]/K_D)]$), where g is the slope of the ramp current, i.e. the conductance, measured between

– 100 and – 60 mV, to normalized data points obtained at more than four different toxin concentrations. This fit indicates that one toxin molecule is sufficient to block the current through the channel. The standard deviations obtained by this fitting routine reflect the uncertainty of the fit. When K_D was inferred from one single concentration the following calculation was used: $K_D = [\text{toxin}] / ((1/y) - 1)$, where y is the fraction of unblocked conductance. The value of each toxin concentration was the mean of at least three measurements \pm SD (where SD represent the standard deviations of the calculated K_D s).

Transfection. pcDNA3/Hygro vector containing the entire coding sequence of hIKCa1 was co-transfected together with a GFP expressing construct into tsA cells using FuGene6 Transfection Reagent (Roche, Germany), and currents were measured 2–3 days after transfection.

Mutagenesis. The H236C hIKCa1 channel mutant was generated with the QuickChange™ site-directed mutagenesis kit (Stratagene, Germany), and the mutation was confirmed by sequencing (GATC, Germany).

Docking. For the docking of MTX into the hIKCa1 channel vestibule we used the crystal structure of the KcsA channel (Doyle et al., 1998). The homology model of the hIKCa1 channel was made by replacing the KcsA amino acids with the equivalent amino acids from the hIKCa1 channel sequence. The docking was performed manually by fitting the MTX into the channel pore similarly to the docking of KTX into mKv1.1 channel vestibule described by Wrisch and Grissmer (2000). The program used for docking was RasMol v2. The orientation of the toxin into the channel was deduced from the changes in affinity of MTX mutants for the wild type hIKCa1 channel (Castle et al., 2003).

3. Results

To characterize the effect of extracellularly applied MTX on currents through hIKCa1 channels, we measured, using the whole-cell mode of the patch-clamp technique, currents in the hIKCa1 transfected tsA cells in response to depolarizing voltage ramps from – 120 to 0 mV for

400 ms, in the absence and presence of MTX (Fig. 3B). The addition of 1 nM MTX to the external mammalian Ringer solution resulted in almost 50% reduction of the slope current. To further quantify the blocking potency of MTX on hIKCa1 channels, we applied to the external Ringer solution increasing MTX concentrations and we plotted the normalized conductance ($g_{\text{MTX}}/g_{\text{control}}$) against the used concentrations (Fig. 4). We deduced the dissociation constant (K_D) by fitting a modified Hill equation to the data points as described in Section 2. The fit gave a K_D value of 0.81 ± 0.08 nM when normal Ringer solution with pH₀ 7.4 was used. Interestingly, by using normal Ringer solutions with pH₀ values of 6.2, 8.3 or 9.1, we obtained a decrease of MTX affinity for hIKCa1 channels, 1 nM MTX blocking less than 50% (Fig. 3A, C and D). This decrease in binding affinity of MTX at a pH₀ lower or higher than 7.4 was very unusual since previous reports showed that the blocking activity of different peptides is either increased (Deutsch et al., 1991; Perez-Cornejo et al., 1998) or decreased (Wrisch and Grissmer, 2000) by the increase in pH₀. The dose-response curves from Fig. 4 show that MTX affinity for hIKCa1 is shifted towards lower concentrations when normal Ringer with pH₀ 6.2, 8.3 or 9.1 was used. The fits reveal a K_D of 1.21 ± 0.05 , 1.94 ± 0.19 , and 2.59 ± 0.18 nM for MTX in normal Ringer with pH₀ 6.2, 8.3, and 9.1, respectively. Because these results are in agreement with the K_D values calculated from single concentrations (Section 2) (K_D values are 1.3 ± 0.4 ($n = 24$), 0.9 ± 0.5 ($n = 24$), 1.2 ± 0.9 ($n = 15$), and 2.6 ± 0.9 nM ($n = 22$) in normal Ringer with pH₀ 6.2, 7.4, 8.3, and 9.1, respectively) we further used this method for deducing the K_D values.

