Title:
Differential sensitivity of Kir2 inward rectifier potassium channels to a mitochondrial uncoupler: identification of a regulatory site*.

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Running title:
Regulation of \( K_{ir} \)2 channels

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non-standard abbreviations used in the paper
FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone
PIP\(_2\), phosphatidylinositol 4,5-bisphosphate
Abstract

The aim of this study was to gain insight into the mechanism by which members of the K_{ir} subfamily are differentially sensitive to agents that inhibit mitochondrial function by identifying responsible site(s) in K_{ir} proteins. K_{ir} channels were expressed in *Xenopus* oocytes and assayed by two-electrode voltage-clamp and patch-clamp. Incubation of oocytes in carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, inhibited K_{ir}2.2 and K_{ir}2.3, but not K_{ir}2.1. Replacement of the first 44 amino acids of K_{ir}2.2 or the first 19 of K_{ir}2.3 with the first 45 of K_{ir}2.1 did not affect the sensitivity of the channels to FCCP. Contrastingly, a larger substitution of K_{ir}2.1 N-terminal sequence (1-78) into K_{ir}2.2 or K_{ir}2.3 produced channels that were resistant to FCCP. Sequence alignment between residues 46 and 78 (K_{ir}2.1 numbering) revealed four residues that are the same in K_{ir}2.2 and K_{ir}2.3 but different in K_{ir}2.1. Each of these four residues in the resistant chimera was converted back to the K_{ir}2.2/K_{ir}2.3 amino acid. Three of the mutants (D51N, I59A, G65S) were not sensitive to FCCP, but the H53Q mutant was sensitive. K_{ir}2.1-H53A and K_{ir}2.1-H53E were also sensitive. In contrast, K_{ir}2.1-H53R and K_{ir}2.1-H53K were resistant. K_{ir}2.2 and K_{ir}2.3 currents recovered during perfusion of inside-out patches from FCCP-treated oocytes. FCCP was without effect on K_{ir}2.2 and K_{ir}2.3 when applied directly to inside-out patches. Together, these results suggest inhibition of K_{ir}2.2 and K_{ir}2.3 by a ligand that bears a positive charge and is produced by an intracellular action of FCCP.
Mitochondrial dysfunction is increasingly recognized as an important aspect of the pathophysiology of several neurological and cardiovascular disorders, including Alzheimer’s disease (Baloyannis et al., 2004), Parkinson’s disease (Fiskum et al., 2003), heart failure (Marin-Garcia et al., 2001), myocardial and cerebral ischemia (Sadek et al., 2003; Sims and Anderson, 2002) and potentially, epilepsy (Patel, 2002). Alteration of electrical excitability is central to many of these diseases, leading to either neuronal excitotoxicity or cardiac arrhythmia (Doble, 1999; Janse, 2004). Inward rectifier K$^+$ channels play a special role in controlling membrane excitability, so inhibition of their activity would be expected to have a proexcitotoxic or proarrhythmic effect. Indeed, the reduction of cardiac inward rectifier activity seen in heart failure (Beuckelmann et al., 1993; Han et al., 2001; Kaab et al., 1996; Lodge and Normandin, 1997) contributes to the increased risk of arrhythmogenic triggered activity arising from afterdepolarizations (Pogwizd et al., 2001).

We previously investigated the possibility that inward rectifier K$^+$ channels provide a link between mitochondrial dysfunction and membrane excitability, and found that members of the K$_{ir}$2 subfamily are differentially sensitive to agents that inhibit mitochondrial function (Collins and Larson, 2002). The aim of this study was to gain some insight into the nature of the mechanism by identifying the site(s) in the K$_{ir}$2 proteins that are responsible for this differential sensitivity.
The membrane topology and subunit stoichiometry originally proposed by Ho et al. (Ho et al., 1993) and Kubo et al. (Kubo et al., 1993) for inward rectifier K⁺ channels is now well accepted, especially in light of the recent determination of the crystal structure of an inward rectifier-type channel protein from a prokaryote (Kuo et al., 2003). Thus, inward rectifier channels are composed of four subunits, each of which has two transmembrane α-helices (M1 and M2) and an extracellular reentrant helix-loop (H5) that forms the selectivity filter. The amino and carboxyl termini are both intracellular. Here we show that the sensitivity of Kᵢₑ₂ channels to a mitochondrial uncoupler depends on the charge of a specific residue in the N-terminal domain, and provide evidence for the role of a ligand that bears a positive charge.
Materials and Methods

