0026-895X/05/6704-1214–1220$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 67:1214–1220, 2005

Vol. 67, No. 4
8292/1197658
Printed in U.S.A.

Differential Sensitivity of $K_{ir}2$ Inward-Rectifier Potassium Channels to a Mitochondrial Uncoupler: Identification of a Regulatory Site

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Received October 14, 2004; accepted January 3, 2005

ABSTRACT

The aim of this study was to gain insight into the mechanism by which members of the $K_{ir}2$ subfamily are differentially sensitive to agents that inhibit mitochondrial function by identifying responsible site(s) in $K_{ir}2$ proteins. $K_{ir}2$ channels were expressed in *Xenopus laevis* 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. In contrast, 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, and 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 (Sims and Anderson, 2002; Sadek et al., 2003), 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 the 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; Kaab et al., 1996; Lodge and Normandin, 1997; Han et al., 2001) contributes to the increased risk of arrhythmogenic triggered activity arising from afterdepolarizations (Pogwizd et al., 2001).

We investigated previously 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. (1993) and 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 $K_{ir}2$ channels to a mitochondrial uncoupler depends on the charge of a specific residue in the

This work was supported by an award from the American Heart Association.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.008292.

ABBREVIATIONS: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, PIP_2, phosphatidylinositol 4,5-bisphosphate; PIPES, 1,4-piperazinediethanesulfonic acid.

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 laevis* 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 (Epicenter Technologies, 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, Corvallis, OR). Plasmids were linearized with NheI, and cRNA was transcribed in vitro with T7 RNA polymerase (mMessage mMachine; Ambion, Austin, TX). RNA yield and integrity were assessed by agarose-ethidium bromide gel electrophoresis.

Oocyte Isolation. Stage V to VI oocytes were surgically removed from *X. laevis* frogs (Nasco, Fort Atkinson, WI) under anesthesia (0.03% benzocaine for 10–15 min) and incubated with 1 mg/ml collagenase (type CLS3; Worthington Biochemicals, Freehold, NJ) for 2 h at 22°C in 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 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 $MgCl_2$, 1.8 mM $CaCl_2$, and 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 h later (Nanoliter; World Precision Instruments, Sarasota, FL) with 50 nl of nuclease-free water containing amounts of RNA that gave similar expression levels.

Two-Electrode Voltage Clamp. Inward-rectifier currents were recorded 1 to 2 days after injection using a TEC-03 amplifier (NPI Electronic GmbH, Tamm, Germany) controlled by Pulse 8.4 software (Heka, Southboro, MA) via an ITC-16 computer interface (InstruTECH Corporation, Port Washington, NY). Currents were filtered at 500 Hz and digitized at 1 kHz. Data were analyzed using Pulse 8.4 and Prism 3.02 (GraphPad Software Inc., 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 to 1.5 MΩ when filled with 3 M 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 $MgCl_2$, and 5 mM HEPES, pH 7.4).

Patch Clamp. Inward-rectifier currents were recorded in giant cell-attached and inside-out patches from *X. laevis* oocytes (Hilgemann, 1995) 2 to 4 days after injection with $K_{ir}2$ cRNA. The patch-clamp amplifier was an Axopatch 200B (Axon Instruments Inc., 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 to 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; MP Biomedicals, Irvine, CA) was dissolved in dimethyl sulfoxide to 100 mM. This stock solution was diluted into experimental solutions as indicated in the figure legends. ATP (dipotassium salt) was purchased from MP Biomedicals. Phosphatidylinositol 4,5-bisphosphate (PIP_2) was purchased from EMD Biosciences (San Diego, CA) and prepared according to the methods of Rohacs et al. (2002). PIP_2 was dispersed in water at a concentration of 1 mM and then sonicated for 25 min 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

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

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

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

The results shown in Fig. 2 suggested that glutamine at position 53 ($K_{ir}2.1$ numbering) is a specific requirement for sensitivity of $K_{ir}2$ 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 the mutation of histidine 53 in $K_{ir}2.1$ to alanine (H53A) or glutamate (H53E) also produced FCCP-

sensitive channels. In contrast, $K_{ir}2.1$ mutants in which histidine 53 was changed to arginine (H53R) or lysine (H53K) retained their resistance to FCCP (Fig. 3).

