Novel Insights into the Structural Basis of pH-Sensitivity in Inward Rectifier K⁺ Channels Kir2.3

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Key Words

Oocyte • IRK • Hydrogen bond • Model • Cross linking
• Protonation • Channel

thus reducing ion permeation. The data provide molecular insight into the unique pH regulation of inward rectifier channels.

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Abstract

The Kir2 channels belong to a family of potassium selective channels with characteristic strong inward rectification. Heteromeric assemblies of Kir2.1, Kir2.2 and Kir2.3 channels underly membrane potential stabilizing currents in ventricular myocytes, neurons and skeletal muscle. Kir2 channels differ substantially in their sensitivity to extracellular pH. The extracellular histidine Kir2.3(H117) contributes to the pH dependence of K-channels containing Kir2.3. Here, we study the possibility of intramolecular interactions of the residue Kir2.3(H117) with conserved cysteines in close proximity to the selectivity filter. We engineered a cobalt coordination site and reduction/oxidation sensitivity in Kir2.3 by introduction of a cysteine into the putatively hydrogen bonding residue (Kir2.3(H117C)) confirming that this residue is in proximity to Kir2.3(C141). Using SCAM we determined the location of the Kir2.3(H117) in the outer pore mouth and incorporated these data into a 3D model. We conclude that formation of a hydrogen bond at low pH may stabilize the outer pore domain to favour the selectivity filter in a slightly distorted conformation

Introduction

Kir2 channels (KCNJ, IRK) play critical roles in the regulation of resting membrane potential of excitable cells including cardiac myocytes, skeletal muscle and neuronal tissue [1]. Kir2 channels are highly expressed in the heart, where they appear to act as a major determinant of late repolarization (phase 3) and of the resting membrane potential (phase 4) [1]. Cardiac Kir2.1, Kir2.2 and Kir2.3 are believed to form heteromeric channels to conduct I_{K1} [2]. A gain of function mutation causes atrial fibrillation [3]. I_{K1} is decreased in cardiac failure [4-7] which may compromise stability of the resting membrane potential and repolarisation. Further, upregulation of I_{K1} may have stabilizing effects on ventricular tachyarrhythmias [8]. Conversely, Kir2 channels may provide a functional link between mitochondrial dysfunction and membrane excitability [9, 10].

In sheep cardiac myocytes sensitivity to external pH is transferred by the Kir2.3 subunit to cardiac Kir2.1/2.3

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heteromeric channels [11]. Shortly after cloning of Kir2.3 the pH dependency was suggested to result from titration of cysteines modulated by histidine 117 in the extracellular pore region of human Kir2.3 channels leading to reduced single channel conductance [12]. Unfortunately, mutation of the possible titrated cysteines Cys114 or Cys 146 lead to non-functional mutants and could thus not be directly tested for their pH dependence. The homologous site to Kir2.3(H117) is Kir2.1(E125) and contributes to the Ba²⁺ and Mg²⁺ sensitivity in Kir2.1 channels [13]. However, the extracellular mouth of Kir2 channels lacks a known 3D-molecular basis as all assumptions rely on a weak homology on the primary structure (amino acid homology) to the solved crystal structures of the channels KcsA, MthK, Kv1.2 and KirBac.

Here, we studied the effects of charges on the residues Kir2.3-His117 and Kir2.1-Glu125. We exchanged residue Kir2.3-His117 and the homologous residue Kir2.1-Glu125 (pK of ~4.4 under standard conditions) by positively charged Arg (pK of ~12.0 under standard conditions), neutral Cys (thiol pK of ~9.0-9.5 under standard conditions) and Ala. Further, we mutated His117 to negatively charged Glu and introduced a His into Kir2.1 in position 125. We showed that introduction of a charged residue is not sufficient to reduce or increase Kir2 currents excluding a simple electrostatic effect by protonation of His117 as the basis of pH modulation in Kir2.3. On the contrary, the pH-effect in Kir2.3 seems to depend on a hydrogen bond formation between a histidine and a cysteine close to the selectivity filter. Mutation of Kir2.3(H117C) resulted in Co²⁺-sensitive channels indicating that Co²⁺ may be coordinated between the native and the engineered cysteines. This supports the idea that the two residues are in spatial proximity to allow a hydrogen bond formation. Using SCAM we analysed the distance of the residue in position 117 to an engineered TEA-binding site enabling 3D-modelling of the outer pore of Kir2.3 channels.

