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Inward rectification in K_{ATP} channels: a pH switch in the pore

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Inward-rectifier potassium channels (K_{ir} channels) stabilize the resting membrane potential and set a threshold for excitation in many types of cell. This function arises from voltage-dependent rectification of these channels due to blockage by intracellular polyamines. In all K_{ir} channels studied to date, the voltage-dependence of rectification is either strong or weak. Here we show that in cardiac as well as in cloned K_{ATP} channels ($K_{ir}6.2$ + sulfonylurea receptor) polyamine-mediated rectification is not fixed but changes with intracellular pH in the physiological range: inward-rectification is prominent at basic pH, while at acidic pH rectification is very weak. The pH-dependence of polyamine block is specific for K_{ATP} as shown in experiments with other K_{ir} channels. Systematic mutagenesis revealed a titratable C-terminal histidine residue (H216) in $K_{ir}6.2$ to be the structural determinant, and electrostatic interaction between this residue and polyamines was shown to be the molecular mechanism underlying pH-dependent rectification. This pH-dependent block of K_{ATP} channels may represent a novel and direct link between excitation and intracellular pH.

Keywords: K_{ATP} channels/pH/polyamines/protonation

Introduction

Inward-rectifier potassium channels (K_{ir} channels) stabilize the membrane potential (E_M) near the K^+ reversal potential (E_K) in excitable and non-excitable cells (Hille, 1992). This function arises from the high K^+ conductance mediated by these channels around E_K and at a limited voltage range positive to E_K. Further depolarization decreases K^+ conductance, thereby switching off its stabilizing effect on E_M (Fakler *et al*., 1995). The range of membrane potential over which K_{ir} channels stabilize E_M depends on their voltage-dependence of rectification which may be strong or mild (Hille, 1992) and is predominantly due to a voltage-dependent pore block by the intracellular polyamines spermine (SPM) and spermidine (SPD) (Fakler *et al*., 1994, 1995; Ficker *et al*., 1994; Lopatin *et al*., 1994). Accordingly, strong rectifiers maintain their stabilizing effect over a voltage range of $\leqslant$50 mV positive to E_K, while mild rectifiers retain their stabilizing effect virtually over the whole physiological voltage range.

During the past several years, many cDNAs encoding distinct K_{ir} subunits have been isolated and structural comparisons suggest that they may be subdivided into seven subfamilies (K_{ir} 1–7; Doupnik *et al*., 1995; Krapivinsky *et al*., 1998). Functional expression of K_{ir} family members showed that they either exhibit strong or weak voltage-dependent rectification (for review see Doupnik *et al*., 1995; Fakler and Ruppersberg, 1996; Nichols and Lopatin, 1997). Sequence comparison between the prototypes of strong and weak rectifiers, $K_{ir}2.1$ (IRK1; Kubo *et al*., 1993) and $K_{ir}1.1$ (ROMK1; Ho *et al*., 1993), led to identification of two residues defining the voltage-dependence of polyamine block: negatively charged glutamate or aspartate residues in the second transmembrane segment (M2-site; Fakler *et al*., 1994; Lu and MacKinnon, 1994; Stanfield *et al*., 1994; Wible *et al*., 1994) and in the cytoplasmic C-terminus (C-terminal site; Taglialatela *et al*., 1995; Yang *et al*., 1995). Exchange of the neutral M2-site in the weakly rectifying $K_{ir}1.1$ to a negatively charged residue converted this channel into a strong inward-rectifier, while neutralizing both sites in $K_{ir}2.1$ greatly reduced SPM-mediated rectification in these channels.

ATP-sensitive K^+ channels (K_{ATP} channels) are weak rectifiers which are expressed in a wide variety of tissues including pancreatic β cells, cardiac myocytes, skeletal muscle and brain and serve to couple metabolic state to excitability (for review see Ashcroft, 1988). K_{ATP} channels are assembled from the sulfonylurea receptor (SUR1 or SUR2A, B), a member of the superfamily of ATP-binding cassette proteins (Aguilar-Bryan *et al*., 1995) and a K_{ir} subunit ($K_{ir}6.1$, $K_{ir}6.2$; Inagaki *et al*., 1995; Sakura *et al*., 1995). While SUR has been identified as the regulatory subunit of K_{ATP} which confers sensitivity to sulfonylureas, channel openers and Mg-ADP (Inagaki *et al*., 1995, 1996; Nichols *et al*., 1996; Gribble *et al*., 1997; Shyng *et al*., 1997b) $K_{ir}6.x$ acts as the pore-forming subunit of the channel complex (Shyng and Nichols, 1997; Shyng *et al*., 1997a; Tucker *et al*., 1997). $K_{ir}6.2$ does not contain either site for SPM block in its primary sequence, explaining the weak rectification observed in K_{ATP} channels in many studies.