Figure 4 shows that $K_{ir}2.2$ and $K_{ir}2.3$ could recover from inhibition by FCCP. These experiments were conducted in oocytes that were expressing $K_{ir}2.2$ or $K_{ir}2.3$ and were incubated in 10 μM FCCP for 1 h. 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

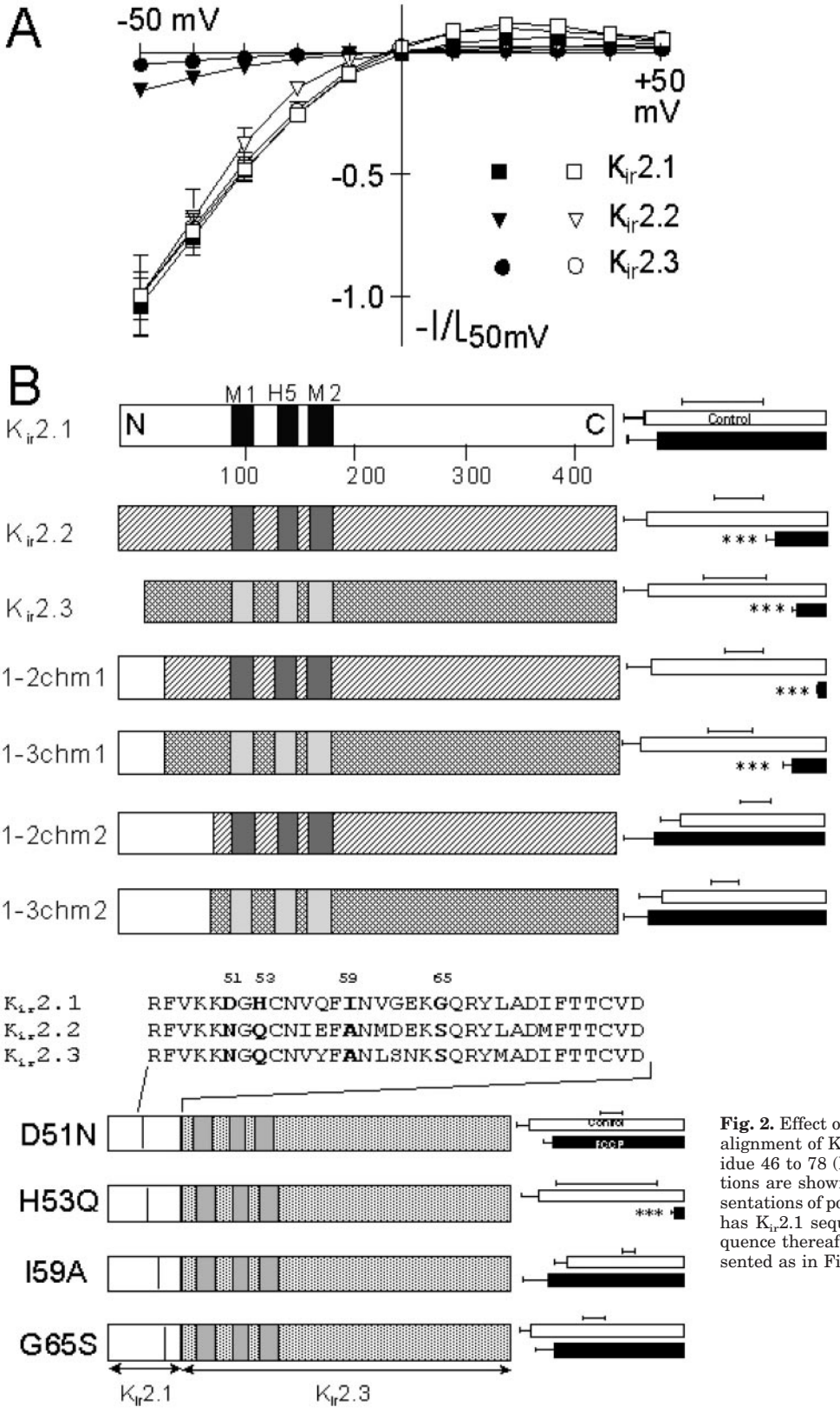

Fig. 1. Effect of FCCP on $K_{ir}2$ channels and chimeras expressed in *X. laevis* oocytes. Inward-rectifier K^+ currents were recorded by two-electrode voltage clamp after incubation in ND-96 (control) or for 90 min in ND-96 with 10 μM FCCP. A, current-voltage relationships recorded from oocytes expressing $K_{ir}2.1$ (squares), $K_{ir}2.2$ (triangles), or $K_{ir}2.3$ (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 $K_{ir}2$ channel sequences (left) and their sensitivity to FCCP (right). N, amino terminal of the sequence; C, carboxyl terminal. M1, M2, and H5 represent the first and second transmembrane α helices and the re-entrant pore helix-loop, respectively. White and black correspond to $K_{ir}2.1$ sequence, dark gray and diagonal correspond to $K_{ir}2.2$ sequence, and light gray and crosshatch correspond to $K_{ir}2.3$ sequence. Right, bars represent inward-rectifier current recorded at −50 mV membrane potential in control conditions (□) or after FCCP treatment (■). Bar length is mean current ($n = 6$); error bars represent S.E.M. The calibration line represents 5 μA. ***, $p < 0.001$ for control versus FCCP by t test.

