Regulation of a mammalian *Shaker*-related potassium channel, *h*Kv1.5, by extracellular potassium and pH

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Abstract **Using the whole-cell recording mode of the patch-clamp technique we studied the effects of removal of extracellular potassium, $[K^+]_o$, on a mammalian *Shaker*-related K^+ channel, *h*Kv1.5. In the absence of $[K^+]_o$, current through *h*Kv1.5 was similar to currents obtained in the presence of 4.5 mM $[K^+]_o$. This observation was not expected as earlier results had suggested that either positively charged residues or the presence of a nitrogen-containing residue at the external TEA^+ binding site (R487 in *h*Kv1.5) caused current loss upon removal of $[K^+]_o$. However, the current loss in *h*Kv1.5 was observed when the extracellular pH, pH_o, was reduced from 7.4 to 6.0, a behavior similar to that observed previously for current through *m*Kv1.3 with a histidine at the equivalent position (H404). These observations suggested that the charge at R487 in *h*Kv1.5 channels was influenced by other amino acids in the vicinity. Replacement of a histidine at position 463 in *h*Kv1.5 by glycine confirmed this hypothesis making this H463G mutant channel sensitive to removal of $[K^+]_o$ even at pH_o 7.4. We conclude that the protonation of H463 at pH 7.4 might induce a pK_a shift of R487 that influences the effective charge at this position leading to a not fully protonated arginine. Furthermore, we assume that the charge at position 487 in *h*Kv1.5 can directly or indirectly disturb the occupation of a K^+ binding site within the channel pore possibly by electrostatic interaction. This in turn might interfere with the concerted transition of K^+ ions resulting in a loss of K^+ conduction.** **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: *Shaker*-related K^+ channel; Patch clamp technique; K^+ dependence; pH dependence

1. Introduction

The proper physiological function of excitable tissues in mammals depends critically upon the extracellular potassium concentration, $[K^+]_o$. Small changes in $[K^+]_o$ can lead to changes in membrane potential, to electrical instability and epileptiform seizures [1–4]. These changes might occur through the modulation of K^+ channels by $[K^+]_o$ thereby modulating the firing pattern of neurons as a function of $[K^+]_o$ [3]. One candidate of such a K^+ dependent K^+ channel is the voltage-gated, rapidly inactivating Kv1.4 channel endogenously expressed in neurons of the hippocampus [3,5,6]. Mutagenesis work on this channel [3] as well as on Kv1.3 [7] revealed that the position of the channel known to be the binding site for extracellular TEA^+ (position 533 in *r*Kv1.4; position 404 in *m*Kv1.3) was responsible for the sensitivity to $[K^+]_o$. Uncharged amino acid residues at this position (Y or V), as is found in Kv1.1 and Kv1.2 channels, made the channels insensitive to removal of $[K^+]_o$, whereas charged amino acid residues (K or R at pH_o 7.4 or H at pH_o 6.0) rendered the channel sensitive to removal of $[K^+]_o$. In addition, channels containing the neutral, polar residue asparagine (N) at this position were also sensitive to removal of $[K^+]_o$ indicating it is not only charge but also the presence of a nitrogen-containing group at this position that might create an unstable channel in the absence of $[K^+]_o$. The current loss upon removal of $[K^+]_o$ is apparently not due to fast inactivation in the absence of $[K^+]_o$ but due to a new channel conformation, a non-conducting state of the channel induced by the reduction of $[K^+]_o$ distinct from the inactivated state as shown previously [7]. One possibility to explain the instability of the channel in the absence of $[K^+]_o$ is that the pore region collapses upon removal of $[K^+]_o$ resulting in a conformational change of the channel that does not allow for ion flux anymore [8,9].

In this paper we present evidence that other amino acids in the vicinity of the extracellular TEA^+ binding site can influence the charge at this position thereby modulating the sensitivity of the channel to removal of $[K^+]_o$.

2. Materials and methods

2.1. Cell culture

Rat basophilic leukemia (RBL-2H3) cells were maintained in Eagle's minimal essential medium (EMEM, from Life Technologies, Karlsruhe, Germany) supplemented with 1 mM L-glutamine (Biochrom, Berlin, Germany) and 10% heat-inactivated fetal calf serum (PAA Laboratories, Linz, Austria), in a humidified 5% CO_2 incubator at 37°C.