In this study we show that polyamine-mediated rectification in K_{ATP} channels is not fixed but controlled by the intracellular pH (pH_i), and we demonstrate the molecular mechanism underlying this phenomenon.

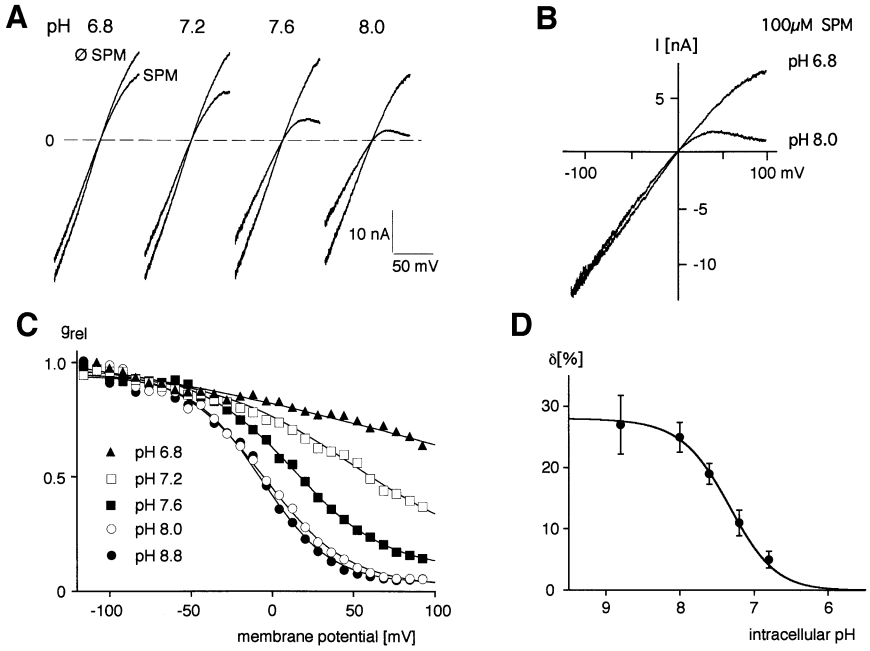

Fig. 1. Block of K_{ATP} channels by SPM is determined by the intracellular pH. (**A**) Current mediated by $K_{ir}6.2$ / SUR1 channels as response to 3 s voltage ramps from –120 to +100 mV measured in giant inside-out patches from *Xenopus* oocytes. pH_i was changed from 6.8 to 8.0 as indicated and I–Vs were recorded in the absence and presence of 100 μM SPM. Zero current level as indicated. (**B**) I–Vs from (A) recorded in the presence of SPM for pH_i 6.8 and 8.0, scaled to the current amplitude at –120 mV for better comparison. (**C**) g–V plots obtained from I–Vs as in (A) but with 500 μM SPM. Lines represent fit of a single Boltzmann function to the data points (see Materials and methods); fit parameters were $V_{1/2}$ /V_s: –7/21 mV (pH 8.8), –5/26 mV (pH 8.0), 13/29 mV (pH 7.6), 50/43 mV (pH 7.2), 133/148 mV (pH 6.8). (**D**) Electrical distance (δ) obtained from fits as in (C) plotted as a function of pH_i (data points are mean ± SD of five experiments). Line is fit of a logistic function to the data; $pH_{\delta 1/2}$ and Hill coefficient were 7.3 and 1.3.

Results

Polyamine-mediated rectification of K_{ATP} is controlled by pH_i

Rectification properties of K_{ATP} channels were investigated in giant inside-out patches from *Xenopus* oocytes coexpressing $K_{ir}6.2$ and SUR1 subunits. As illustrated in Figure 1, inward-rectification induced by 100 μM SPM was strongly dependent on pH_i: while rectification was weak at neutral pH_i, it increased considerably upon alkalinization (Figure 1A and B). In the absence of SPM, the current–voltage relation (I–V) was linear and showed little change with pH_i. However, at pH_is <6.8 and >8.0 substantial reduction of the current amplitude was seen (data not shown) as reported before (Misler *et al.*, 1989; Proks *et al.*, 1994).

For a more quantitative analysis, I–Vs were transformed to conductance–voltage plots (g–V; see Materials and methods) and fitted with a single Boltzmann function:

$$g_{rel} = g(V)/g_o = \{1+\exp[V-V_{1/2}] / V_s\}^{-1} \qquad (1)$$

where g_o is the conductance in the absence of SPM, $V_{1/2}$ is the voltage for half-maximal SPM block and V_s is the voltage required for an e-fold change in conductance.