Materials and Methods

Molecular biology

Molecular biological procedures were performed as described previously [14]. Sequences of cDNA clones were: 1) *Cavia porcellus* Kir2.1 AF187872 [KCNJ2] and 2) *Homo sapiens* Kir2.3 U07364 [KCNJ4]. The Quick change mutagenesis system (Stratagene, Scotland) was used for site-directed mutagenesis in the two clones. The resulting constructs were verified by automated DNA sequencing. Kir2 cDNA constructs in pSGEM were linearized with NheI, and cRNA was synthesized by *in*

vitro transcription (mMessage mMachine T7 kit, Ambion, Applied Biosystems, Germany). cRNA concentrations were determined by photospectrometry and transcript quality was checked by agarose gel electrophoresis. Enzymes were purchased by NEB (New England Biolabs, MA, USA).

Two-electrode voltage clamp technique (TEVC)

TEVC was performed as previously reported [14]. Briefly, *Xenopus laevis* ovaries were retrieved from tricaine anesthetized animals, oocytes were collagenase-treated (1 mg/ml, type II, Worthington, USA) in ND96 Ca²⁺-free solution (in mM: NaCl 96, KCl 2, MgCl₂ 1, HEPES 5, pH 7.4) for about 120 min and subsequently stored in ND96 (in mM: NaCl 96, KCl 2, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, pH 7.4) along with sodium pyruvate (275 mg/l), theophylline (90 mg/l) and gentamycin (50 mg/l) at 17-18°C. For characterization of wild type and mutant Kir2 channels, each oocyte was injected with 1 ng cRNA. Standard TEVC recordings were performed at 22-23°C with a Turbo Tec-10CX Turbo Tec-03 (NPI, Germany) amplifier, an ITC-16 interface combined with pCLAMP 8.0 software (Axon Instruments Inc. / Molecular Devices, USA) and analyzed with Clampfit 8.0 and Origin 6.0 (Additive, Germany). Macroscopic currents were recorded 2 days after injections. Pipettes were filled with 3 M KCl and had resistances of 0.4 – 1.0 MΩ. Proper voltage clamp was provided using the amplifiers integrator and visual control of the PI-controller. Leak currents were not subtracted. Recording solution KD60 contained (in mM): NaCl 38, KCl 60, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, pH 7.4. Chemicals were from Sigma-Aldrich Chemie GmbH (Germany) if not stated otherwise. Student's *t* test was used to test for statistical significance, which was assumed at *p* < 0.05 (indicated by asterisk).

SCAM

The combination of site-specific cysteine mutagenesis and usage of MTS reagents is a powerful technique for probing the structure and function of proteins. Introduction of individual cysteine residues at various positions in an ion channel, and then testing the chemical modification of an introduced cysteine may produce a measurable change in the function of the channel, which can be monitored by electrophysiological recording, thereby affording information concerning the time-course, state dependence and membrane-sidedness of the accessibility of that cysteine. This approach applied to membrane-spanning segments of ion channels and transport proteins is called the substituted-cysteine-accessibility method (SCAM). MTS reagents were purchased from Toronto biochemicals (Canada) and stored at 100 mM dissolved in DMSO or H₂O. MTS-solutions were freshly prepared from 100 mM stock solutions and oocytes were incubated in MTS reagent containing solutions (1 mM MTS reagent in ND96) for 5 minutes at room temperature and subsequently washed 10 times in ND96 to remove unbound MTS reagents before recording. Here, we tested for chemical modification of one or two adjacent cysteines by single sulfhydryl active reagents or double sulfhydryl active reagents putatively modifying one cysteine or linking two cysteines respectively, resulting in functional effects.

Table 1. Alignment of KcsA and Kir2.3 M1-M2 region (generated by CLUSTAL W (1.82) multiple sequence alignment). The helical regions are underlined, the selectivity filter sequence is in italicised letters and the putative hydrogen bonding residues in Kir2.3 and the homologous residues in the other channel sequences are bold.