2.2. Electrophysiology

Patch clamp recordings were performed in the whole-cell mode on cRNA-injected RBL cells [10] as described earlier [7,11,12]. RBL cells were chosen because of the apparent lack of endogenous voltage dependent conductances. Microelectrodes were pulled from glass capillaries (Clark Electromedical Instruments, Reading, UK), coated with Sylgard (Dow Corning Corp., Midland, MI, USA), and fire-polished to resistances of 2–5 MΩ. All experiments were done at room temperature (21–25°C). The bath solution contained (in mM): 160 NaCl, 4.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and either 10 MES (pH 6.0) or 10 HEPES (pH 7.4), adjusted to pH 6.0 or 7.4 with NaOH (290–320 mOsm) or 164.5 NaCl, 0 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, adjusted to either pH 7.4 or 6.0 with NaOH (290–320 mOsm). The pipet solution contained (in mM): 134 KF, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 10 EGTA,

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adjusted to pH 7.2 with KOH (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. 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 and leak currents using templates created by averaging and scaling 10 current traces elicited during hyperpolarizing voltage pulses (P/10 procedure). All illustrated results were obtained at least in triplicate.

2.3. Expression

The plasmid encoding the human Kv1.5 channel was a friendly gift from Dr. L. Philipson (University of Chicago, Chicago, IL, USA). The QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to generate point mutations encoding the amino acid exchanges. RNA was transcribed from cDNA and injected into RBL cells. RBL cells are electrophysiologically well characterized and do not express detectable voltage-gated outward rectifying K^+ channels [13]. The cRNA was diluted with 0.5% fluorescein-5-isothiocyanate (FITC) dextran MW 10 000 in 100 mM KCl to a final concentration of 0.5–1 mg/ml. The cRNA/FITC-solution was filled into injection capillaries (Femtotips, Eppendorf, Germany) and RBL cells were injected using an Eppendorf microinjection system (Micromanipulator 5171 and Transjector 5246, Eppendorf, Germany) as described [11,12,14]. 2–6 h later, injected cells were identified by fluorescence and whole-cell currents were investigated.

3. Results

We have demonstrated earlier [7] that replacing the histidine at position 404 in *m*Kv1.3 (equivalent to position 449 in *Shaker*, see Fig. 1) by a positively charged arginine rendered the channel very sensitive to removal of $[K^+]_o$. This observation was in agreement with earlier findings by Pardo et al. (1992) on *r*Kv1.4 and our own findings on *m*Kv1.4 that the presence of a charged lysine residue (K533 in *r*Kv1.4 and *m*Kv1.4) at the homolog position was responsible for this effect. In keeping with this notion, current through wild type *h*Kv1.5 channels, with a positively charged arginine at the homolog position (R487), should be similarly sensitive to removal of $[K^+]_o$. Unexpectedly, current through wild type *h*Kv1.5 channels were hardly affected by a reduction in $[K^+]_o$, at least at pH_o 7.4. This can be seen on the left side of Fig. 2 which shows whole-cell currents elicited with 200 ms