Subcloning, mutagenesis and in vitro transcription. Complementary DNAs encoding \( K_{ir}2.1 \) (IRK1) (Kubo et al., 1993), \( K_{ir}2.2 \) (MB-IRK2) (Takahashi et al., 1994) and \( K_{ir}2.3 \) (MB-IRK3) (Kurachi and Takahashi, 1996) were subcloned into the *Xenopus* expression vector pGEMHE (Liman et al., 1992). Point mutants and novel restriction sites were introduced by the QuikChange method (Stratagene, La Jolla, CA). Chimeras were constructed by first introducing a unique silent restriction site into the \( K_{ir}2.1 \) coding sequence, and at the equivalent site in the \( K_{ir}2.2 \) or \( K_{ir}2.3 \) coding sequence. These plasmids were then digested with the appropriate restriction enzyme and BamHI (5' to the coding sequence) or NheI (3' to the coding sequence). The resulting DNA fragments were separated by agarose gel electrophoresis and purified with the MiniElute Gel Extraction Kit (Qiagen, Valencia, CA). Fragments were then ligated using the Fast-Link kit (Epicentre, Madison, WI) to form plasmids containing the novel chimeras. All chimeras and point mutants were confirmed by DNA sequencing (Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University). Plasmids were linearized with NheI, and cRNA was transcribed *in vitro* with T7 RNA polymerase (mMessage mMachines, Ambion, Austin, TX). RNA yield and integrity was assessed by agarose-ethidium bromide gel electrophoresis.

Oocyte isolation. Stage V-VI oocytes were surgically removed from *Xenopus laevis* frogs (Nasco) under anesthesia (0.03% benzocaine for 10-15 minutes) and incubated with 1 mg/ml collagenase (Worthington, type CLS3) for 2 hours at 22° C in 96 mM
NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.4, with agitation, to remove connective tissue. Oocytes were then washed several times with 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, pH 7.4 (ND-96) and stored in the same solution at 18° C. Both solutions also contained 100 µg/ml streptomycin and 60 µg/ml ampicillin. Oocytes were injected up to 24 hours later (Nanoliter; World Precision Instruments, Sarasota, FL) with 50 nl nuclease-free water containing amounts of RNA that gave similar expression levels.

**Two-electrode voltage clamp.** Inward rectifier currents were recorded 1-2 days after injection, using a TEC-03 amplifier (npi, Tamm, Germany) controlled by Pulse 8.4 software (Heka, Southboro, MA) via an ITC-16 computer interface (Instrutech, Long Island, NY). Currents were filtered at 500 Hz and digitized at 1 kHz. Data were analyzed using Pulse 8.4 and Prism 3.02 (GraphPad, San Diego, CA). Microelectrode pipettes were prepared from thin-walled, 1.5 mm outer diameter borosilicate glass capillaries (TW150F-3; World Precision Instruments) on a microprocessor-controlled puller (PUL-100; World Precision Instruments), and had resistances of 0.5-1.5 MΩ when filled with 3M KCl. Oocytes were placed in a 100 µl volume recording chamber that was continuously perfused at a rate of approximately 1.2 ml/min with '90K' solution: 90 mM KCl/KOH, 3 mM MgCl₂, 5 mM HEPES, pH 7.4.

**Patch-clamp.** Inward rectifier currents were recorded in 'giant' cell-attached and inside-out patches from *Xenopus* oocytes (Hilgemann, 1995), 2-4 days after injection with Kᵢ₂.
cRNA. The patch-clamp amplifier was an Axopatch 200B (Axon Instruments, Union City, CA). Currents were filtered at 1 kHz, and data were acquired at 5 kHz with a Digidata 1320A computer interface and pClamp 8 software (Axon Instruments). Data were analyzed using pClamp 8 and Prism 3.02. Patch pipettes had inner tip diameters of 20-25 μm. The composition of recording solutions is given in the figure legends.

**Materials**

Restriction enzymes were purchased from MBI Fermentas (Hanover, MD) or New England Biolabs (Beverly, MA). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP; ICN, Costa Mesa, CA) was dissolved in dimethylsulfoxide (DMSO) to 100 mM. This stock solution was diluted into experimental solutions as indicated in figure legends. ATP (dipotassium salt) was purchased from ICN Biomedicals (Aurora, OH).

Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) was purchased from EMD Biosciences (San Diego, CA) and prepared according to Rohacs et al. (Rohacs et al., 2002). PIP$_2$ was dispersed in water at a concentration of 1 mM, then sonicated for 25 minutes on ice in a Sonic 100W (Fisher Scientific International; Hampton, NH) at 50% power. After sonication the sample was divided into 33 μl aliquots and stored at -80°C. The suspension was injected directly into the oocyte. Thawed aliquots were used on the same day, and unused material was discarded.
Results

Figure 1A shows the effect of FCCP, a mitochondrial uncoupler (Guerrieri et al., 1976), on three different members of the K\textsubscript{i,2} inward rectifier K\textsuperscript{+} channel subfamily. Current-voltage relationships were obtained by two-electrode voltage-clamp after incubation for 90 minutes in 10 μM FCCP or in control conditions. The figure demonstrates that K\textsubscript{i,2.2} and K\textsubscript{i,2.3} were inhibited by FCCP, whereas K\textsubscript{i,2.1} was not.

Figure 1B shows the effect of FCCP on chimeric channels in which part of the N-terminal domain of K\textsubscript{i,2.2} or K\textsubscript{i,2.3} was replaced with the equivalent sequence from K\textsubscript{i,2.1}. The horizontal bars on the right of the figure represent the K\textsuperscript{+} current recorded at a membrane potential of -50 mV after control (open bars) or FCCP treatment (filled bars). Comparison of these data for 1-2chm1 and 1-3chm1 with the data for wild-type K\textsubscript{i,2.1}, K\textsubscript{i,2.2} and K\textsubscript{i,2.3} shows that replacement of the first 44 amino acids of K\textsubscript{i,2.2} or the first 19 amino acids of K\textsubscript{i,2.3} with the first 45 amino acids of K\textsubscript{i,2.1} did not affect the sensitivity of the channels to FCCP. In contrast, a larger substitution of K\textsubscript{i,2.1} N-terminal sequence (78 amino acid residues) into K\textsubscript{i,2.2} (1-2chm2) or K\textsubscript{i,2.3} (1-3chm2) produced channels that were resistant to FCCP (compare 1-2chm2 and 1-3chm2 ± FCCP data with K\textsubscript{i,2.1} ± FCCP data). Therefore, replacement of most of the N-terminal domain of K\textsubscript{i,2.2} or K\textsubscript{i,2.3} with K\textsubscript{i,2.1} sequence eliminated the sensitivity to FCCP.

The data presented in figure 1B suggested that sequence differences between residues 46 and 78 (K\textsubscript{i,2.1} numbering) affect the sensitivity of K\textsubscript{i,2} channels to FCCP.
Examination of the sequence alignment for this segment revealed four residues that are the same in K\textsubscript{ir}2.2 and K\textsubscript{ir}2.3 but different in K\textsubscript{ir}2.1 (shown in bold in the upper part of figure 2). Point mutations were made in 1-3chm2 so that one of these four residues was converted back to the K\textsubscript{ir}2.2/K\textsubscript{ir}2.3 amino acid. These four mutants are represented schematically in figure 2. The bars on the right of the figure represent the K\textsuperscript{+} current recorded at -50 mV in control conditions (open bars) or after FCCP treatment (filled bars). Three of the mutants (D51N, I59A, G65S) were not sensitive to FCCP. In contrast, the H53Q mutant, in which the histidine residue at position 53 (K\textsubscript{ir}2.1 numbering) was changed to glutamine, was inhibited by FCCP.

The results shown in figure 2 suggested that glutamine at position 53 (K\textsubscript{ir}2.1 numbering) is a specific requirement for sensitivity of K\textsubscript{ir}2 channels to FCCP, or that histidine specifically produces resistance to FCCP. Figure 3 shows that glutamine does not have a specific role in conferring sensitivity to FCCP, because mutation of histidine 53 in K\textsubscript{ir}2.1 to alanine (H53A) or glutamate (H53E) also produced FCCP-sensitive channels. In contrast, K\textsubscript{ir}2.1 mutants in which histidine 53 was changed to arginine (H53R) or lysine (H53K) retained their resistance to FCCP (figure 3).

