Supplemental Material for Wojtovich *et al.* "A novel mitochondrial K_{ATP} channel assay"

METHODS

Animal models: Sprague-Dawley rats, 200-225 grams, were purchased from Harlan (Indianapolis, IN) and housed on a 12 hr. light/dark cycle with food and water available *ad libitum*. All procedures were performed in accordance with the US National Institutes of Health "*Guide for the care and use of laboratory animals*", and were approved by the University of Rochester's Committee on Animal Resources (protocol # 2007-087).

Reagents: Atpenin A5 (AA5) was from Axxora LLC (San Diego, CA), and was added from stock solutions in DMSO such that the final concentration of vehicle was <0.1%. Phosphatidylinositol-4,5-bisphosphate (PIP_2), 1.2-dioctanoyl glycerol (DOG), and 1,2-dipalmitoyl glycerol (DPG) (Avanti Polar Lipids; Alabaster, AL) stock solutions (500 µg/mL; ≥250 µL) were dispersed in water by sonication on ice using a Sonifier model 450 (Branson, Danbury CT) sonicator with microtip probe, for 30 min (intensity output 3, duty cycle 25%, cycle time 1 s.) as previously described.[1] Lipid working stocks were made fresh just prior to experiments and discarded after use. BTC-AM was obtained from Invitrogen (Carlsbad, California). BTC-AM stock solutions were prepared at a concentration of 4 mmol/L in DMSO. Aliquots were stored at -20°C for up to two weeks and discarded after use. Pluronic F-127 (0.05% w/v final) was added and mixed with BTC-AM, and this solution was added to the mitochondrial suspension. All steps involving BTC-AM or BTC-AM loaded mitochondria were preformed in the dark. Unless otherwise stated all other chemicals were of the highest grade obtainable from Sigma (St. Louis, MO).

Cardiac mitochondria: In order to ensure mK_{ATP} channel activity, mitochondria were rapidly isolated by differential centrifugation in sucrose-based buffer (heart mitochondria isolation media, HMIM: 300 mmol/L sucrose, 20 mmol/L Tris, 2 mmol/L EGTA, pH 7.35 at 4°C) as previously described.[2] Protein concentration was determined by the Folin-phenol method.[3]

mK_{ATP} swelling assay: The activity of mK_{ATP} was monitored spectrophotometrically at 520 nm, as the light scatter (absorbance) change due to K^+ uptake and swelling, as previously described,[2] within 1.5 hr of mitochondrial isolation. Briefly, mitochondria (0.25 mg/mL) were added rapidly to a stirring cuvette containing mK_{ATP} swelling buffer (100 mmol/L KCl, 10 mmol/L HEPES, 2 mmol/L succinate, 2 mmol/L $MgCl_2$, 2 mmol/L KH_2PO_4, 1 µg/mL oligomycin, pH 7.2 at 37°C). The change in A_{520} was monitored using a Beckman DU800 spectrophotometer.

Thallium flux assay: Mitochondria were isolated essentially as described for mK_{ATP} activity assays in heart mitochondria isolation media (HMIM: 300 mmol/L sucrose, 20 mmol/L Tris, 2 mmol/L EGTA, pH 7.35 at 4°C) with a some modifications.[2] A homogenized rat heart was centrifuged at 600 x *g* for 5 min. at 4°C, the resulting supernatant was filtered (300 µm plastic mesh) and centrifuged for 10 min. at 7000 x *g* at 4°C. The mitochondria-enriched pellet was resuspended in 800 µl of HMIM and placed in an open stirring cuvette. The mitochondria were incubated with 20µmol/L BTC-AM and 0.05% Pluronic F-127 (w/v) for 10 min. at room temperature. Following the incubation, the mitochondria were diluted with 35 ml of HMIM and centrifuged at 7000 x *g* for 5 min. at 4°C. The resulting pellet was again resuspended in 35 ml of HMIM and centrifuged at 7000 x *g* for 5 min. at 4°C. The final pellet was resuspended in HMIM and protein was determined via Folin-phenol method.[3] BTC-AM loaded mitochondria (~0.3 mg/ml) were added to a stirring cuvette containing in chloride free thallium assay buffer (195 mmol/L mannitol, 10 mmol/L HEPES, 2 mmol/L $MgSO_4$, 2 mmol/L Na_2HPO_4, 2 mmol/L succinate and 1 µg/ml oligomycin, pH7.2 at 37°C). Tested compounds were present prior to the