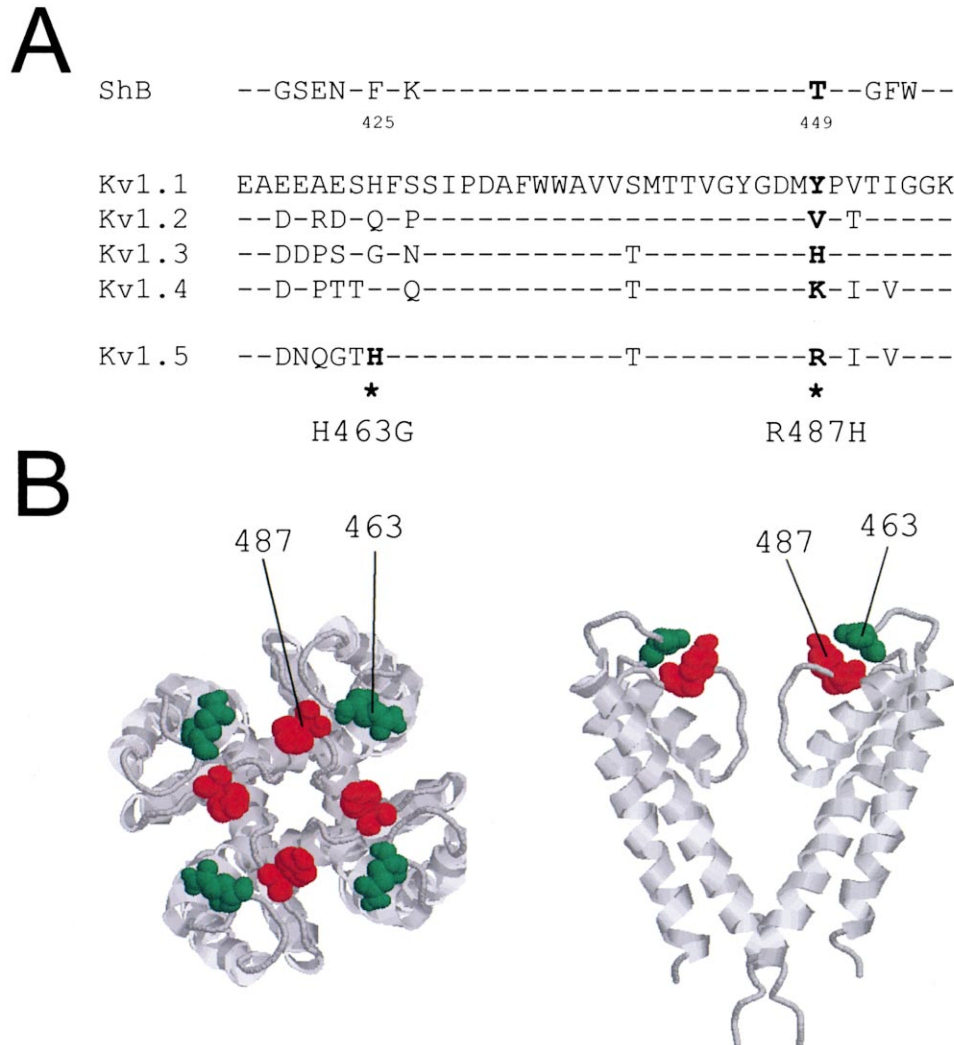

Fig. 1. Putative pore region of voltage-gated K^+ channels. A: Amino acid alignment of the putative 'P-region' of *Shaker*-related potassium channels. The amino acid positions in the different channels responsible for external TEA interaction (position 449 in *Shaker*) are highlighted. B: Top and side view of the *KcsA* channel. Equivalent positions at which amino acid changes were introduced are shown in green (463 equivalent position) and red (487 equivalent position).

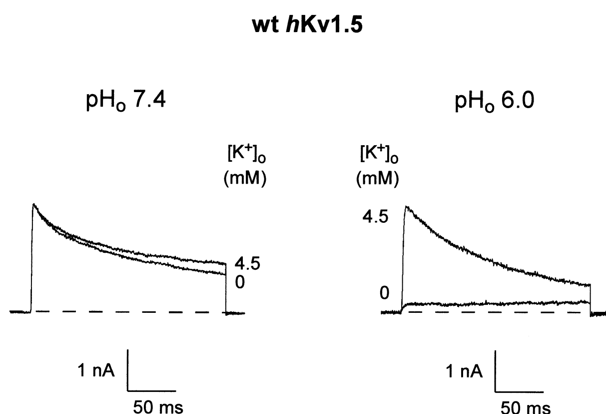

Fig. 2. pH and K$^+$ dependence of wild type (wt) *h*Kv1.5 channels with and without 4.5 mM $[K^+]_o$ at pH_o 7.4 and pH_o 6.0. Currents were elicited by 200 ms depolarizing voltage steps from a holding potential of −80 mV to a test potential of 40 mV in the presence (4.5 mM) or absence of extracellular K$^+$ at pH_o 7.4 (left) and at pH_o 6 (right).

depolarizing voltage steps from −80 to 40 mV in the presence (4.5 mM) and absence of $[K^+]_o$ at pH_o 7.4. Removal of $[K^+]_o$ neither changed the peak current amplitude nor significantly the time course of current decay during depolarization. In contrast, at pH_o 6.0 (Fig. 2, right) current through wild type *h*Kv1.5 channels became very sensitive to removal of $[K^+]_o$. The suppression of current upon removal of $[K^+]_o$ at pH_o 6.0 was almost complete, i.e. peak current amplitude was reduced to ~10% of the peak current amplitude obtained in the presence of K$^+$ (4.5 mM). This behavior (of *h*Kv1.5 at pH_o 6.0) was similar to the behavior observed for *m* and *r*Kv1.4 as well as for the H404R mutant of *m*Kv1.3 at pH_o 7.4 [3,7]. In addition, current through wild type *m*Kv1.3 channels, with a protonable histidine (H404) at the equivalent position, showed a similar reduction of current upon removal of $[K^+]_o$ at pH_o 6.0 [7].