hKir2.3	MLMIFSAFLVSWLFFGLLFWCIAFFHGDLEASPGVPAAGGPAAGGGG	
gpKir2.1	MLVIFCLAFVLSWLFEGCVFWLIALHGLDLA	
gpKir2.2	MLLIFSLAFSLASWLLFGVIFWVIAVAGDLEP	
gpKir2.4	MCLLFSCSFLASWLLFGLAFWLIASLHGLDAA	
hKir1.1_rom-k1	KMTIFITAFLGSWFFFGLLWYAVAYIHKDLPEF	
KcsA	-----ALHWRAGAATVLLVIVLLAGSYLA-----	
KirBac1.1	WRDLYYWALKVSWPVFFASLAAFLVNNLTFLALL	
KirBac3.1	LDDHYHDLTLVSWPVFITLITGLYLVNTALFALA-----	
	M1-segment	
hKir2.3	AAPVAPKPCIMHVNGFLGAFLEFSVETQTITIGYGFRCVTEECPLAVIAV	
gpKir2.1	SKES--KACVSEVNSFTAAFLFSIETQTITIGYGFRCVTEECPIAVFMV	
gpKir2.2	AENRGRTPCVMQVHGFMAAFLFSIETQTITIGYGLRCVTEECPIAVFMV	
gpKir2.4	PEPP--APCFSHVASFLAAFLFALETQTSIGYGVRSVTEECPIAAVA	
hKir1.1_rom-k1	HPSANHTPCVENINGLTSAFLFSLETQITIGYGFRCVTEECQATAIFLL	
KcsA	-----VLAERGAPGAQLITYPRALWWSVETATTVGYGDLYP--VTLWGRCA	
KirBac1.1	YQLGDAPIANQSPPGFVGAFFFSVETLATTVGYGFRCV--QTVYAHATA	
KirBac3.1	YLACGDVLENARPGSFTDAFFFSVQTMATITIGYGLIP--IGPLANTLV	
	Pore helix	M2-segment
hKir2.3	VVQSIIVGCVIDSFMIGTIMAKMARP	121
gpKir2.1	VFQSIIVGCIIDAFIIGAVMAKMAKP	103
gpKir2.2	VAQSIIVGCIIDSFMIGAIMAKMARP	105
gpKir2.4	VLQCIAGCVLDAFVVGAVMAKMAKP	103
hKir1.1_rom-k1	IFQSIILGVIINSFMCGAILAKISRP	106
KcsA	VVVMVAGITSGFLVTAALATWFEVGR	95
KirBac1.1	TLEIFVGMGSIALSTGLVFARFAR-	104
KirBac3.1	TLEALCGMLGLAFAASLIYARETRP	105
	M2-segment	

Fig. 1. Different pH sensitivities of Kir2 channels. A, Effect of pH_{external} as indicated on Kir2.1 and Kir2.3 channel currents elicited during 3sec voltage steps from -140 mV to $+40$ mV in oocytes. The zero current levels are indicated by arrows. B, Current amplitudes were analyzed at the end of pulses at -120 mV and normalized to the currents at pH 8 ($n=7$). Data for Kir2.3 were fitted to a Hill-equation with a Hill-coefficient of 1. C, Kir2.3 currents were analyzed at the end of pulses at voltages indicated (all $n=7$). Mean values are given with errors as SEM.

Molecular modeling

The X-ray structures of KcsA [15] and KirBac3.1 [16] served as structural template for Kir2.3 channels M1/H5/M2 domain, respectively. We started our 3D-modelling approach based on homology of Kir2.3 and the coordinates of KirBac3.1 (1XI4) and KcsA (1r3jC) [16]. The KirBac3.1 and KcsA coordinates were downloaded from the RCSB protein data bank (<http://www.rcsb.org/pdb/home/home.do>). An alignment including Kir2.3, KirBac3.1 and KcsA is shown in Table 1. Molecular modeling was performed using the Swiss model and DeepView / Swiss-PdpViewer v3.7. The binding sites of K^+ ions in the selectivity filter are numbered from 0 to 5 starting from the extracellular side. Positions 0, 2 and 4 were loaded with K^+ ions during energy optimization procedures. The models were energy minimized using GROMOS 96 empirical force field at standard settings implemented in the DeepView / Swiss-PdpViewer v3.7 [17, 18]. H-bonds were computed at pH7.

Results

Closely related Kir potassium channels differ in their pH_{external} sensitivity

Kir2.1 and Kir2.3 channels were expressed in *Xenopus laevis* oocytes and currents were recorded in KD60 at different pH. Decreasing the pH from 8 to 6

resulted in about 45% reduction of Kir2.3 channel inward current whereas the IC_{50} (the half point of inhibition) was at about $pH = 6.8$ (Fig. 1A,B). The inhibition was reversible. The inward currents inhibition was voltage independent at voltages in the range of -60 to -140 mV (Fig. 1C). No clear pH effects were found for Kir2.1 channels recorded under similar conditions (Fig. 1A-C).

Histidine 117 is involved in Kir2.3 pH_{external} sensitivity

Based on its physico-chemical features histidines ($pK_a \sim 6.08$) are good candidates to confer sensitivity to external acidic pH, although the exact pK_a of histidines may be modulated by the protein environment. Typically, at neutral pH nitrogen atoms in the side chains of histidines

Fig. 2. Identification of the pH sensing residue in Kir2.3 channels. A, Alignment of Kir2 channel protein sequences of the H5 pore loop. The position of the selectivity filter sequence is underlined, the position of the putative pH site is boxed and the residues homologous to the TEA binding site in other potassium channels are marked black. The position of the residues within the outer pore domain are depicted at the right. B, Wild type and mutant Kir2.3 channels were expressed and their channel currents were tested at -120 mV at various pH (n=3-7). C, Analysis of tail currents as in Fig. 1 was performed (n=3-7). The zero current levels are indicated by arrows. Data as mean $\pm$ SEM.

show relatively low affinity to protons but become ionized and thus positively charged at lower pH values. Alignment of protein sequences spanning the transmembrane regions of all Kir2 channels revealed that pH sensitive Kir2.3 but not pH insensitive Kir2.1 and Kir2.2 channels harbour a histidine at a homologous position (Fig. 2A).