addition of thallium. An initial baseline of 10 sec was collected prior to the addition of the 2 mmol/L $TlSO_4$ stimulus via a syringe port to the stirring cuvette. The change in fluorescence was monitored in an Varian Cary Eclipse spectrofluorimeter (Ex 488 ± 2.5 nm, Em 525 ± 20 nm) and normalized to the 10 s. of baseline. Experiments utilizing this approach were completed within 1.5 hours of isolation unless otherwise noted. Note that the assay medium did not contain potassium; osmotic support was provided by sucrose during the mitochondrial isolation and by mannitol during the assay.

Perfused hearts: Isolated rat hearts were retrograde (Langendorff) perfused with Krebs Henseleit Buffer (KH: 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 25 mmol/L $NaHCO_3$, 1.2 mmol/L KH_2PO_4, 11 mmol/L glucose, 2.5 mmol/L $CaCl_2$, gassed with 95% O_2, 5% CO_2) in constant flow mode, essentially as described.[4] After 20 min. equilibration, hearts were subjected to one of the following protocols: (i) Ischemia Reperfusion (IR) alone, comprising 15 min. perfusion, 20 min. vehicle (water) infusion, 30 s. wash-out, then 25 min. global ischemia followed by 120 min. reperfusion; (ii) Ischemic Preconditioning (IPC) + IR, comprising 5 min vehicle infusion, 3 x 5 min. global ischemia interspersed with 5 min. reperfusion, with vehicle, then 30 s. wash-out, then IR as above; (iii) Fluoxetine (FLX) + IPC + IR, comprising 5 min FLX infusion, 3 x 5 min. global ischemia interspersed with 5 min. reperfusion, with FLX, then 30 s. wash-out, then IR as above. (iv) FLX + IR, comprising 15 min. perfusion, 20 min. FLX infusion, 30 s. wash-out, then IR as above; (v) Zimelidine (ZM) + IPC + IR, comprising 5 min ZM infusion, 3 x 5 min. global ischemia interspersed with 5 min. reperfusion, with ZM, then 30 s. wash-out, then IR as above.

Both FLX and ZM stock solutions (10mmol/L) were dissolved in water and made fresh before each experiment. FCCP stock solution was dissolved in ethanol. FCCP, FLX, ZM, and vehicle were infused into the perfusion cannula just above the aorta, with the final injection level being 0.01% of the total perfusion volume, which did not affect cardiac function. After following the above protocols, hearts were sliced transversely into 2 mm slices and stained in 1 % (w/v) tetrazolium chloride (TTC) in 100 mmol/L sodium phosphate buffer (pH 7.4) at 37°C for 20 min. Slices were fixed for 24 hr. in 10% neutral buffered formalin. The slices were then placed between glass gel plates, and scanned. The image was then analyzed using ImageJ software in order to quantify the infarct (white) vs. live (red) tissue area. Data was expressed as a percent of area at risk (100% in this global ischemia model).

Molecular Biology: Kir4.1 and Kir6.2Δ36 were subcloned into the pGEMSH vector (modified from pGEMHE vector[5] for oocyte expression.[6]

Electrophysiology: Xenopus oocytes were harvested, dissociated and defolliculated by collagenase type I (Sigma) treatment.[7] Oocytes were injected with 4-6 ng cRNA and were incubated at 18°C for 2-5 days in OR2 solution containing (in mmol/L): 82.5 NaCl, 15 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES,with pH adjusted to 7.5 with NaOH. Two-electrode voltage clamp (TEVC) was used to measure whole-cell currents in response to a series of 20 mV steps from -140 to +80 mV from a holding potential of either -80 mV (Kir4.1) or 0 mV (Kir6.2) (GeneClamp 500B, 1322A, Digidata interface, pCLAMP9 software, Axon Instruments). The standard bath solution contained for Kir4.1 experiments contained (in mmol/L): 4 KCl, 106 NaCl, 2 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES with pH adjusted to 7.4 with KOH. For Kir6.2 experiments solutions contained (in mmol/L): 110 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 5 HEPES with pH adjusted to 7.4 with KOH. Pipettes were filled with 2.5% agarose containing 3 mol/L KCl and had resistances between 0.1 and 0.4 MΩ. Oocytes were incubated for 1 hour in FLX-containing bath solution at room temperature and currents were measured 1 min after oocyte impalement. Ba^{+2}-sensitive currents were determined using bath solution containing 5 mmol/L $BaCl_2$.