One possible explanation for this apparent discrepancy could be that the effective charge of the arginine at position 487, especially in *h*Kv1.5, might be influenced by another amino acid in the vicinity. As a first test of this idea, we tried to recreate the situation seen in *m*Kv1.3 (H404) where the channels became more sensitive upon removal of $[K^+]_o$ by changing the pH_o from 7.4 to 6.0. Therefore we replaced R487 in *h*Kv1.5 by a histidine. The resulting R487H mutant *h*Kv1.5 channels carry now the identical residue at the corresponding position in *m*Kv1.3 and consequently should behave in a similar way regarding the pH_o and $[K^+]_o$ dependence. Fig. 3 shows the result of an identical experiment as shown in Fig. 2 except this time using the mutant R487H *h*Kv1.5 channels. Similar to Fig. 2, currents through the mutant R487H *h*Kv1.5 channels were hardly affected by a reduction in $[K^+]_o$ at pH_o 7.4 (Fig. 3, left), with the exception of a small change in inactivation. Peak current amplitude, elicited with 200 ms depolarizing voltage steps from −80 to 40 mV, was reduced less than 10% upon removal of $[K^+]_o$ and the time course of current decay during depolarization was only a little bit faster in the absence of $[K^+]_o$ compared to when 4.5 mM K$^+$ was present. Similar to the results shown in Fig. 2 (right) for current through wild type *h*Kv1.5 channels at pH_o 6.0, currents through the mutant R487H *h*Kv1.5 channels became also very sensitive to removal of $[K^+]_o$ at pH_o 6.0 (Fig. 3,

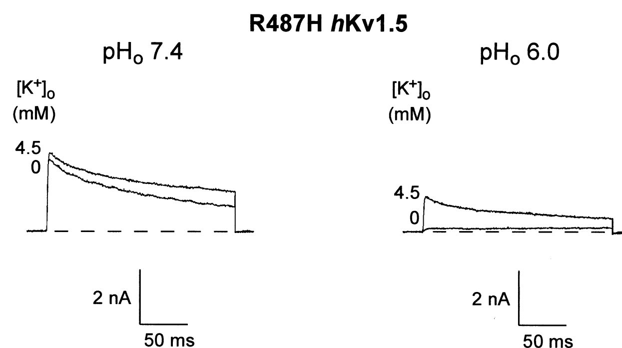

Fig. 3. pH_o and K$^+$ dependence of the R487H mutant *h*Kv1.5 channels with and without 4.5 mM $[K^+]_o$ at pH_o 7.4 and pH_o 6.0. Currents were elicited under identical conditions as described in the legend to Fig. 2.

right). Peak current amplitude through the mutant R487H *h*Kv1.5 channels was reduced to ~10% of the peak current amplitude obtained in the presence of K$^+$ (4.5 mM). This behavior seems to be identical to what was observed for current through wild type *h*Kv1.5 channels indicating that the charge of the histidine at position 487 acts similar as the charge of the arginine at the same position. Comparing these results to those obtained with *m*Kv1.3 [7] showed three differences: (a) changing pH_o from 7.4 to 6.0 reduced peak currents through mutant R487H *h*Kv1.5 channels by ~50% (in the presence of 4.5 mM K$^+$), while having little effect on peak current through *m*Kv1.3 [7]; (b) the reduction of peak current amplitude upon removal of $[K^+]_o$ is much weaker at pH_o 7.4 for the mutant R487H *h*Kv1.5 channels compared to the situation observed in *m*Kv1.3 [7]; (c) the reduction of peak current amplitude upon removal of $[K^+]_o$ is much stronger at pH_o 6.0 for the mutant R487H *h*Kv1.5 channels compared to the situation observed in *m*Kv1.3 [7].