Histidine 117 is located close to the putative selectivity filter and therefore is a good candidate to confer pH_{external} sensitivity to Kir2.3 channels. In line with this, Coulter et al. suggested an important role of His117 in human Kir2.3 [12]. To elucidate the structural determinants of pH sensitivity we studied a series of mutations in the Kir2.3 residue His117. In order to determine, if charges in position 117 are affecting ion conduction and rectification, we introduced a positively charged basic arginine, a neutral alanine or a negatively charged glutamic acid resulting in mutants H117R, H117A and H117E. The substitution of H117 by positively charged arginine resulted in channels with decreased currents compared with H117A and H117E. All mutant channels were insensitive to external pH in the range tested (Fig. 2B). Further, Kir2.3wt containing H117 showed currents which were about the size of H117R at low pH and currents about the size of H117A or H117E at high pH. These findings seem inconsistent with the idea of histidine being charged at low external pH to exert a simple electrostatic effect (Fig. 2C): in case of electrostatic effects neutral alanine substitutions can be expected to have intermediate effects between positively charged arginines and negatively charged glutamates. These data suggest that the exact physico-chemical nature of histidine rather than the charge is required for the pH effect (Fig. 2C).

Introduction of histidine into Kir2.1 introduces only mild pH sensitivity

To corroborate our findings, we introduced the identified amino acid histidine into the Kir2.1 channel subunit resulting in Kir2.1-E125H. In addition, we generated the mutant channels Kir2.1-E125A and Kir2.1-

Fig. 3. Generation of mild pH sensitive Kir2.1. A, Representative current traces at pH 6 and pH 8 are shown. The zero current levels are indicated by arrows. B, Wild type and mutant Kir2.1 channels were expressed in oocytes and their channel currents were determined at -120 mV and varying extracellular pH (n=3-4). Data as mean $\pm$ SEM.

Fig. 4. Introduction of Co^{2+} coordination sites into Kir2.3. Wild type and mutant Kir2.3 channels were expressed and their sensitivity to 5 mM CoCl_2 was tested ($n=4-6$). Currents were analysed at the end of 3 second pulses. The results are represented as mean $\pm$ SEM. The position of the residues within the outer pore domain and the resulting putative Co^{2+} coordination site are depicted beneath.

E125R. These channels were expressed and their sensitivities to different pH values were tested. The Kir2.1-E125R channels generated the smallest and Kir2.1-E125A the largest channel currents. However, the difference was comparatively small (maximally only about 25 % differences in current amplitude). Further, we found only weak pH sensitivity in Kir2.1-E125H channels (only about 15 % difference in current amplitude between $I_{\text{pH}5}$ vs. $I_{\text{pH}8}$) (Fig. 3A,B).

Construction of Co^{2+} -coordination sites in Kir2.3

Coulter et al. [12] raised the idea that cysteines may interact with the inhibiting hydrogens since the pH effects could be mimicked with inhibition by Zn^{2+} which could bind to cysteines/histidines. In order to determine the molecular distances between the residues Kir2.3(117) and putatively H-interacting cysteines (TEA site) we employed the mutant Kir2.3(H117C). We applied CoCl_2 to test if the native cysteines and the engineered cysteines coordinate cobalt. If the engineered cysteines and the TEA-site cysteins are sufficiently close enough to allow Co^{2+} coordination then the channels may exert functional effects. Co^{2+} was chosen because the commonly used Cd^{2+} interacts with homocysteines to inhibit Kir2.3 channels containing cysteines (H117C/C141Y, C141, H117C/C141), but Co^{2+} does not interact with homocysteines. Indeed, application of Co^{2+} strongly reduces currents in mutant Kir2.3(H117C), whereas no effect could be seen in wt channels (Fig. 4). Kir2.3(H117C/C141Y) were insensitive to 5 mM CoCl_2 (data not shown).