Fig. 2. Effect of point mutations on FCCP sensitivity. Top, alignment of $K_{ir}2.1$, $K_{ir}2.2$, and $K_{ir}2.3$ sequences from residue 46 to 78 ($K_{ir}2.1$ numbering). Positions of point mutations are shown in boldface. Bottom left, schematic representations of point mutants in the 1–3chm2 chimera, which has $K_{ir}2.1$ sequence from residues 1 to 78 and $K_{ir}2.3$ sequence thereafter. Bottom right, data obtained and represented as in Fig. 1.

broken lines represent the period of time during which the patches of membrane were excised into the inside-out configuration. The excision of intact "giant" patches (Hilgemann, 1995) takes longer than the excision of conventional patches. After excision, the inside-out patches were perfused with FVPP solution (40 mM KCl, 75 mM potassium gluconate, 5 mM potassium fluoride, 0.1 mM sodium vanadate, 10 mM potassium pyrophosphate, 1 mM EGTA, 0.2 mM ADP, 10 mM HEPES, 10 mM glucose, and 0.1 spermine, pH 7.4) (see Fig. 4 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 (data 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 Fig. 5. The figure shows superimposed current traces recorded in inside-out patches from *X. laevis* 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 PIP_2. In this experiment, oocytes were preinjected with 50 nl of water, 1 mM PIP_2, or 50 mM ATP before 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 preinjection 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-