In order to test if the reduced blocking effect of MTX in normal Ringer solution with pH₀ values other than 7.4 is due to the different used buffers, we repeated the experiments in normal Ringer solution with the pH₀ adjusted to different values using the same buffer (MES, HEPES, or TRIS). For the reliability of these measurements, the pH₀ values were measured before and after the experiments. The K_D values revealed the same blocking tendency of MTX on hIKCa1 channels as shown above, the block being strongest at pH₀

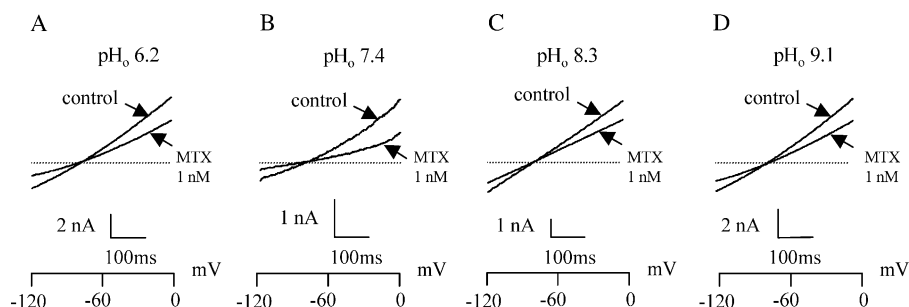


Fig. 3. pH₀-dependent block of extracellularly applied MTX on currents through hIKCa1 channels. Currents were elicited by 400 ms voltage ramps from – 120 to 0 mV, every 30 s. Currents were measured in normal Ringer solution with pH₀ 6.2 (A), 7.4 (B), 8.3 (C), and 9.1 (D), before (control) and after application of 1 nM MTX.

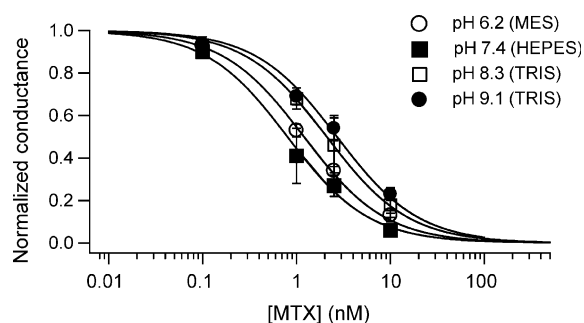


Fig. 4. Dose–response curve for MTX to block current through hIKCa1 channels. The dissociation constant (K_D), deduced by fitting a modified Hill equation ($g_{\text{MTX}}/g_{\text{control}} = 1/(1 + ([\text{toxin}]/K_D)^n)$) to the data points (each data point is the mean of 3–9 experiments), revealed values of 1.21 ± 0.05 , 0.81 ± 0.08 , 1.94 ± 0.19 , and 2.59 ± 0.18 nM when normal Ringer solution with pH_0 6.2, 7.4, 8.3 or 9.1, respectively, was used.

7.4, and decreased by lowering or increasing the pH_0 (Table 1). Since the buffer capacity of the different used buffers depend on their pK_a -value, we can only be sure that the pH_0 is buffered appropriately by those buffers that have their pK_a value close to the buffered pH_0 . Therefore, for further calculations we used the K_D values obtained at pH_0 6.2 from those experiments that used MES as pH_0 buffer (pK_a 6.1), at pH_0 7.4 from those experiments that used HEPES (pK_a 7.55), and at pH 8.3 and 9.1 from those experiments that used TRIS (pK_a 8.3) as pH_0 buffer.

At low pH_0 (6.2), the reduced affinity of MTX for hIKCa1 channels might be due to the protonation of an amino acid from the toxin or the channel that would repel another positively charged amino acid. At high pH_0 (8.3, and 9.1), the loss in the binding affinity of MTX must be due to other mechanisms, possibly a conformational change of the toxin in basic solution as has been suggested by Kharrat et al. (1996) using CD spectroscopy (for details, see Section 4). Previous studies (Perez-Cornejo et al., 1998; Bretschneider et al., 1999; Wrisch and Grissmer, 2000; Thompson and Begenisich, 2000) had shown that an exposed histidine in either the toxin or the channel, titrated with different pH_0 s, changed the binding affinity of the toxin to the channel. Therefore, we investigated if the histidine situated in the external pore region of the hIKCa1 channel (His236), is responsible for the pH_0 dependence of the MTX block. For this purpose we replaced His236 with a smaller amino acid,