Construction of putative disulfide bonds in Kir2.3

To further support the idea of cysteines interacting with the inhibiting hydrogens [12] we tested wildtype and mutant Kir2.3 channels. In order to confirm that the residues Kir2.3(117) and a putatively H-interacting cysteines (TEA site) are within atomic distances we probed the channels with tert-Butyl hydroperoxide (tbHO_2) and DL-Dithiothreitol (DTT). The reducing agent DTT may break and the oxidizing agent tbHO_2 may support formation of disulfide bonds. Consistent with the idea of spatial proximity of the residues, the channels

Fig. 5. tbHO_2 reduces and DTT increases Kir2.3 current. Wild type and mutant Kir2.3 channels were expressed and their sensitivity to 2 mM tbHO_2 and 10 mM DTT was tested. Wild type and mutant Kir2.3 channels were expressed and perfused with 2 mM tbHO_2 and 10 mM DTT. Kir channel currents elicited during 3 second voltage steps from -140 mV to $+40$ mV in oocytes. Currents were analysed at the end of pulses. The results are represented as mean $\pm$ SEM ($n=5-6$). Data are represented as mean $\pm$ SEM ($n=5-6$).

containing only one cysteine (Kir2.3wt and Kir2.3(H117C/C141Y)) were largely insensitive to the agents. However, the channel containing cysteines at both putatively partnering positions (Kir2.3(H117C/C141Y)) was suppressed by tbHO_2 and stimulated by DTT, in line with the idea of S-S bonds formation to inhibit currents (Fig. 5). Like the Co^{2+} -results (Fig.4) these findings also support the idea that the cyteine residues are in close proximity.

Fig. 6. Introduction of TEA sensitivity into Kir2.3 to measure distances between cysteines and TEA sites in mutant Kir2.3 channels. A, Wild type and mutant Kir2.3 channels were expressed and their inhibition in percent by externally applied TEA were determined at -120 mV ($n=3-7$). Data as mean $\pm$ SEM. The data were fitted by a Hill equation. IC_{50} value of Kir2.3-H117C/C141Y was $51.9 \pm 5.2 \mu\text{M}$ (Hill coefficient = 1). The position of the residues within the outer pore domain and the resulting putative TEA binding site are depicted in the inset. B, I/V curves of Kir2 channel currents analysed at the end of 3 sec pulses ($n=4-9$). The oocytes tested with MTS reagents were incubated in 1 mM MTS reagent for 5 minutes at room temperature. Oocytes were washed ten times with ND96 before recordings. Molecular structure of MTS reagents and their respective sizes (in Å) are indicated in the box. Data as mean $\pm$ SEM.

Introduction of TEA binding sites into Kir2.3 channels and analysis of the distances between residue Kir2.3-H117C and the TEA binding site

The gross pore structure of Kir2 potassium channels is believed to be similar to the pores of other K^+ channels where information of solved crystal structures exists [19]. In several Kv channels an extracellular TEA high affinity binding site is reported with the requirement of a tyrosine or valine at a homologous position close to the outer mouth opening of the selectivity filter. Kv channels lacking these

Fig. 7. Measurement of distances between native and engineered cysteines in Kir2.3 channels. A, Molecular structure of MTS reagents and their respective length (in Å) are indicated. B, I/V curves of Kir2 channel currents with or without MTS-linker-MTS were analysed at the end of 3 sec pulses ($n=4-9$). The oocytes tested with MTS-linker-MTS reagents were incubated in 1 mM of the respective MTS-linker-MTS reagent for 5 minutes at room temperature. Oocytes were then washed ten times with ND96 before recordings. Abscissae indicate voltages. Data are represented as mean $\pm$ SEM.

residues can easily be sensitized by mutation of residues to tyrosines or valines. Here, we mutated the respective residue to tyrosine generating the mutant Kir2.3(C141Y). In addition, we mutated the pH sensing site in Kir2.3 to cysteine. We applied TEA in concentrations from 3 μM to 10 mM. Wild type Kir2.3 and single mutant channels Kir2.3(H117C) and Kir2.3(C141Y) were largely insensitive to TEA up to 3 mM. The mutant channels Kir2.3(H117C/C141Y) were sensitive to TEA, however, the inhibition was only partial, reaching 55% block and showing IC_{50} values of about 51 μM , respectively (Fig. 6). The relative small cyteine ($86\text{\AA}^3$) in Kir2.3(H117C) possibly allows access of TEA to the TEA binding site, whereas the $32\text{\AA}^3$ larger histidines ($118\text{\AA}^3$) may restrict access to the TEA binding sites in the Kir2.3 channels.