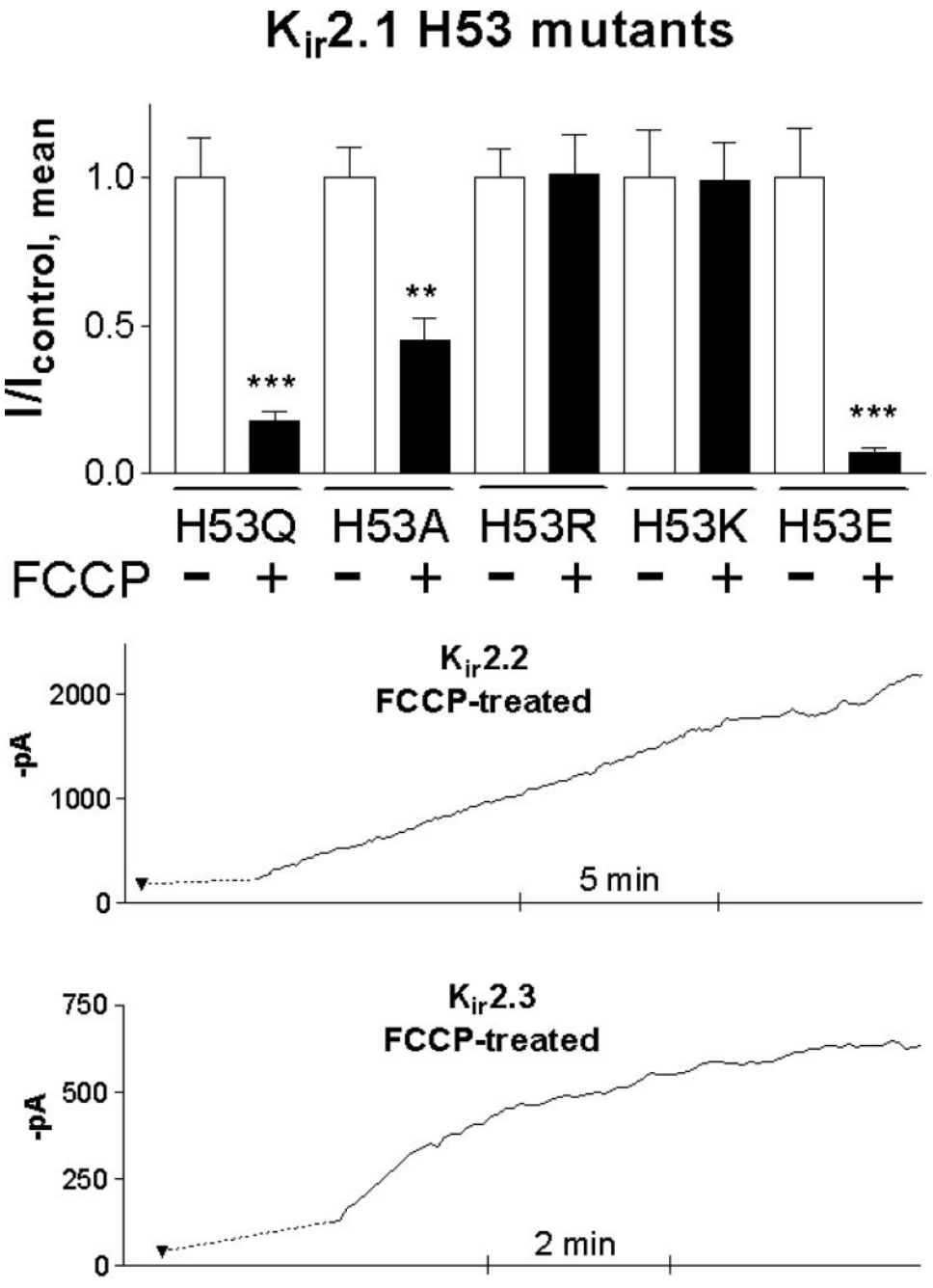

Fig. 3. Sensitivity of $K_{ir}2.1$ to FCCP is influenced by the charge on residue 53. Residue 53 in $K_{ir}2.1$ was mutated to glutamine (H53Q), alanine (H53A), arginine (H53R), lysine (H53K), or glutamate (H53E). Mutant channels were expressed in *X. laevis* oocytes and inward-rectifier current was recorded by two-electrode voltage clamp after incubation in ND-96 (control; □) or for 90 min in ND-96 with 10 μM FCCP (■). Data are currents recorded at −50 mV, normalized to the mean control current for each mutant. ⋆⋆, $p < 0.01$; ⋆⋆⋆, $p < 0.001$ by t test ($n = 6$).