Taken together this would suggests that a simple titration of the amino acid at position 487 in *h*Kv1.5 cannot explain the loss of current upon removal of $[K^+]_o$. Other mechanisms or amino acids that are different in *m*Kv1.3 – might influence the titration, i.e. the amount of charge, at this position.

One possible candidate that could influence the effective charge at position 487 in *h*Kv1.5 channels could be the histidine at position 463. This histidine is not present in *m*Kv1.3 but is present in Kv1.1 (H355) and has been shown [15] to be able to influence the effective TEA$^+$ concentration at the ex-

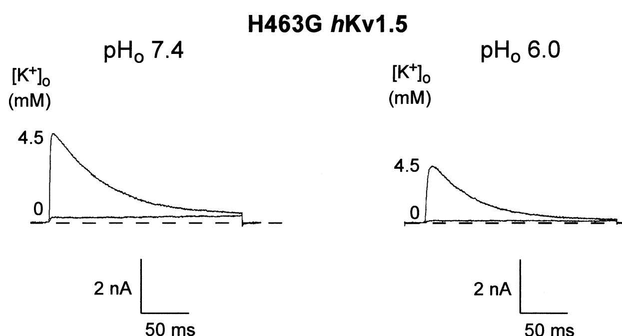

Fig. 4. pH_o and K$^+$ dependence of the H463G mutant *h*Kv1.5 channels with and without 4.5 mM $[K^+]_o$ at pH_o 7.4 and pH_o 6.0. Currents were elicited under identical conditions as described in the legend to Fig. 2.

ternal TEA^+ receptor site (position 379 in Kv1.1, position 487 in *h*Kv1.5). If the titration of this histidine can influence the charge at position 487, then replacement of this histidine by a non-titratable amino acid, for example glycine, should make the channel sensitive to the removal of $[K^+]_o$ independent of the pH_o. To test this idea, we generated exactly this mutant channel (H463G *h*Kv1.5) and performed the same experiments as shown for the wild type *h*Kv1.5 in Fig. 2 and the mutant R487H *h*Kv1.5 channel in Fig. 3. The result of such an experiment with H463G mutant *h*Kv1.5 channels is shown in Fig. 4. It shows that this mutant channel is indeed very sensitive to the removal of $[K^+]_o$ at pH_o 7.4 and also at pH_o 6.0. This behavior is similar to experiments with the H404R mutant channel of *m*Kv1.3 [7] and suggested to us that the effective charge at R487 in *h*Kv1.5 channels can be influenced by the charge at position 463.

4. Discussion

Shaker-related K^+ channels are able to sense the external $[K^+]_o$ if they carry a positively charged (K at pH_o 7.4; or H at pH_o 6.0) or a nitrogen-containing amino acid (N) at the homolog position to 82 in *KcsA* or 449 in *Shaker* [7]. This position comprises part of the external TEA^+ binding site [16,17] and is also involved in mediating C-type inactivation. For example the substitution of R487Y in *h*Kv1.5 conferred increased sensitivity to extracellular TEA^+ [18] comparable to that found in *Shaker* T449Y [16] or other channels carrying a Y at that position [19,20].

We wanted to know why *h*Kv1.5, with a positively charged R at the external TEA^+ binding site, was unable to sense $[K^+]_o$ at pH_o 7.4 and was able to do so at pH_o 6.0. By introducing amino acid substitutions by point mutations we could show that the charge at R487 is dominated by another charge in the vicinity. The amino acid in question seems to be H463 whose protonation apparently can influence the charge at R487 presumably through a pK_a shift.