Fig. 8. Homology models of Kir2.3 channels. Channel models of Kir2.3 derived from the KirBac3.1 X-ray structures. The Kir2.3 models were several times energy minimized *in vacuo* using GROMOS96 empirical force field. Side views of two subunits. Close-ups of two subunits in stereo view. Residues H117 and C141 are shown in stick representation and are coloured in CPK code. The backbone of selectivity filter residues TIGYG is shown in CPK colour code. The same residues backbones in the position without the hydrogen bond between H117 and C141 are presented superposed in orange. Potassium ion binding sites are shown filled with green coloured K⁺ ions and numbered from 0 to 5 starting from the extracellular side.

Since the structural basis of the external TEA binding site is known for KcsA channels [20, 21] we aimed to measure the distance between the cysteine-mutated pH sensing residues in Kir2.3 and the respective engineered TEA-binding sites by using SCAM technique. We have employed a family of alkyl-triethylammonium derivatives (MTS-ET, MTS-PTvEA and MTS-TEAH) in which the length of a C-chain is altered. The respective sizes of the MTS reagents are given in brackets (Fig. 6A). We applied MTS-alkyl-linker-TEA compounds with linkers of variable length aiming to build disulfide bridges of MTS-reagents with the cysteines at sites Kir2.3-117. If the alkyl linkers between the respective MTS and the TEA are sufficiently long to allow TEA binding, then the channels are expected to be partially blocked. In our experiments MTS-ET, MTS-PTvEA and MTS-TEAH were used. All three MTS-linker-TEA reagents substantially and irreversibly (no washout) inhibited the double mutant Kir2 channel currents but not Kir2.3-C141Y channels (Fig. 6B), indicating that the linker chain of MTS-ET is sufficiently long to allow binding to the engineered TEA binding site. This may correspond to a distance between Kir2.3-H117C and Kir2.3-C141Y of =8.9 Å.

Determination of distances between native and engineered cysteines in Kir2.3

By using MTS-linker-MTS reagents we aimed to determine the distance between the cysteines at the pH sites. Binding of MTS-linker-MTS to two cysteines in adjacent subunits may result in marked functional effects [22]. Molecular gating in Kir2 channels occurs and both, selectivity filter rearrangements and M2 motion have been proposed to occur (reviewed by Loussouarn et al. 2002). Thus, locking of channels in a particular state may be possible, as recently described for ligand gated GluR channels [22, 23]. Kir2.3 without a cysteine at either the

pH-site or close to the selectivity filter are insensitive to MTS-reagents. Mutant Kir2.3 channels containing a single cysteine were inhibited by the MTS-linker-MTS reagents (Fig. 7). Whereas all MTS-linker-MTS reagents inhibited Kir2.3-wt (contains cyteine at 141) with similar potency, the inhibition of Kir2.3-H117C/C141Y depended largely on the linker length: the shorter the linker the stronger the inhibition possibly by stronger constraints on channels structure.

An interpretation of these data is that binding of cysteines of two subunits to MTS-linker-MTS with shorter length may exert constraints on the channels resulting in channel inhibition. On the other hand, either binding of MTS-linker-MTS to only one subunit or to two subunits with long linkers could be insufficient to restrict Kir2.3 channel structure and therefore may not result in marked functional effects. Following these arguments the data can be interpreted as Kir2.3 cysteines 117 are separated by about 10-12 Å. However, further interpretations of these data are possible and cannot be excluded.

Molecular modeling of the Kir2.3 outer pore domain

Generally, it is believed that potassium channels including Kir2 channels have a highly conserved pore domain structure. We aimed to incorporate our experimental results in Kir2.3 channel models. Therefore, we started our 3D-modelling approach based on homology of Kir2.3 and the KcsA and BirBac3.1 coordinates [15, 16]. According to this approach, tetrameric KcsA like Kir2.3 channels consist of a tetrameric assembly of subunits with two transmembrane spanning regions lining the pore helix and a selectivity filter characteristic for potassium channels. We built the homology models and modified the models satisfying our experimental results.

Discussion

In this study, we focused on the difference of sensitivity to inhibition by low external pH among members of the Kir2 channel family and identified a pH sensing residue in the outer pore domain.

Different pH sensitivity of Kir2 channels depends on a histidine in the outer pore domain

In Kv1 channels (Kv1.1, 1.4, 1.5, 1.7 and 1.10) sensitivity to external pH is realized by binding of a proton to a conserved histidine. However, the pH_{external} sensitivity seems to require the respective histidine and additional charged residues in the outer pore domain of Kv1 channels [24].