Fig. 4. Recovery of $K_{ir}2.2$ and $K_{ir}2.3$ in inside-out patches. *X. laevis* oocytes expressing $K_{ir}2.2$ or $K_{ir}2.3$ were treated with 10 μM FCCP in ND-96 for 1 h, and 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: 40 mM KCl, 75 mM potassium gluconate, 5 mM KF, 0.1 mM $NaVO_3$, 10 mM potassium pyrophosphate, 1 mM EGTA, 0.1 mM spermine, and 10 mM HEPES, pH 7.4. Traces represent inward currents recorded at −50 mV membrane potential. Leak current was monitored at 50 mV and was stable and negligible throughout. ▼, cell-attached configuration. The broken 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 $K_{ir}2.2$ experiment (electrode resistance, 830 kΩ) and approximately 0.5 mV at the end of the $K_{ir}2.3$ experiment (electrode resistance, 780 kΩ).

histidine mutations were made at position 52 in $K_{ir}2.2$ (Q52H) and position 27 in $K_{ir}2.3$ (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 (Heytler and Prichard, 1962; Guerrieri et al., 1976). 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 (Fig. 4). ATP is

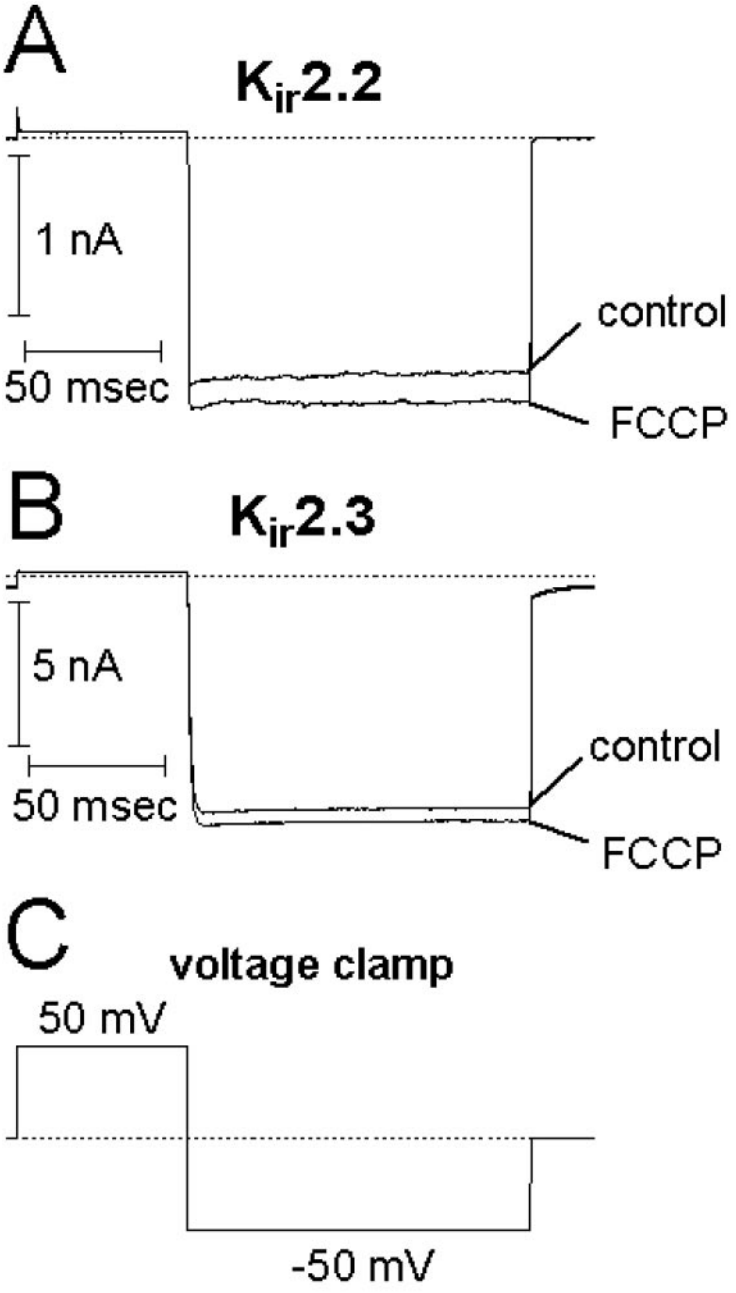

Fig. 5. No direct inhibition of FCCP on $K_{ir}2.2$ or $K_{ir}2.3$. Inward-rectifier currents were recorded by patch clamp in *X. laevis* oocytes expressing $K_{ir}2.2$ or $K_{ir}2.3$. The same FVPP-type solution was used in the recording dish and in patch pipettes, and for perfusing inside-out patches: 40 mM KCl, 75 mM potassium gluconate, 5 mM KF, 0.1 mM $NaVO_3$, 10 mM sodium pyrophosphate, 1 mM EGTA, 10 mM glucose, 0.1 mM spermine, and 10 mM PIPES, pH 7.4. Traces represent currents recorded in inside-out patches from oocytes expressing $K_{ir}2.2$ (A) and $K_{ir}2.3$ (B) while applying the voltage-clamp protocol shown in C before (control) and during the 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 caused by series resistance was approximately 1 mV in A (electrode resistance, 800 kΩ) and approximately 5 mV in B (electrode resistance, 640 kΩ).

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 preinjection of oocytes with PIP_2 or ATP did not attenuate the inhibition of $K_{ir}2.2$ or $K_{ir}2.3$ by FCCP (Fig. 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 Fig. 4 support such a mechanism, because perfusion of inside-out membrane patches from FCCP-treated oocytes resulted in the recovery of $K_{ir}2.2$ and $K_{ir}2.3$ currents.

Residues other than residue 53 ($K_{ir}2.1$ numbering) would inevitably 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 (Fig. 7). On the other hand, 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.

From 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; Kaab et al., 1996; Lodge and Normandin, 1997; Han et al., 2001), 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 mitochon-

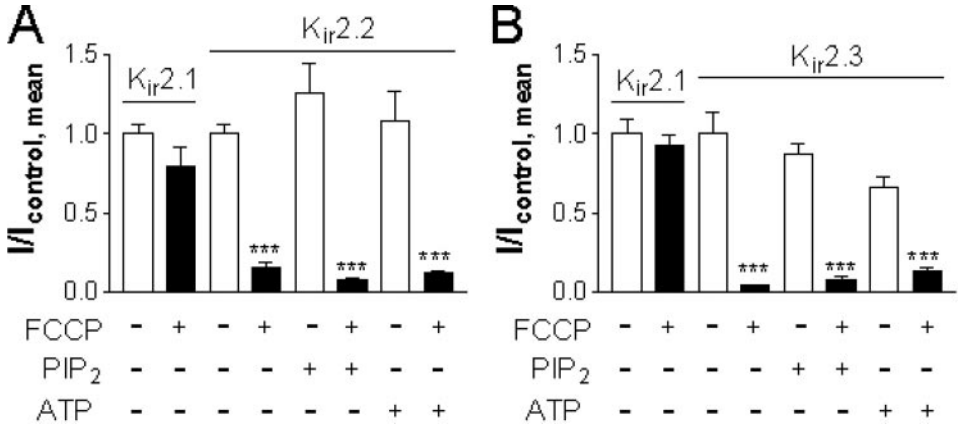

Fig. 6. No effect of ATP or PIP_2 on the inhibition of $K_{ir}2.2$ and $K_{ir}2.3$ by FCCP. *X. laevis* oocytes expressing $K_{ir}2.2$ (A) or $K_{ir}2.3$ (B) were injected with 50 nl of water (columns 1–4), 1 mM PIP_2 (columns 5 and 6), or 50 mM ATP (columns 7 and 8), incubated in ND-96 for 60 min (ATP) or 30 to 60 min (PIP_2), then incubated for a further 90 min in ND-96 (control; □) or ND-96 with 10 μM FCCP (■). 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. ★★★, $p < 0.001$ by t test (n = 5–10).