Figure 4 shows that K\textsubscript{ir}2.2 and K\textsubscript{ir}2.3 could recover from inhibition by FCCP. These experiments were conducted in oocytes that were expressing K\textsubscript{ir}2.2 or K\textsubscript{ir}2.3, and incubated in 10 µM FCCP for 1 hour. The inward rectifier current was recorded by patch-clamp. The inverted triangles represent the current recorded at -50 mV in the cell-
attached patch configuration at the beginning of the experiment. The dashed lines represent the period of time during which the patches of membrane were excised into the inside-out configuration. (Excision of intact 'giant' patches (Hilgemann, 1995) takes longer than excision of conventional patches.) Following excision, the inside-out patches were perfused with 'FVPP' solution (see figure legend). As can be seen in the figure, $K_{ir}^{2.2}$ and $K_{ir}^{2.3}$ currents (solid lines) increased with time of perfusion. The leak current (not shown) was stable and negligible throughout.

FCCP did not inhibit $K_{ir}^{2.2}$ and $K_{ir}^{2.3}$ when applied directly to the cytoplasmic surface of membrane patches, as shown in figure 5. The figure shows superimposed current traces recorded in inside-out patches from *Xenopus* oocytes expressing $K_{ir}^{2.2}$ or $K_{ir}^{2.3}$ during perfusion with FVPP solution (control) or 10 µM FCCP in FVPP.

Figure 6 shows that the inhibition of $K_{ir}^{2.2}$ and $K_{ir}^{2.3}$ by FCCP was not attenuated by ATP or phosphatidylinositol 4,5-bisphosphate (PIP$_2$). In this experiment oocytes were pre-injected with 50 nl water, 1 mM PIP$_2$, or 50 mM ATP prior to incubation in FCCP. Comparison of the open and closed bars shows that the inhibition of $K_{ir}^{2.2}$ and $K_{ir}^{2.3}$ by FCCP after PIP$_2$ or ATP pre-injection was equivalent to the inhibition in control conditions.

According to sequence alignments, the equivalent position to histidine 53 in $K_{ir}^{2.1}$ is position 52 in $K_{ir}^{2.2}$ and position 27 in $K_{ir}^{2.3}$. To investigate whether a histidine at this
position is sufficient to confer resistance to FCCP, glutamine-to-histidine mutations were made at position 52 in Kᵢᵣ2.2 (Q52H) and position 27 in Kᵢᵣ2.3 (Q27H). Figure 7 shows that these mutants were inhibited by FCCP (compare open and filled bars).
Discussion

The data presented here show that the resistance to FCCP of $K_{ir}$2.1 in comparison to $K_{ir}$2.2 and $K_{ir}$2.3 was eliminated by changing the histidine residue at position 53 to a neutral or negatively charged residue. In contrast, resistance to FCCP was retained when the histidine was changed to arginine or lysine. Thus, the presence of a positively charged residue at position 53 is required for resistance to FCCP, rather than a requirement for histidine per se.

FCCP uncouples oxidative phosphorylation from electron transport by acting as a proton ionophore in the inner mitochondrial membrane (Guerrieri et al., 1976; Heytler and Prichard, 1962). This action depolarizes the mitochondria (Aronis et al., 2002), thereby inhibiting ATP production (Luo et al., 1997). Inward rectifier $K^+$ channels are known to be activated by PIP$_2$ (Hilgemann and Ball, 1996; Huang et al., 1998; Rohacs et al., 1999), and neutralization of histidine 53 was found to decrease $K_{ir}$2.1’s affinity for PIP$_2$ (Lopes et al., 2002). This led us to hypothesize a mechanism in which FCCP depletes ATP, leading in turn to a depletion of PIP$_2$ because of continued lipid phosphatase activity in the face of decreased phosphorylation of inositol phospholipids. Depletion of PIP$_2$ would then lead to inward rectifier $K^+$ channel inhibition. However, this hypothesis is not supported by our data, because $K_{ir}$2.2 and $K_{ir}$2.3 currents recovered from inhibition by FCCP when inside-out patches were perfused with a solution that did not contain ATP (figure 4). ATP is required for the regeneration of PIP$_2$ in inside-out patches (Hilgemann and Ball, 1996; Huang et al., 1998). Furthermore, $K_{ir}$2.1 and $K_{ir}$2.2
have similar affinities for PIP$_2$ (Du et al., 2004), and pre-injection of oocytes with PIP$_2$ or ATP did not attenuate the inhibition of K$_{ir}$2.2 or K$_{ir}$2.3 by FCCP (figure 6).