4.1. Amino acids involved in K^+ binding in hKv1.5

Three permeant ion binding sites were found in the pore of *KcsA*, two in the selectivity filter and one in the internal pore cavity [21]. The backbone carbonyl oxygens of the highly conserved T/SXGYG act as a selectivity filter for K^+ ions [21,22]. Because of the high similarity between *KcsA* and the *Shaker*-like potassium channels within the P-region and the fact that both channels are selective for potassium one can assume that the structure of the *KcsA* pore in general reflects the structure of the pore of other potassium selective channels. We think that the outer most K^+ binding site might be responsible for the observed effects. This position has been described as the external lock-in site [23–25] that is highly selective for K^+ and has to be very close to the extracellular solution due to the weak voltage dependency. Since this external lock-in site has a relatively high affinity to bind K^+ it might be likely that a potassium ion would be at this site most of the time and one could imagine that the occupancy of this site is a prerequisite for a functional channel. According to Vergara et al. ([26]) the K_d for the external K^+ binding site is in the low micromolar range ($\sim$2.7 μM) determined for the external lock-in site of Ca^{2+}-activated K^+ channel (BK_{Ca}) [23,24,26]; indicating that even under low $[K^+]_o$ this binding site would be occupied. In addition, the occupancy of this site might have effects on other channel properties such as toxin binding, C-type inactivation or deactivation. Experimental evidence that $[K^+]_o$ did influence ligand binding came from toxin binding experiments. For Shk-Lys22, a modified sea anemone toxin, it was found that the sensitivity differed in 4.5 compared to 164.5 mM $[K^+]_o$ [27]. The authors proposed that the occupancy of the external K^+ binding site appeared to destabilize the interaction of the native toxin with the channel via electrostatic repulsion. This was also found for Lys27 of kaliotoxin [28] and Lys27 of agitoxin [29]. In addition, in the Kv2.1 channel the removal of $[K^+]_o$ (also in the absence of $[K^+]_i$) resulted in a channel conformation that was insensitive to block by TEA^+ [30] indicating conformational rearrangements of the external vestibule of the channel upon removal of $[K^+]_o$. Since this TEA^+ sensitivity in the absence of $[K^+]_o$ was almost restored by replacing a lysine at position 356 in Kv2.1 (equivalent to H463 in *h*Kv1.5) with a glycine the authors suggested that the conformational changes that occurred upon removal of $[K^+]_o$ would extend all the way to the outer edge of the external vestibule.

Other experiments demonstrated that $[K^+]_o$ has also an influence on C-type inactivation [31–34], recovery from inactivation [35] as well as deactivation [31,36,37] possibly also via the occupancy of the external lock-in site described above.

4.2. pK_a shift of R487 through H463

It is easily conceivable that the occupancy of this external K^+ binding site might depend on the charge at R487. As illustrated in Fig. 1B R487 is located $\sim$7 Å (Cα–Cα) away from the tyrosine of the selectivity filter according to the *KcsA* crystal data [21] and therefore close to the external look-in site. The positive charge at position 487 might be able to push K^+ out of this external lock-in site via electrostatic repulsion and the pore might then collapse due to the lack of a stabilizing K^+ at that site. H463 is $\sim$8 Å (Cα–Cα) away from R487 and the charge of this histidine could influence the effective charge at position R487 or the conformation of the K^+ binding site thereby having an indirect effect on the occupancy of the external K^+ binding site.

H463 (equivalent to position 58 in *KcsA*, 425 in *Shaker* or 355 in Kv1.1) is located in the turret (amino acids 457–466 in *h*Kv1.5; amino acids 52–61 in *KcsA*; amino acids 419–428 in *Shaker*) $\sim$13.5 Å away from the Y of the selectivity filter (Fig. 1B) because of the highly flexible structure of the turret it is possible that this loop is located closer to the middle of the pore as has been suggested earlier for Kv1.3 and Kv1.1 [38]. The equivalent position to H463 has been reported to influence the TEA^+ sensitivity of *h*Kv1.1 channels [15] and also confers sensitivity to removal of $[K^+]_o$ (in the absence of $[K^+]_i$) for Kv2.1 channels [30]. Both channels, *h*Kv1.5 as well as Kv2.1 do have a H or a K at an equivalent position to 58 in *KcsA* or 425 in *Shaker* and we assume that an electrostatic effect in both cases may account for the effect of $[K^+]_o$ removal. A shift in the pK_a of R487 might explain why *h*Kv1.5 was not able to sense $[K^+]_o$. Arginine side chains do have an intrinsic pK_a value of $\sim$12. This should result in a full protonation of this arginine at pH_o 7.4. Our results indicate, however, that the arginine at position 487 might have a lower pK_a value due to the presence of H463. Evidence from other systems might support this idea. For example, the pK_a value of a lysine residue can exhibit low pK_a values if another charge is close by. This is the case for one of the active site

lysines in acetoacetate decarboxylase whose pK_a value is 6.0 [39] or in the ovotransferrin structure [40]. In addition, there are several well documented cases for which an electrostatic mechanism, operating over distances up to 15 Å, can influence the affinity of a charged ligand binding to a protein [41]. From calbindin for example it is known that negatively charged residues near but not at the Ca^{2+} binding sites of calbindin [42] and subtilisin perturb the pK_a of the active site histidine by a simple electrostatic effect [41].