Figure 1C shows representative g–Vs obtained from an experiment with 500 μM SPM, and pH_i varied between 6.8 and 8.8. Under these conditions, g–Vs were shifted by as much as 124 mV upon changing pH_i from 6.8 ($V_{1/2}$ = 111 ± 35 mV) to 8.8 ($V_{1/2}$ = –13 ± 5 mV), and V_s decreased >5-fold from 120 ± 35 mV to 22 ± 4 mV (n = 5). Assuming a Woodhull model (Woodhull, 1973), the V_s values can be correlated with the product of the valence of the blocker and the fraction of the transmembrane electrical field (δ) the blocker crosses to reach its binding site inside the pore (see Materials and methods). As shown in Figure 1D, the calculated δ ranged from 5% at pH_i 6.8 to 27% at pH_i 8.8. Moreover, the resulting δ–pH_i relation could be approximated by a logistic function with a Hill coefficent of 1.3, a pH_i for half-maximal δ ($pH_{\delta 1/2}$) of 7.3, and asymptotic values for δ of 28 and 0%. This suggests that SPM enters the pore only at basic but not at acidic pH_i and that the fraction of channels that can be blocked by SPM changes with pH_i.

Rectification in cardiac K_{ATP} channels is pH dependent

pH-dependence of SPM-mediated rectification was also observed for K_{ATP} channels in excised patches from murine cardiomyocytes (n = 8). The I–Vs recorded under symmetrical K^+ conditions (Figure 2A) were identical with the results for the cloned K_{ATP} channels presented in Figure 1B. To demonstrate directly the effect of a pH_i change on the amplitude of the K_{ATP} current under more physiological conditions, experiments were repeated in assymmetrical K^+ in the presence of 100 μM SPM. As shown in Figure 2B and C, the switch in pH_i from 8.0 to 6.8 led to a substantial increase of the K_{ATP}-mediated current for potentials positive to E_K (approximately –75 mV in these experiments). Similar to SPM block in other K_{ir} channels (Hagiwara *et al.*, 1976; Nichols and Lopatin, 1997; Oliver *et al.*, 1998), rectification in K_{ATP} channels is shifted to more negative potentials upon reduction of the extracellular K^+ concentration (Figure 2A and C).

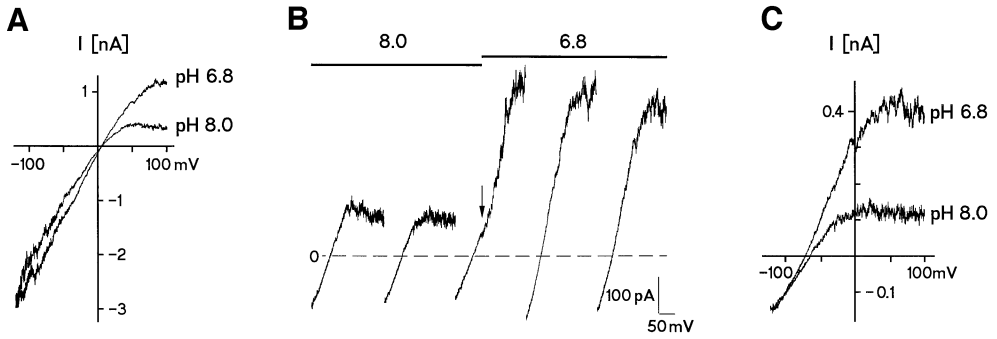

Fig. 2. pH_i-dependent rectification in cardiac K_{ATP} channels. (**A**) Current mediated by cardiac K_{ATP} channels in response to 3 s voltage ramps as in Figure 1A measured in inside-out patches from murine cardiomyocytes at the pH_i values indicated; K^+ concentration in the patch pipette was 155 mM and 120 mM on the cytoplasmic side of the membrane. (**B**) Experiment as in (A) but with 5 mM K^+ in the pipette. Arrow indicates the change in pH_i. Time and current scale as indicated. (**C**) Superposition of traces 2 and 5 from the experiment in (B) scaled to the current measured at –120 mV at pH_i 6.8 for better comparison. No leakage subtraction was performed.