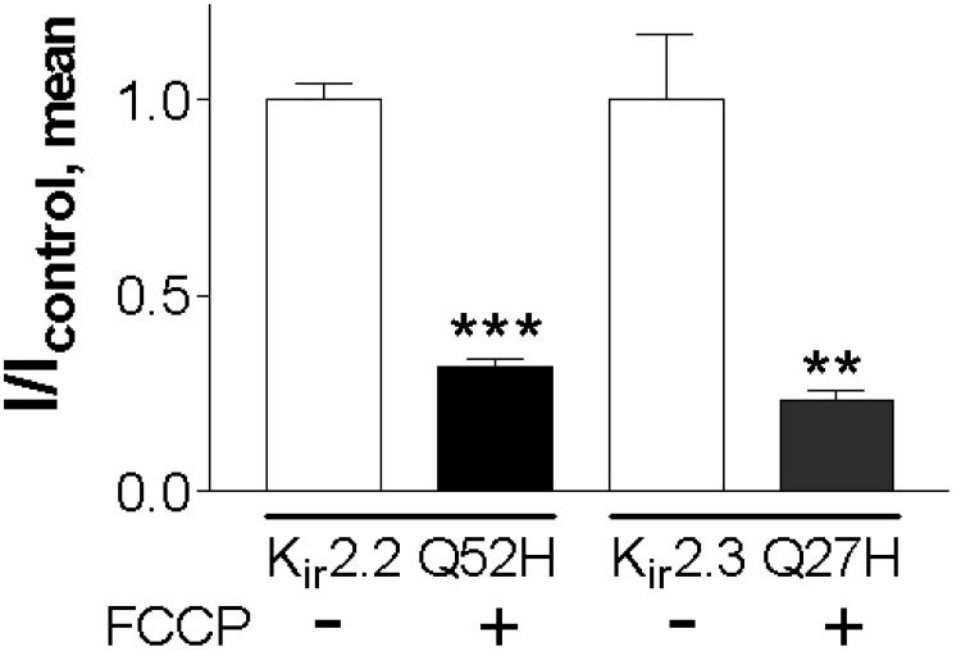

Fig. 7. A histidine residue at position 53 ($K_{ir}2.1$ numbering) is not sufficient to make $K_{ir}2.2$ or $K_{ir}2.3$ resistant to FCCP. The glutamine residue at the position equivalent to $K_{ir}2.1$ residue 53 was mutated to histidine in $K_{ir}2.2$ (position 52) and $K_{ir}2.3$ (position 27). These constructs were expressed in *X. laevis* oocytes, which were then incubated for 90 min in ND-96 with 10 μM FCCP (■) or ND-96 (control; □). 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. ★★, $p < 0.01$; ★★★, $p < 0.001$ by t test (n = 6).

drial 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 caused by 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 (Zaritsky et al., 2001; Preisig Muller et al., 2002; 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 (Doble, 1999; Patel, 2002; Sims and Anderson, 2002; Fiskum et al., 2003; Baloyannis et al., 2004). 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 (Morishige et al., 1993; Falk et al., 1995; Horio et al., 1996; Karschin et al., 1996) raises the possibility of an important role for them in the pathophysiology of these diseases.

Acknowledgments

We thank Drs. Lily Jan and Yoshihisa Kurachi for cDNA clones of IRK1, MB-IRK2, and MB-IRK3. We also thank Dr. Diomedes Logothetis for sharing data before publication. We also thank Zac Blackwood, Cory Rahn, Tong Shen, and Tam To for technical assistance.

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