4.3. Alternative possibilities to explain the $[K^+]_o$ dependence of hKv1.5

Other factors that might influence the charge at R487 besides H463 could be the net charge in the outer pore. This could play a role especially in explaining the differences in the behavior upon removal of $[K^+]_o$ in Kv1.5 compared to Kv1.4. Both channels do express a positively charged amino acid at the external TEA^+ binding site (R487 in *h*Kv1.5 and K533 in *r*Kv1.4) and also have a histidine at equivalent positions in the outer pore region (H463 in *h*Kv1.5 and H509 in *r*Kv1.4). They should therefore behave identically upon removal of $[K^+]_o$ if (a) K533 behaves similar to R487 and (b) if only these two positions (R487 and H463 in *h*Kv1.5 and K533 and H509 in rKv1.4) were important for the $[K^+]_o$ effect. From earlier experiments [3,7] and those presented in this paper it is obvious that *h*Kv1.5 and Kv1.4 behave differently in terms of current loss upon removal of $[K^+]_o$. Assuming that lysine and arginine at the external TEA^+ binding site have similar properties other factors might also contribute to the charge at this site. A comparison of the amino acid composition in the region of the turret (*h*Kv1.5 459-NQG-462 compared to 505-EPT-508, the equivalent region in Kv1.4) show that Kv1.4 has an additional negative charge that could neutralize H509 thereby removing the influence of this amino acid on the charge at K533. Therefore one could assume that conformational rearrangements of the external vestibule upon removal of $[K^+]_o$ are compensated differently in Kv1.4 and Kv1.5 channels perhaps because of structural differences of the turret regions of those two Kv channels.

4.4. Physiological consequences

The K^+ channel *h*Kv1.5 is expressed in human heart muscle [43] and might be involved in the repolarization of the atrial action potential of the human heart. Kv channels might therefore be targets in pathological conditions that disturb acid–base balance like myocardial ischemia, [44] which are often accompanied by acidosis and a rise in $[K^+]_o$. Steidl and Yool [45] for example found that *h*Kv1.5 channels were modulated by external acidification and hypothesized that this *h*Kv1.5 modulation might have dramatic effects on action potential repolarization, duration, and therefore contractility under acid conditions. In contrast, we could show that under normal $[K^+]_o$, in the presence of 4.5 mM $[K^+]_o$, *h*Kv1.5 peak current is not pH_o dependent. Only under low $[K^+]_o$ conditions we observe a pH_o dependence of the current through *h*Kv1.5. The discrepancy between the experiments by Steidl and Yool [46] and our results could be due to the use of different external solution as well as the difference in the expression system [45]. Therefore we conclude that only under rare conditions, for example in diseases were patients do have a pathophysiologically reduced serum potassium level, like it is known from patients with hypokalemic periodic paralysis (HypoPP), the *h*Kv1.5 might be involved in cardiac arrhythmia under acid conditions.

4.5. Conclusion

In conclusion, we have shown that current through the human Kv1.5 channel was only reduced at low pH_o under low $[K^+]_o$ conditions. The presence of 4.5 mM K^+ is sufficient to prevent this reduction. We could verify that the interaction of amino acids in *h*Kv1.5 channels at position 463 and 487 (*KcsA* position 58 and 82, respectively) were crucial to the integrity of the external potassium binding site. Assuming that by lowering $[K^+]_o$ the external K^+ binding site is less often occupied, this renders the channel into a pH_o sensitive channel. If additional forces act on a potassium ion sitting in the binding site like a positive charge at the TEA^+ binding site (R487 in *h*Kv1.5) – also under the influence of another nearby charge (H463 in *h*Kv1.5) – this would push the potassium ion out of its binding site. Therefore the individual structure of the turret especially the nature of the residues at position 58 and 82 in *KcsA* are major determinants for modulation of an external K^+ binding site. For the Kv1.5 pore H463 might be able to influence the effective charge at R487 thereby influencing indirectly or directly the occupation of the K^+ binding site.

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