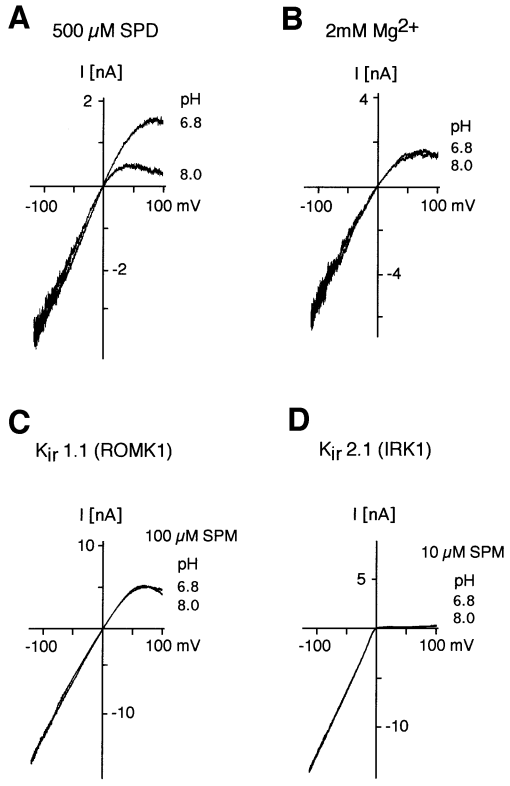

Fig. 3. pH_i-dependent rectification discriminates between K_{ir} subtypes and blocking molecules. (**A**, **B**) Block of K_{ATP} channels by 500 μM SPD exhibits pH_i-dependence (A), while block by Mg^{2+} did not (B). (**C**, **D**) Polyamine-mediated rectification of $K_{ir}1.1$ (C) and $K_{ir}2.1$ (D) channels is independent of pH_i. Conditions and voltage protocol as in Figure 1. Current amplitude at pH_i 6.8 was scaled to that measured at –120 mV at a pH_i of 8.0 for better comparison.

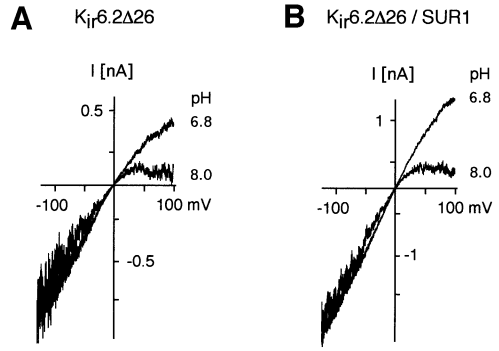

Fig. 4. pH_i-dependent rectification is determined by the $K_{ir}6.2$ subunit of K_{ATP} channels. SPM block of $K_{ir}6.2\Delta26$ channels expressed either alone (**A**) or coexpressed with SUR1 (**B**) at the pH_i values indicated.

pH-dependent rectification is specific for polyamines and the $K_{ir}6.2$ subunit

The pH-dependence of rectification was further investigated by testing various blocking molecules, as well as other K_{ir} subunits. As shown in Figure 3A, pore block by SPD (500 μM) was pH-dependent similar to SPM, while Mg^{2+} block (2 mM) was almost independent of pH_i (Figure 3B), as was block by tetraethylammonium (TEA, 10 mM, not shown). Thus, pH_i seems to modulate a site which is specific for polyamines.

Experiments with $K_{ir}1.1$ (ROMK1) and $K_{ir}2.1$ (IRK1), the prototypes for strongly and weakly rectifying K_{ir} channels, revealed no pH-dependence in SPM block for either subtype (Figure 3C and D). Together, these findings suggest that pH-dependence of rectification is a characteristic feature for the block of K_{ATP} channels by the polyamines SPM and SPD.

Since K_{ATP} channels are heteromers of $K_{ir}6.2$ and SUR, involvement of either subunit in pH-dependent polyamine block was tested. These experiments were performed with a C-terminal deletion mutant of $K_{ir}6.2$ ($K_{ir}6.2\Delta26$), which forms functional channels in the absence of SUR (Tucker *et al.*, 1997). As shown in Figure 4, pH-dependence of SPM block was the same whether Kir6.2Δ26 was expressed alone or coexpressed with SUR1, suggesting that the pH-modulatory site resides in the pore-forming $K_{ir}6.2$ subunit. In agreement, we found that pH-dependence of SPM block did not differ between $K_{ir}6.2$ coexpressed with SUR1 or SUR2A (data not shown).

Mutagenesis identified H216 as the molecular determinant for pH_i-dependent polyamine block

The δ–pH_i-relation with a $pH_{\delta1/2}$ of 7.3 (Figure 1D) led to the assumption that a histidine (H) residue in $K_{ir}6.2$ may underlie the pH-dependence of rectification. Therefore, all nine intracellular histidines were substituted by residues with non-titratable sidechains (Figure 5, upper panel). As shown in Figure 5, mutation of H216 to glutamine (Q) resulted in channels for which SPM rectification was strong, but independent of pH_i (Figure 5, lower panel). All other mutations exhibited pH-dependence of polyamine-mediated rectification similar to wild-type (WT) channels.