cystein, and we measured currents through hIKCa1_H236C channels in the absence and the presence of 1 nM MTX, at different pH_0 s. From the block we calculated the K_D values for MTX block on current through hIKCa1_H236C channels. The calculated K_D value at pH_0 7.4 is 1 ± 0.3 nM ($n = 5$) which is almost identical to the one calculated for the block of MTX on current through the wild type hIKCa1 channel (Fig. 5A). This indicates that at pH_0 7.4 the amino acid His236 from the channel is not involved in MTX binding. By lowering the pH_0 we obtained a similar loss in MTX binding affinity as for the wild type hIKCa1 channel ($K_D = 1.4 \pm 0.7$ nM ($n = 4$)), although the change in this case is not significant. These results might suggest that by protonation, His236 might repel a positively charged amino acid from MTX, making the block at this pH_0 value weaker. At high pH_0 (8.3 and 9.1), the K_D values are 1.4 ± 0.6 ($n = 4$), and 1.5 ± 0.3 nM ($n = 6$). The blocking potency of MTX on current through hIKCa1_H236C mutant channel at these basic pH_0 s is lower than in normal Ringer solution with pH_0 7.4, but not significant (Fig. 5A). This increase in the binding affinity of MTX for the hIKCa1_H236C channel mutant at higher pH_0 values compared with the wild type hIKCa1 channel is interesting since the histidine is not usually charged at basic pH_0 , suggesting that these results cannot be explained through the electrostatic repulsion of the positively charged amino acids from the toxin by the His236 (Section 4).

In order to see if this amino acid is important in the binding of other scorpion toxins, we used CTX a bigger scorpion toxin that might reach this residue. A concentration of 5 nM of CTX applied to the external normal Ringer solution with pH_0 7.4 was able to reduce about 50% of the current through hIKCa1 channels. The K_D revealed a half-blocking concentration of 3.3 ± 1.4 nM ($n = 8$), which is in agree with previous studies (Rauer et al., 2000; Logsdon et al., 1997; Fanger et al., 1999). Using normal Ringer solutions with pH_0 6.2, 8.3, and 9.1 we obtained K_D values of 4.6 ± 1.8 ($n = 4$), 4.3 ± 0.3 ($n = 3$), and 4.2 ± 1.8 nM ($n = 5$), respectively. Fig. 5B shows no pH_0 dependent block of CTX on current through hIKCa1 channels. Interestingly, by replacing the His236 with cystein, the binding affinity of CTX increased, the calculated K_D values being 1.6 ± 0.3 ($n = 3$), 1.4 ± 0.7 ($n = 4$), 1.3 ± 0.6 ($n = 4$), and 1.2 ± 0.3 nM ($n = 4$), in normal Ringer solution with pH_0 6.2, 7.4, 8.3, and 9.1, respectively.

Table 1
Dependency of the K_D (nM) values using different pH_0 buffers

Used buffer	pH_0 6.2	pH_0 7.4	pH_0 8.3	pH_0 9.1
MES	1.3 ± 0.3 ($n = 24$)	1 ± 0.2 ($n = 3$)	1.5 ± 0.9 ($n = 3$)	1.4 ± 0.5 ($n = 3$)
HEPES	1.4 ± 0.4 ($n = 6$)	0.9 ± 0.5 ($n = 24$)	1.35 ± 0.6 ($n = 8$)	1.3 ± 0.6 ($n = 8$)
TRIS	1.6 ± 0.4 ($n = 3$)	1.5 ± 0.7 ($n = 3$)	2 ± 0.9 ($n = 15$)	2.6 ± 0.9 ($n = 22$)

K_D values were calculated from single concentrations as described in Section 2 for the block of MTX on current through hIKCa1 channels in normal Ringer solutions with different pH_0 . The numbers represent mean \pm SD.