Kir2 channels were reported to exert different pH sensitivities. Kir2.3 channels can transfer their pH sensitivity to Kir2.1/Kir2.3 heteromeric channels [11]. Here, we show that Kir2.3 channels, but not Kir2.1 channels are inhibited by low/acidic extracellular pH (Figure 1). In this study the half maximal inhibition of Kir2.3 is close to pH 6.8, close to the typical pK_a of single histidines in aqueous solution. In contrast, Coulter et al. report a pK for human Kir2.3 of 7.4 [12]. The origin of differences in the pK values are not clear. Also, the absolute inhibition of Kir2.3 by external pH described by Coulter et al. is larger (inward currents: Coulter pH 6.6 vs. pH 8 = 33 % vs. 100 %; in this study pH 6 vs. pH 8 = 55% vs. 100%) [12]. Coulter et al. raise the possibility that the residues Cys114 or Cys145 may be titratable residues but could not prove this hypothesis, because these mutants were nonfunctional [12]. We also generated these mutants hKir2.3(C114A) and hKir2.3(C145A), but in our hands these mutant channels were nonfunctional as well (data not shown) making it impossible to study the hypothesis.

In an alignment of the outer pore domain of Kir2 channels we identified a histidine, which is present in pH sensitive channels, but absent in largely pH_{external} insensitive Kir2s (labelled with pH site, Fig. 2A). This residue was also identified by Coulter et al. as a major pH sensitivity determinant [12]. We observed that mutagenesis of this residue in Kir2.3 diminished pH sensitivity. The importance of this residue was confirmed by further mutagenesis. The positively charged residue arginine resulted in channels with lower macroscopic currents (about the size of Kir2.3 at pH 6), whereas neutral alanine and negatively charged glutamic acid showed similarly larger currents about the size of Kir2.3 at pH 8 (Fig. 2B). The fact that neutral alanine does not result in intermediate effects is

inconsistent with a simple charge effect. Interestingly, Coulter et al. report that mutation of the homologous residue in the human Kir2.3 to glutamine, asparagine and lysine results in pH-sensitive mutants whereas mutants incorporating asparagine and glutamate (functional in case of glutamate Kir2.3 background, Fig. 2B,C) were non-functional [12]. Obviously, the exact physico-chemical nature of the histidine and not simply a charge is crucial for channel inhibition at low pH.

To corroborate our Kir2.3 results we introduced similar amino acid substitutions into Kir2.1 at a putatively homologous position. Kir2.1 channels acquired mild pH sensitivity with introduction of a histidine, smaller currents with basic arginine and larger currents with neutral alanine or acidic glutamate. Noteworthy, although present in Kir2.1 the size of effects of both, charge of residues and pH dependency of the Kir2.1-E125H mutant are much smaller compared to effects in Kir2.3. Possibly, the outer pore structure of Kir2.1 and Kir2.3 are structurally similar but not identical.

Residues 117 and 141 are in atomic proximity

Cysteines in spatial proximity can form binding sites for divalent cations like Zn²⁺, Cd²⁺ or Co²⁺. Therefore, engineered cysteine pairs in a protein can be used to study atomic distances between the respective residues by application of e.g. CoCl₂. In our experiments Kir2.3 channels gain Co²⁺ sensitivity by introduction of a cysteine at residue 117. This sensitivity can be disrupted by further mutation of the endogenous cysteine at the residue 141 (TEA site) resulting in mutant Kir2.3-H117C/C141Y. These findings indicate that the cysteines in both positions, 117 and 141, are required for Co²⁺ coordination (Fig. 4). A second experimental approach to study colocalisation of two residues is to form/disrupt disulfide bonds by application of tBH₂O₂ and DTT. We found that cysteines at both positions (117 and 141) are required for tBH₂O₂ and observed DTT sensitivity indicative of disulfide bond modification (Fig. 5). Summarising, we present two lines of evidence that residues 117 and 141 are within atomic distance to each other possibly allowing the formation of the proposed hydrogen bond between the two residues.

The residue 117 (pH site) is located in a crevice close to the selectivity filter

Extracellular tetraethylammonium (TEA) binds in a series of potassium channels to four aromatic tyrosine residues at the extracellular mouth of the selectivity filter of tetrameric potassium channels [20]. An engineered TEA binding site can be used as an electrostatic calliper

to measure the size of the outer pore opening [25]. Further, commercial TEA-MTS reagents with linkers of variable length can be used to measure the distance between TEA binding sites and cysteine residues. In order to explore the size of the outer pore structure we used SCAM and an engineered TEA binding site. We mutated the pH site in Kir2.3 to cysteines resulting in mutant Kir2.3-H117C. Further, we engineered TEA binding sites into the Kir2.3 channels. The resulting mutant channels showed high sensitivity towards TEA with IC_{50} values of about 51 μ M in Kir2.3-H117C/C141Y (Fig. 6). However, the mutant channels were not fully blocked by high concentrations of TEA. Both, the unusually high TEA sensitivity and the partial block indicates structural differences between Kir2.3 and Kv channels, which are typically blocked with IC_{50} values around 300-500 μ M [25]. Surprisingly, Kir2.3-C141Y is insensitive to TEA (data not shown) making electrostatic calliper measurements of the outer pore opening impossible [25]. We therefore used the TEA-linker-MTS reagents to estimate the distance between TEA binding sites and cysteine residues in Kir2.3-H117C/C141Y (Fig. 6). We found that MTSET like MTPtrEA and MTS-TEAH inhibited mutant channels substantially, suggesting that the length of the short C3-linker in MTSET is sufficient to bridge the distance ($\sim 8.9\text{\AA}$) between cysteines and the TEA binding sites. This indicates a substantially smaller outer opening compared to KcsA or Kv1.2.