A possible explanation for the observed influence of the side-chain charge of residue 53 on the sensitivity of the channel to FCCP is that this charge alters the affinity of a binding site for an inhibitory ligand. Such a mechanism predicts that the inhibitory effect of FCCP will be reversed if the putative ligand is washed away from the intracellular surface of the membrane. The data presented in figure 4 support such a mechanism, because perfusion of inside-out membrane patches from FCCP-treated oocytes resulted in recovery of K$_{ir}$2.2 and K$_{ir}$2.3 currents. Inevitably, other residues besides residue 53 (K$_{ir}$2.1 numbering) would be involved in forming the putative regulatory site. Such residues could be variant between K$_{ir}$2.1 and K$_{ir}$2.2/K$_{ir}$2.3 because mutation of the glutamine residue at the position equivalent to K$_{ir}$2.1 His53 (residue 52 in K$_{ir}$2.2; residue 27 in K$_{ir}$2.3) to histidine was insufficient to convert K$_{ir}$2.2 and K$_{ir}$2.3 to FCCP-resistant channels (figure 7). Alternatively, FCCP could exert its effect via a second binding site on K$_{ir}$2.2 and K$_{ir}$2.3. Further mutagenesis studies will address this issue.

Based on the results presented here, we speculate that mitochondrial dysfunction in heart failure (Marin-Garcia et al., 2001) produces an inward rectifier K$^+$ channel inhibitor that is responsible for reducing the cardiac inward rectifier current (Beuckelmann et al.,
1993; Han et al., 2001; Kaab et al., 1996; Lodge and Normandin, 1997), thereby contributing to an increased risk of arrhythmia (Pogwizd et al., 2001). Furthermore, we speculate that the inhibitor bears a positive charge, because the presence of a positive charge in or near the binding site would be expected to decrease the affinity of the channel for the positively charged moiety of the inhibitory ligand. As reported previously, the inhibitory effect of FCCP cannot be entirely accounted for by intracellular acidification (Collins and Larson, 2005).

Gene knockout studies indicate that \( K_{ir}2.1 \) is an essential component of the cardiac inward rectifier current (Zaritsky et al., 2001). As shown here and previously (Collins and Larson, 2002), \( K_{ir}2.1 \) is relatively insensitive to mitochondrial inhibitors. Therefore, if the cardiac inward rectifier channels were all \( K_{ir}2.1 \) homomultimers, it would be less likely that the inhibition of inward rectifier activity in heart failure was because of mitochondrial dysfunction. However, evidence in the literature suggests that a significant proportion of cardiac inward rectifiers are \( K_{ir}2.1-K_{ir}2.2 \) or \( K_{ir}2.1-K_{ir}2.3 \) heteromultimers (Preisig Muller et al., 2002; Zaritsky et al., 2001; Zobel et al., 2003), which are sensitive to FCCP (Collins and Larson, 2005). \( K_{ir}2.1 \), \( K_{ir}2.2 \) and \( K_{ir}2.3 \) are all transcribed in human heart (Wang et al., 1998), so it is likely that a significant proportion of inward rectifier \( K^+ \) channels in human cardiac myocytes are \( K_{ir}2.1-K_{ir}2.2 \) and/or \( K_{ir}2.1-K_{ir}2.3 \) heteromultimers that may be sensitive to mitochondrial dysfunction.
Several neurological disorders are characterized by neuronal excitotoxicity and mitochondrial dysfunction (Baloyannis et al., 2004; Doble, 1999; Fiskum et al., 2003; Patel, 2002; Sims and Anderson, 2002). Albeit the importance of inward rectifier K\(^+\) channels relative to other types of K\(^+\) channel for the control of neuronal excitability is unclear, their widespread expression in the brain (Falk et al., 1995; Horio et al., 1996; Karschin et al., 1996; Morishige et al., 1993) raises the possibility of an important role for them in the pathophysiology of these diseases.
Acknowledgments