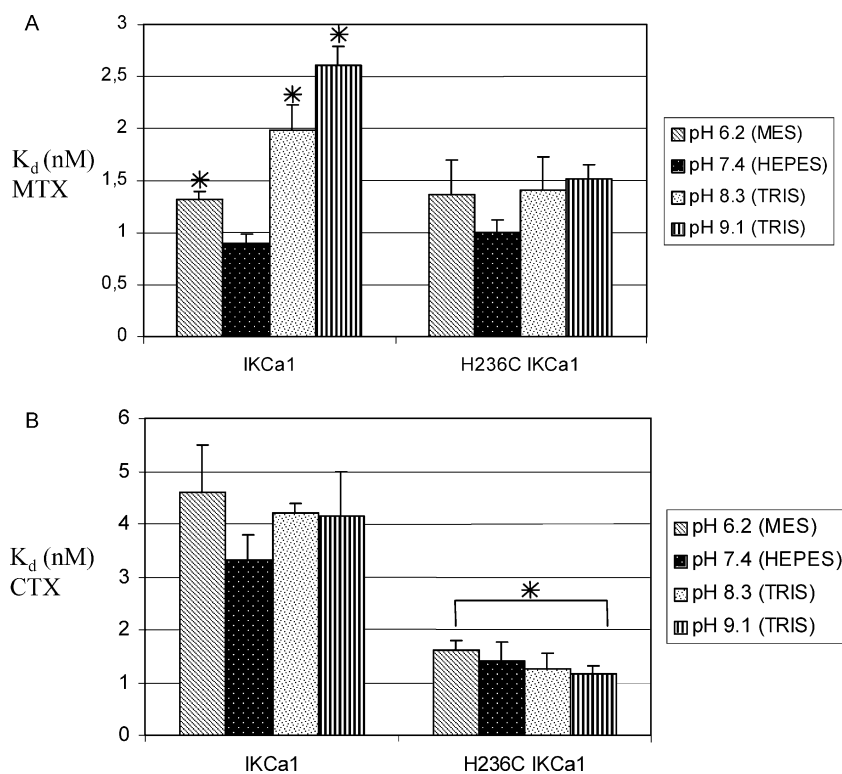


Fig. 5. pH_0 dependency of MTX (A) and CTX (B) block on current through hIKCa1_H236C channels compared with the hIKCa1 channels (K_D values are obtained as described in Section 2). Student's *t*-tests were performed and the differences were considered significant when $p < 0.01$ and indicated by an asterisk.

These values indicate that the replacement of His236 with cysteine, made the block of CTX for current through hIKCa1_H236C channel mutant more efficient. This increase in affinity came almost exclusively through a faster association rate of the toxin for the hIKCa1_H236C mutant channel compared to the wt channel. Using the time course of block and unblock of CTX we could calculate association and dissociation rates for the CTX block on wt hIKCa1 as well as the hIKCa1_H236C mutant channel. Dissociation rates were identical for both channels ($\sim 0.02 \text{ s}^{-1}$) where association rates were faster for the mutant channel.

4. Discussion

We report here that the block of MTX, but not of CTX on current through hIKCa1 channels is pH_0 dependent. The pH_0 dependency of the block is apparently not due to the different buffers used for preparing the normal Ringer solution. The replacement of hIKCa1 histidine 236 with a smaller amino acid, cysteine, did not change MTX binding affinity, and partially reduced the pH_0 dependency of the block. In contrast to MTX, the hIKCa1_H236C mutation leads to an increase in CTX binding affinity.

Since the reports made by now indicate either an increase (Deutsch et al., 1991; Perez-Cornejo et al., 1998) or a decrease (Wrisch and Grissmer, 2000) of peptide binding affinities by an increase in pH_0 , we think there are two different mechanisms that might explain the pH_0 dependency of MTX block on current through hIKCa1 channels, one that works at low pH_0 (6.2), and one that works at high pH_0 (8.3, and 9.1, respectively).

We suggest that at pH_0 6.2 the protonated His236 from the hIKCa1 channel might repel the positively charged Lys27 from MTX, and as a consequence results in a lowered binding affinity (Fig. 6A). The replacement of this histidine with a smaller and neutral amino acid, cysteine, decreases the pH_0 dependency of the block of MTX at pH_0 6.2. Similarly, Bretschneider et al. (1999), have shown using normal Ringer solution with acidic pH_0 that the protonation of a histidine in the mKv1.1 channel lowered the tetraethylammonium (TEA^+) binding affinity. Perez-Cornejo et al. (1998) also reported that a change in pH_0 from 7 to 5.5 made the ShakerB channel mutant F425H 200 fold less sensitive to CTX. In contrast, KTX blocked mKv1.1 with higher affinity at low pH_0 . This effect was due to a histidine situated in the toxin which, by protonation, interacted more efficient with the negatively charged amino acids from the outer pore region of the channel (Wrisch and Grissmer, 2000).