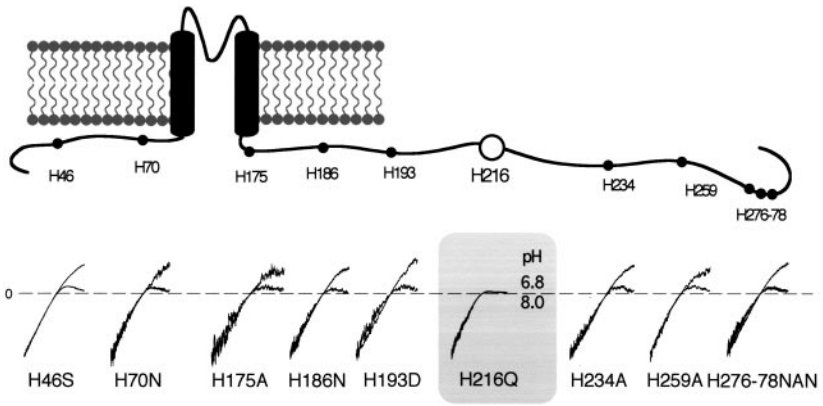

Fig. 5. His216 is the molecular determinant for pH_i-dependent SPM block. Membrane topology (upper panel) of $K_{ir}6.2$ with positions of intracellular histidine residues. I–V_s (lower panel) as in Figure 1 for wild-type or mutant $K_{ir}6.2$ as indicated. Mutations were either introduced into $K_{ir}6.2$ (H70N, H186N, H193D, H216Q, H259A and H276–78NAN) or into $K_{ir}6.2\Delta26$ (H46S, H175A, H234A). In all experiments K_{ir} subunits were coexpressed with SUR1. Note that only the H216Q mutant showed pH-independent SPM block.

These results indicate that H216 is the residue controlling pH-dependent rectification and suggest that protonation–deprotonation of H216 determines the ability of channels to be blocked by polyamines.

Mechanism underlying pH-dependence of polyamine block

Protonation of H216 may modulate polyamine block either by direct electrostatic interaction with the blocker or by allosterically affecting the polyamine-binding site. To test for electrostatic interaction, H216 was further mutated to a positively charged lysine (H216K) or arginine (H216R) as well as to a negatively charged glutamate (H216E).

For H216K and H216R mutant channels, 100 μM SPM produced a very weak and almost voltage-independent block ($\delta < 5\%$) similar to WT channels at pH 6.8 (Figure 6B and D). In contrast, H216Q and H216E resulted in channels that exhibit SPM block with a voltage-dependence similar to that seen in WT channels at pH_i 8.0 (V_s: 21 ± 3 mV and 27 ± 4 mV, $n = 4$; Figure 6A, C and D). The voltage required for half-maximal block in H216E was shifted to more negative potentials by ~45 mV (at pH_i 8.0) with respect to the neutral H216Q mutant (Figure 6A and C). This reflects a considerable increase in affinity for SPM and supports direct electrostatic interaction between polyamines and the residue at position 216. Based on these findings, an allosteric mechanism seems rather unlikely since a negative charge should not affect SPM affinity if the site of protonation was different from the polyamine-binding site.

As expected for non-titratable sidechains, neither mutant showed pH-dependence of SPM-mediated rectification, although in H216E $V_{1/2}$ was somehow shifted when pH_i was changed from 6.8 to 8.0 (Figure 6C). Moreover, block by SPM was incomplete in this mutant resulting in a residual conductance of 10–20% (Figure 6C). Incomplete block has been reported previously for Mg^{2+} block in the strong rectifier channel $K_{ir}2.1$ (Fakler *et al.*, 1995; Yang *et al.*, 1995).

In summary, a positively charged residue at position 216 prevents pore block by polyamines, while a neutral residue permits polyamine-mediated rectification (Figure 6). A negatively charged residue further increases sensitivity for SPM, indicating direct electrostatic interaction between residue 216 and the positively charged polyamines.

Discussion

The functional role of K_{ir} channels critically relies on their degree of rectification (Nichols and Lopatin, 1997). Strong rectifiers are essential for stabilizing the resting membrane potential and setting a sharp threshold for excitation as they conduct almost no current at depolarized potentials. Weak rectifiers conduct substantial outward current at depolarized potentials and are, therefore, expected to contribute to repolarization of action potentials (AP). Cells are supposed to carefully adjust rectification properties because they largely determine their electrical behaviour, such as duration and frequency of AP, for example, in cardiac myocytes or bursting pattern such as in neurons or pancreatic β cells (Hille, 1992). Adjustments of rectification may occur in several ways. First, the ratio between strongly and weakly rectifying K_{ir}s could be altered either by a change in expression or a change in open probability of functional channels as seen for muscarinic K_{ir} channels in cardiac myocytes. Secondly, the intracellular free concentrations of polyamines could be varied as recently suggested (Bianchi *et al.*, 1996; Shyng *et al.*, 1996). Finally, the rectification properties of K_{ir} channels could be directly regulated by intracellular factors. Our findings are the first indication that the affinity of a K_{ir} channel for polyamines may be modulated by intracellular pH.