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References


Footnotes

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Figure 1. Effect of FCCP on $K_{ir2}$ channels and chimeras expressed in *Xenopus* oocytes. Inward rectifier $K^+$ currents were recorded by two-electrode voltage clamp after incubation in ND-96 (control) or for 90 minutes in ND-96 with 10 µM FCCP. **A:** Current-voltage relationships recorded from oocytes expressing $K_{ir2.1}$ (squares), $K_{ir2.2}$ (triangles) or $K_{ir2.3}$ (circles), in control conditions (open symbols) or after FCCP treatment (filled symbols). Data were normalized to the current recorded at a membrane potential of -50 mV. Inward current is presented as negative. Data are mean ± s.e.m., n = 6. Error bars smaller than the size of the symbol are not shown. **B:** Schematic representations of wild-type and chimeric $K_{ir2}$ channel sequences (left) and their sensitivity to FCCP (right). 'N' indicates the amino terminal of the sequence; 'C' represents the carboxyl terminal. Filled boxes labeled 'M1', 'M2' and 'H5' represent the first and second transmembrane alpha helices and the re-entrant pore helix-loop, respectively. White and black fills correspond to $K_{ir2.1}$ sequence, dark grey and diagonal correspond to $K_{ir2.2}$ sequence, and light grey and cross-hatch correspond to $K_{ir2.3}$ sequence. Bars on the right of the figure represent inward rectifier current recorded at -50 mV membrane potential in control conditions (open bars) or after FCCP treatment (filled bars). Bar length is mean current (n = 6); error bars are s.e.m. The calibration line represents 5 µA. "***" represents p <.001 for control vs FCCP by t-test.
Figure 2. Effect of point mutations on FCCP sensitivity. **Top:** Alignment of \( \text{K}_{\text{ir}}2.1 \), \( \text{K}_{\text{ir}}2.2 \) and \( \text{K}_{\text{ir}}2.3 \) sequences from residue 46 to residue 78 \((\text{K}_{\text{ir}}2.1 \text{ numbering})\). Positions of point mutations are shown in bold. **Bottom, left:** Schematic representations of point mutants in the 1-3chm2 chimera, which has \( \text{K}_{\text{ir}}2.1 \) sequence from residues 1-78 and \( \text{K}_{\text{ir}}2.3 \) sequence thereafter. **Bottom, right:** Data obtained and represented as in figure 1.

Figure 3. Sensitivity of \( \text{K}_{\text{ir}}2.1 \) to FCCP is influenced by the charge on residue 53. Residue 53 in \( \text{K}_{\text{ir}}2.1 \) was mutated to glutamine (H53Q), alanine (H53A), arginine (H53R), lysine (H53K) or glutamate (H53E). Mutant channels were expressed in *Xenopus* oocytes and inward rectifier current was recorded by two-electrode voltage-clamp after incubation in ND-96 (control; open bars) or for 90 minutes in ND-96 with 10 \( \mu \text{M} \) FCCP (filled bars). Data are currents recorded at -50 mV, normalized to the mean control current for each mutant. **p < .01; ***p < .001 by t-test \((n = 6)\).

Figure 4. Recovery of \( \text{K}_{\text{ir}}2.2 \) and \( \text{K}_{\text{ir}}2.3 \) in inside-out patches. *Xenopus* oocytes expressing \( \text{K}_{\text{ir}}2.2 \) or \( \text{K}_{\text{ir}}2.3 \) were treated with 10 \( \mu \text{M} \) FCCP in ND-96 for 1 hour, then inward rectifier currents were recorded by patch-clamp. The same 'FVPP' type solution was used in the recording dish and in patch pipettes, and for perfusing inside-out patches: \((\text{mM})\) 40 KCl, 75 K-gluconate, 5 KF, 0.1 NaVO\(_3\), 10 K-pyrophosphate, 1 EGTA, 0.1 spermine, 10 HEPES, pH 7.4. Traces represent inward currents recorded at -50mV membrane potential. Leak current was monitored at 50 mV, and was stable and
negligible throughout. The inverted triangles indicate the cell-attached configuration. The dashed lines represent the periods during which membrane patches were excised into the inside-out configuration. Data were not acquired during these periods. The solid lines are currents recorded during perfusion of inside-out patches with FVPP. The voltage error because of series resistance was approximately 2 mV at the end of the K\textsubscript{ir}2.2 experiment (electrode resistance: 830 kOhm), and approximately 0.5 mV at the end of the K\textsubscript{ir}2.3 experiment (electrode resistance: 780 kOhm).