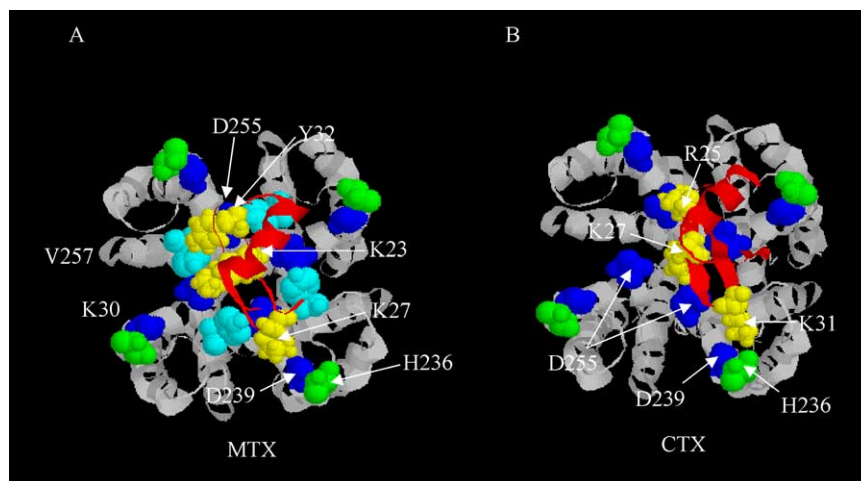


Fig. 6. Docking of MTX and CTX into a homology model of hIKCa1 channel. The docking configuration is shown as top view. The homology structure of hIKCa1 channel is based on the KcsA crystal structure. The channel backbone is represented in gray ribbons, while MTX and CTX are shown as red ribbons. Possibly interacting channel amino acids are represented in blue (Asp239, Asp255), cyan (Val257) and green (His236) as space filled cartoons, while their interacting toxin partners are represented in yellow space filled cartoons (Tyr32, Lys23, Lys27, Lys30 for MTX, and Arg25, Lys27, and Lys31 for CTX). The figure was made using RasMol v2.

At high pH_0 (8.3, or 9.1), even if the blocking potency of MTX for the hIKCa1_H236C channel mutant was higher than for the wild type hIKCa1 channel, we have to keep in mind that at these pH_0 values the histidine is usually not protonated and can not explain the pH_0 dependency of MTX block. We think that the pH_0 dependency of the block in this case is due to the structural modifications that occur in the peptide and/or channel in basic Ringer solution, and that the replacement of the His236 with a smaller amino acid might only create more space for this modified MTX to fit better into the hIKCa1_H236C channel pore. Kharrat et al. (1996) showed using CD spectroscopy that the secondary structure of MTX changed at pH 9.5. Since the percentage of α helical structures increased (26% in pH 7.5 versus 30% in pH 9.5), while the percentage of other structures decreased (34% versus 30%), it could be that the structure of the peptide is slightly changed and makes the interaction of MTX with the hIKCa1 channel weaker. Moreover, Fig. 6A shows that both Lys30, and Lys27 might have an important contribution in the interaction of MTX with the hIKCa1 channel, since they are positively charged amino acids situated in the close proximity of amino acids that correspond to key binding sites in other channels (Aiyar et al., 1996; Gilquin et al., 2002), Val257, Asp255, and Asp239, respectively. Since at pH_0 9.7 the charge of lysine is neutral, we can presume that at least part of the charges of exposed lysines in the toxin are lost, leading to a weaker block of current through the channel at basic pH_0 values.

In contrast to the MTX block, there is no pH_0 dependency of the block of CTX on current through hIKCa1 channels. However, from our data, it seems that His236 is an amino acid that is reached by the CTX (Fig. 6B), and that the replacement of this histidine with

the smaller amino acid, cystein, is helping the peptide toxin to fit better in the channel pore. Previous reports that characterized amino acid interactions of CTX with hIKCa1 channels indicate that the functional dyad of CTX is constituted by the amino acids Lys27 and Arg25. Other amino acids as Lys31, and Lys32, are important for stabilizing CTX binding to this channel. Thermodynamic double-mutant cycle analyses have shown strong coupling between Lys31 and Asp239 from hIKCa1 channel, which would indicate that these two amino acids are situated, when bound, in close proximity with each other (Rauer et al., 2000). Since His236 is situated in the proximity of Asp239, it might be that Lys31 from CTX comes in its way to the channel pore close to His236, but it is situated too far from His236 when bound. This might explain why at pH_0 6.2 we do not see a pH_0 dependent block of CTX on current through hIKCa1 channel, but that by replacing the His236 with Cys, the affinity of CTX is increased.

5. Conclusions

Our findings suggest differences in the binding and sensitivity to pH_0 changes of MTX and CTX to hIKCa1 channels. We report that the MTX block on current through hIKCa1 channels is pH_0 dependent. At low pH_0 (6.2), this lowering in the binding affinity of MTX could be explained through the protonation of His236 from hIKCa1 channel, which could repel a positively charged amino acid from the toxin. However, at high pH_0 (8.3 and 9.1), we suspect a conformational change of MTX that could make it come in closer proximity with the His236. In comparison with MTX,

CTX block on current through hKCa1 channel is not pH₀ dependent, but the replacement of His236 increases its binding affinity, suggesting that CTX might reach this amino acid. We suppose that the replacement of His236 with cysteine creates more space for the toxin to fit better in the channel pore. These information describe functional and structural differences of two known peptide toxins, and might be helpful for future drug design studies.

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