Polyamine-mediated rectification is controlled by pH_i: biophysical characterization

In K_{ATP} channels polyamine-dependent rectification is controlled by pH_i in the range 6.8–8.0. At pH_i 6.8 polyamines such as SPM and SPD produce only weak and voltage-independent block while at pH_i 8.0 strong inhibition of outward current is seen. The 'blocking site' seems to be selective for polyamines since voltage-dependent block by Mg^{2+} and TEA is not affected by pH_i. pH_i affects the voltage-dependence for SPM block changing the V_s-value of the g–V curve from 120 mV at low pH_i to 22 mV at high pH_i. Based on a Woodhull

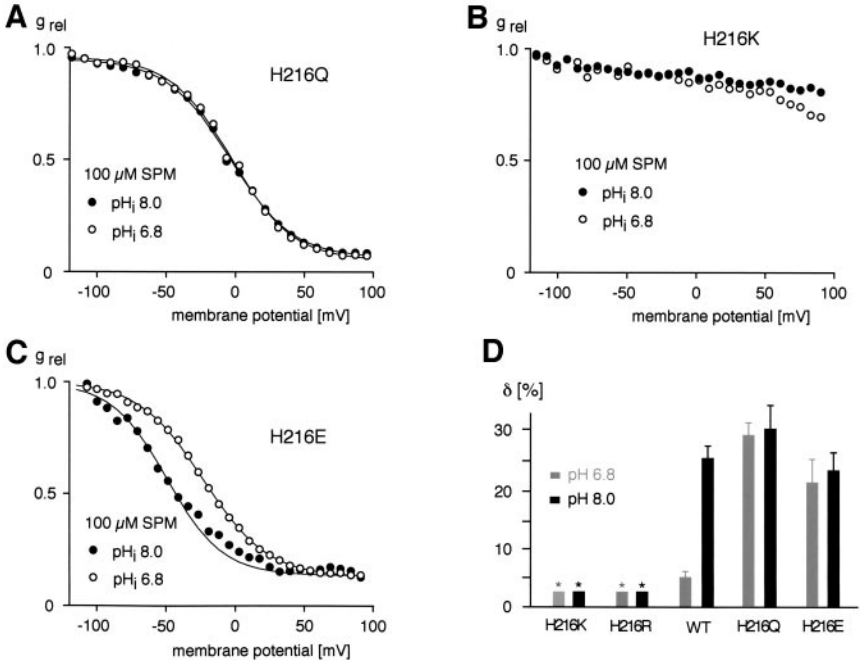

Fig. 6. SPM block is governed by the charge of the residue at position 216 in $K_{ir}6.2$. (A–C) g–V plots of $K_{ir}6.2$ mutant channels that exhibit either a positively charged residue [H261R, (**A**)], a neutral [H261Q, (**B**)] or a negatively charged amino acid [H261R, (**C**)] at postion 216. Lines represent fit of a single Boltzmann function to the data; values for $V_{1/2}$ and V_s ($V_{1/2}$ /V_s) were: –4 mV/22 mV (pH 6.8) and –4/23 mV (pH 8.0) for H216Q and –24/23 mV (pH 6.8) and –50/20 mV (pH 8.0) for H261E. Note that for H216E channels SPM block was incomplete. (**D**) Electrical distance determined for the SPM block with the channels indicated at pH_i of 6.8 and 8.0. Data are mean ± SD of 4–7 experiments; δ-values for H216K/R were estimated to <5% and marked by asterisks.

model, the pH_i modulated binding site for SPM is located at ~28% of the electrical field.

Molecular mechanism for pH_i-dependent polyamine rectification

Systematic mutagenesis uncovered H216 in the C-terminus of $K_{ir}6.2$ as the site of protonation. When H216 is mutated to positively charged residues SPM block is very weak similar to that with low pH_i in WT channels. Neutral residues at position 216 produce strong SPM-mediated rectification as seen with WT channels at high pH_i. Furthermore, when position 216 is mutated to a negatively charged residue, a substantial increase in SPM affinity is seen without changes in the voltage-dependence of block. These findings indicate direct electrostatic interaction between the residue at position 216 and positively charged polyamines and place H216 in or very close to the polyamine-binding site. Accordingly, protonation of H216 electrostatically repels polyamines from their blocking site in the pore, thereby linking polyamine affinity to the pH_i dependent charge of H216 (see cartoon in Figure 7).

Structural implications and relationship to previous work on other K_{ir} channels

Given the fact that H216 is protonated and interacts with blocking particles in the pore it seems likely that the sidechain of this residue lines the pore. The fact that pH_i does not affect K^+ permeation suggests that H216 may not be located in the part of the pore involved in ion selectivity and high-throughput permeation, but rather in the wider entrance of the pore. This is consistent with the estimated electrical distance of ~30%.