Figure 5. No direct inhibition of FCCP on K\textsubscript{ir}2.2 or K\textsubscript{ir}2.3. Inward rectifier currents were recorded by patch-clamp in *Xenopus* oocytes expressing K\textsubscript{ir}2.2 or K\textsubscript{ir}2.3. The same 'FVPP' type solution was used in the recording dish and in patch pipettes, and for perfusing inside-out patches: (mM) 40 KCl, 75 K-gluconate, 5 KF, 0.1 NaVO\textsubscript{3}, 10 Na-pyrophosphate, 1 EGTA, 10 glucose, 0.1 spermine, 10 PIPES, pH 7.4. Traces represent currents recorded in inside-out patches from oocytes expressing K\textsubscript{ir}2.2 (A) and K\textsubscript{ir}2.3 (B) while applying the voltage-clamp protocol shown in C, before (control) and during application of 10 µM FCCP. The slight increase in inward current was seen in control conditions and was probably not caused by FCCP. The voltage error because of series resistance was approximately 1 mV in A (electrode resistance: 800 kOhm), and approximately 5 mV in B (electrode resistance: 640 kOhm).

Figure 6. No effect of ATP or PIP\textsubscript{2} on the inhibition of K\textsubscript{ir}2.2 and K\textsubscript{ir}2.3 by FCCP. *Xenopus* oocytes expressing K\textsubscript{ir}2.2 (A) or K\textsubscript{ir}2.3 (B) were injected with 50 nl water
(columns 1-4), 1 mM PIP$_2$ (columns 5 and 6), or 50 mM ATP (columns 7 and 8), incubated in ND-96 for 60 minutes (ATP) or 30-60 minutes (PIP$_2$), then incubated for a further 90 minutes in ND-96 (control; open bars) or ND-96 with 10 µM FCCP (filled bars). Inward rectifier currents were then recorded in 90K by two-electrode voltage clamp. Data are currents recorded at -50 mV, normalized to the mean control current. ***p <.001 by t-test (n = 5-10).

Figure 7. A histidine residue at position 53 (K$_{ir}$2.1 numbering) is not sufficient to make K$_{ir}$2.2 or K$_{ir}$2.3 resistant to FCCP. The glutamine residue at the position equivalent to K$_{ir}$2.1 residue 53 was mutated to histidine in K$_{ir}$2.2 (position 52) and K$_{ir}$2.3 (position 27). These constructs were expressed in Xenopus oocytes, which were then incubated for 90 minutes in ND-96 with 10 µM FCCP (filled bars) or ND-96 (control; open bars). Inward rectifier currents were then recorded in 90K by two-electrode voltage clamp. Data are currents recorded at -50 mV, normalized to the mean control current. **p <.01; ***p <.001 by t-test (n = 6).
Figure 1
Figure 2

K_{L2.1} RFVKKDGKHCVQFINVGETKQRYLADIFTTCVD
K_{L2.2} RFVKKNGQCNIEFANMDEKSRQYMADIFTTCVD
K_{L2.3} RFVKKNGQCNVYPFANLSNKSQRYMADIFTTCVD

D51N

H53Q

I59A

G65S

K_{M2.1} K_{M2.3}
Figure 3

**K_i**2.1 H53 mutants

\[ \frac{I}{I_{control}}, \text{mean} \]

<table>
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<tr>
<th>H53Q</th>
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Figure 3
Figure 4

\begin{align*}
K_{ir2.2} & \quad \text{FCCP-treated} \\
FCCP & \quad \text{treated} \\
K_{ir2.3} & \quad \text{FCCP-treated} \\
\text{FCCP} & \quad \text{treated}
\end{align*}
Figure 5

A. 

\[ K_{ir2.2} \]

- 1 nA
- 50 msec
- control
- FCCP

B. 

\[ K_{ir2.3} \]

- 5 nA
- 50 msec
- control
- FCCP

C. 

Voltage clamp

- 50 mV
- -50 mV

Figure 5
Figure 6
Figure 7

The diagram shows a graph comparing the relative activity of different protein variants under various conditions.

- **K<sub>i</sub>2.2 Q52H**
  - Without FCCP (-):
  - With FCCP (+):
  - Significance: ***

- **K<sub>i</sub>2.3 Q27H**
  - Without FCCP (-):
  - With FCCP (+):
  - Significance: **