Binding of MTS-linker-MTS to two cysteines in different (possibly adjacent) subunits may result in marked functional effects modulating molecular gating in Kir2 channels (reviewed by Loussouarn et al. [23]), as recently described for ligand gated GluR channels [22]. The results shown in Fig. 7 suggest that Kir2.3 cysteines 117 are separated by about 10-12 $\text{\AA}$. Clearly, further interpretations of these data are possible. However, these findings are consistent with a smaller opening of the extracellular mouth of the selectivity filter and can well be incorporated into a molecular model in line with other experimental data.

The results presented here underscore the importance of the respective residue at homologous positions in Kir2 channels. Five arguments suggest that the pH site residue is indeed localized in a small diameter crevice at the extracellular entrance of the selectivity filter:

I. The unusual extracellular TEA block (Fig. 6A) characterized by the high apparent affinity and partial block suggests that the binding site has a substantially different structure, supposedly with much narrower diameter compared to KcsA or Kv1.2.

II. The narrower outer pore diameter is further suggested by the MTS-linked TEA analogue experiments (Fig. 6B)

III. The MTS-linker-MTS results are consistent with a small diameter outer pore entrance to the extracellular entrance of the selectivity filter (Fig. 7).

IV. The formation of divalent ion binding sites exerting competitive effects: residue E125 in closely related Kir2.1 was reported as a residue relevant for Ba^{2+} inhibition by increasing the local Ba^{2+} concentration in order to increase block at a deeper site within the selectivity filter [26]. This scenario may be enabled by a small diameter crevice.

V. Kir2.1-E125 may be a binding site enabling Ca^{2+} and Mg^{2+} block [13, 26]. Single channel recordings of wild type Kir2.1 showed that Ca^{2+} and Mg^{2+} decrease single channel conductances whereas the sensitivity is not present in Kir2.1-E125Q channels [13]. The authors conclude that extracellular Ca^{2+} and Mg^{2+} compete with potassium ions to decrease conduction which may be favoured in a small diameter crevice.

Incorporation of the results into a structural Kir2.3 homology model

The engineered TEA binding site in Kir2.3 results in channels which can be partially blocked by this compound with comparatively higher apparent affinity. This TEA binding site may be localized in the centre of the longitudinal channel axis. If this is the case then the pH-residue in Kir2 channels is at least $\sim 3\text{\AA}$ closer to the central axis compared to the KcsA crystal structure. Based on homology to the channels KirBac3.1 and KcsA structural Kir2.3 models were built. We incorporate the molecular diameters experimentally determined by the TEA-linker experiments and introduce a hydrogen bond into one of the Kir2.3 models. If we assume that the MTS-linker-MTS reagents bind to engineered cysteines in adjacent subunits then also the determined distance of cystein residues of about 10 $\text{\AA}$ is consistent with the Kir2.3 model presented. By comparison of the energy minimized/optimized Kir2.3 channel model with the hydrogen bond-free energy minimized/optimized Kir2.3 model, differences in the selectivity filter are obvious. Figure 8 shows an example of the KirBac3.1 based Kir2.3 model. In both, KirBac3.1 and KcsA based Kir2.3 channel models without the hydrogen bond have a smaller diameter at the K^+ binding sites 1 - 4. Thus, the formation of the putative hydrogen bond at low pH may constrain the selectivity filter to adopt a less conductive conformation with reduced single channel conductance [12].

What is the physiological significance of the histidine at the pH site in Kir2 channels? pH sensing Kir2.3 residues may regulate cell excitability depending on extracellular pH. Under physiological conditions, the resting membrane potential is normally above the equilibrium potential for potassium. Kir2 channels allow small ion fluxes which are important for the maintenance of the resting membrane potential and thus couple pH sensing to cell excitability.

In conclusion, we were able to identify a putative hydrogen bond that when formed at low pH constrains

the selectivity filter to reduce conductance thereby transferring pH sensitivity to Kir2.3 channels. This mechanism of pH sensitivity may result from a narrow outer pore structure.

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