For $K_{ir}2.1$ (IRK1) channels a C-terminal residue is known to affect pore-block by SPM (Taglialatela *et al*., 1995; Yang *et al*., 1995). When glutamate 224 in $K_{ir}2.1$ was changed to neutral amino acids SPM block was considerably reduced. The homologous residue in $K_{ir}6.2$ is serine 212 a few residues N-terminal to H216. Mutation of S212 to aspartate (S212D) resulted in high affinity for SPM very similar to that seen in H216E (data not shown) suggesting that indeed the region around 216 forms part of the polyamine-binding site in K_{ATP} channels.

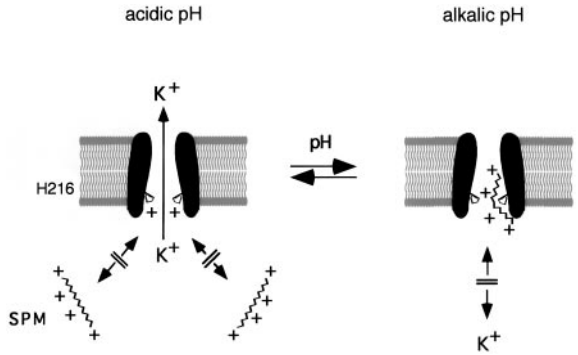

Fig. 7. Model for pH_i-dependent SPM block of K_{ATP} channels. At acidic pH_i protonation of H216 prevents binding of SPM by electrostatic repulsion, while permeation of K^+ ions is not affected. Alkalinization deprotonates H216, permits binding of polyamines to their binding site and thereby blocks the pore in a voltage-dependent manner.

Possible physiological implications

As mentioned above, polyamine-mediated rectification effectively determines cellular excitability by limiting the outward current through K_{ir} channels at potentials positive

to the resting potential (Fakler *et al*., 1995; Oliver *et al*., 1998). The question arises whether the pH-dependent changes in polyamine rectification described in K_{ATP} channels may serve as a tool to regulate excitabilty under physiological conditions.

The free concentrations of SPM and SPD have been estimated to range from 10–200 μM depending on the type of cell (Watanabe *et al*., 1991; Fakler *et al*., 1995) and thus resides in the concentration range for which substantial polyamine block of K_{ATP} channels was observed (Figures 1 and 2).

K_{ATP} channels (K_{ir} 6.2 + SUR1/SUR2A/SUR2B) are expressed in many excitable cells such as cardiac myocytes, skeletal muscle cells, smooth muscle cells, neurons and pancreatic β cells (Ashcroft, 1988; Inagaki *et al*., 1995; Inagaki *et al*., 1996; Aguilar-Bryan *et al*., 1998). The intracellular pH is supposed to be relatively constant at 7.2 and to vary by ~0.1 pH units. Although these changes in pH_i will only lead to small changes in polyamine block, they might be relevant given the fact, that even small changes in K_{ATP}-mediated conductance were reported to be effective in shortening the AP duration (Findlay, 1994). Moreover, changes in pH_i occurring underneath the membrane might be considerably larger than the changes measured in overall pH_i (Thomas and Meech, 1982; Lyall and Biber, 1994).

More importantly, there are many studies reporting substantial variations in pH_i under certain circumstances. For cardiac myocytes it is well established that pH_i may decrease during hypoxia or ischemia by more than one pH unit (Bond *et al*., 1991). In skeletal muscle (Pan *et al*., 1988) and smooth muscle (Harrison *et al*., 1994), pH_i may drop substantially during exhaustive exercise. Also for neurons, sustained activity has been reported to cause intracellular acidification (Thomas and Meech, 1982; Chesler and Kaila, 1992). In all these circumstances, intracellular acidification results from metabolic stress exceeding the compensatory potential of the cell. In these cells a decrease in pH_i would release the polyamine block of K_{ATP} permitting larger outward currents, which in turn should inhibit electrical activity and help protect the cell from energy depletion. Interestingly, it has been reported that glucose metabolism in pancreatic β cells may cause intracellular alkalinization (Lindström and Sehlin, 1984, 1986; Juntti-Berggren *et al*., 1991) which would increase polyamine rectification and thereby facilitate bursting activity and insulin secretion. Taken together, pH_i-dependence of polyamine rectification might add to the well established mechanisms that link K_{ATP} activity to cell metabolism.

Materials and methods

Mutagenesis and cRNA synthesis

Murine K_{ir}6.2 or the truncated isoform ($K_{ir}\Delta C26$) (DDBJ/EMBL/GenBank accession No. D50581; Inagaki *et al*., 1995; Sakura *et al*., 1995; Tucker *et al*., 1997) and rat SUR1 (DDBJ/EMBL/GenBank accession No. L40624; Aguilar-Bryan *et al*., 1995) were used in this study. Site-directed mutagenesis of K_{ir}6.2 was carried out by subcloning the appropriate fragments into the pALTER vector and use of the Altered Sites II protocol (Promega, Madison, WI). For oocyte expression constructs were subcloned into the pBF expression vector (B.Fakler, unpublished data) which provides the 5′ and 3′ untranslated regions of the *Xenopus* β-globin gene. Capped cRNAs specific for SUR1 as well as for K_{ir}6.2 WT and mutant subunits were synthesized *in vitro* using SP6 polymerase (Promega, Heidelberg, Germany) and stored in stock solutions at –70°C.

Preparation and injection of oocytes

Xenopus oocytes were surgically removed from adult females and manually dissected. About 50 nl of a solution containing cRNA specific for SUR1, K_{ir}6.2 WT and K_{ir}6.2 mutant subunits was injected into Dumont stage VI oocytes. Oocytes were treated with collagenase type II (Sigma, 0.5 mg/ml) and incubated at 19°C for 1–3 days prior to use.

Isolation of mouse myocytes

Adult white mice were sacrificed by cervical dislocation. After mounting the heart on a Langendorff apparatus, the organ was perfused for 5 min with a nominally Ca^{2+}-free Tyrode solution containing (mM) 140 NaCl, 5.8 KCl, 0.5 KH_2PO_4, 0.4 Na_2HPO_4, 0.9 $MgSO_4$, 11.1 glucose, 10 HEPES (pH 7.1 with NaOH). Perfusion was continued for 30 min with an enzyme solution prepared from Ca^{2+}-free Tyrode solution by adding 200 mg/l collagenase (type CLS II, Biochrom KG, Berlin, Germany) and defined amounts of Ca^{2+}. The Ca^{2+} concentration was increased in intervals of 5 min by 20 μM to the final concentration of 100 μM. Digestion was stopped by perfusing the hearts for 3 min with KB medium. This medium contained (mM): 50 glutamic acid, 20 HEPES, 20 taurine, 10 glucose, 3 $MgSO_4$, 0.5 EGTA, 30 KCl, 30 KH_2PO_4 pH 7.3 (KOH). In this solution the cells were stored for experimental use. During all perfusion steps the temperature was maintained at 37°C.

Electrophysiology

Giant and macro patch recordings (Fakler *et al*., 1995) in inside-out configuration under voltage-clamp conditions were made at room temperature (~23°C) either on oocytes 3–7 days after injection or on cardiomyocytes 1–8 h after isolation. Pipettes used were made from thick-walled borosilicate glass, had resistances of 0.3–1 MΩ (tip diameter of 5–30 μm) and were filled with (in mM, pH adjusted to 7.4 with KOH) 120 KCl, 10 HEPES and 1.8 $CaCl_2$ (for oocyte recordings) and 155 KCl, 10 HEPES, 10 EGTA, 1 $CaCl_2$ or 5 KCl, 115 NaCl, 10 HEPES, 10 EGTA, 1 $CaCl_2$ (for recordings from cardiomyocytes). Currents recorded in response to voltage ramps of 3 s (–120 to +100 mV) were sampled at 1 kHz with an EPC9 amplifier (HEKA electronics, Lamprecht, Germany), with an analog filter set to 3 kHz (–3 dB) or with a Axopatch 200B amplifier. Voltage was recorded simultaneously at the same rate. SPM-free solution was applied to the cytoplasmic side of excised patches via a multi-barrel pipette and had the following composition: K-Int $_{0\ Mg}$ (in mM, 100 KCl, 10 HEPES, 10 K_2EGTA), total K^+ was 120 mM. pH was adjusted to 8.8 with KOH and subsequently titrated to the pH values indicated with HCl. SPM, SPD and TEA (Sigma, St Louis, MO) were added to K-Int $_{0\ Mg}$ to yield the final concentrations indicated. Mg^{2+} was added to K-Int solution were EGTA was replaced by KCl, to avoid pH_i-dependent changes of the free Mg^{2+} concentration.

Data evaluation

For determination of g–V plots, the current recorded in response to voltage-ramps was divided by the corresponding driving force ($V–E_K$) to yield the respective chord conductance ($E_K = 0$ in symmetrical K^+ solution). The resulting conductance determined in the presence of SPM was normalized with respect to the control conductance (determined in the absence of the blocker) and groups of 100 adjacent current and voltage points were averaged to yield 30 data points for the final g–V. For the conductance value at E_K, which is not defined as chord conductance, a slope-conductance value was calculated from a mono-exponential fit to the neighbouring data points. g–V plots were fitted with a single Boltzmann function by means of a least squares fit (IGOR, WaveMetrics). Electrical distance was calculated according to a Woodhull model (Woodhull, 1973) as $\delta = RT / (zF \cdot V_s)$, with V_s the voltage required for an e-fold change in conductance, z the valence of the blocker (+3 and +4 for SPD and SPM, respectively); R, T and F have their usual meaning.

Computational work was carried out on Macintosh PowerPC 7600/132 Mhz using commercial software (IGOR, WaveMetrics) for fitting.

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