Statistical analysis: In mitochondria or heart experiments, each "N" was an independent heart perfusion or mitochondria isolation from a single animal on one day. Data are presented as mean ± SEM. Statistical differences between groups were determined using ANOVA, with significance defined as $p<0.05$. Hill plots were fitted using Kaleidagraph. In TEVC experiments, the effect of each concentration of FLX was measured on at least 3 oocytes from the same batch and at least 2 batches were used. Error bars represent standard error of the mean (SEM).

NARRATIVE

Online Figure I shows parallel data to that in Figure 2 of the main document, in which the effects of time, PIP_2 and related compounds on mK_{ATP} channel activity were determined. In Figure 2, the novel Tl^+ assay was used, whereas here in Online Figure I the mK_{ATP} was assayed using the classical mK_{ATP} osmotic swelling assay.

Online Figure II shows dose responses of mK_{ATP} channel activity to nucleotides UDP and ATP. In the case of UDP titrations, the channel was first inhibited by ATP, and the ability of UDP to over-ride and open the channel was measured. In the case of ATP, the ability of ATP to close the (constitutively open) channel was measured. Both assay systems (Tl^+ and swelling) yielded comparable results.

Online Figure IIIA shows the dose response to FLX of $K_{IR}4.1$ and $K_{IR}6.2\Delta36$ channels expressed in *Xenopus* oocytes. Online Figure IIIB reproduces the data in Figure 3B of the main manuscript, using the osmotic swelling mK_{ATP} assay instead of the Tl^+ assay. Online Figure IIIC shows the ability of FLX to close the mK_{ATP} channel even in the presence of the agonists AA5 or DZX.

Online Figure IV shows the effects of FLX on cardioprotection elicited by mild uncoupling with FCCP. Contrary to the ability of FLX to block protection by IPC (main manuscript Figure 3), FLX had no effect on FCCP-mediated protection, which occurs via a mechanism that does not involve mK_{ATP}.

Investigation into the modulation of mitochondrial $K^+//H^+$ exchanger (KHE) and its effects on the Tl^+ flux assay. Two inhibitors of KHE are known: quinine and DCCD. However, their use poses some difficulties. First, quinine is reported to inhibit both the KHE (IC_{50} 27 μM; complete inhibition at 500 μM)[8] and the mK_{ATP} (10 μM decreases the open probability by ~50%; complete inhibition at 100μM)[9]. Second, while DCCD does inhibit the KHE (50 nmol/mg complete inhibition), this inhibition requires Mg^{2+} depletion[8]. Despite these concerns, we tested the effect of DCCD (both 50 and 200 nmol/mg) and quinine (500 μM) on the kinetics of the Tl^+ flux assay. The following results were obtained:

(i) The spike intensity was blunted in the presence of 200 nmol/mg DCCD or 500 μM quinine (Ctrl: 31±2.4 fluorescence units; DCCD 26±2.6; Quinine 24±3.0).

(ii) The final plateau was increased in the presence of 200 nmol/mg DCCD, but 500 μM quinine had no effect (Ctrl: 19±3.4; DCCD 27±5.2; Quinine 20±4.8).

(iii) The decay kinetics (slope of the trace from 30 s. to 110 s) were negative and almost zero (-0.014±0.018 units/s.) in controls. With 200 nmol/mg DCCD a small upward slope was observed (0.051±0.038 units/s.) With 500 μM quinine, no change was observed (-0.019±0.040).

Collectively, these results show that quinine was ineffective, even at a high concentration (500 μM). A small effect was observed at the high dose of DCCD (200 nmol/mg), but a lower concentration (50 nmol/mg) was without effect. Despite this trend, the results are not significantly different (N=3). Therefore, the involvement of the KHE in the steady-state fluorescence is currently unclear. It is anticipated that the future availability of better KHE inhibitors may render the assay suitable to the study of KHE kinetics.

Ability of Mg/ATP to restore mK_{ATP} channel run down: In a recent study[10], the authors recovered mK_{ATP} activity in a lipid bilayer system by adding Mg/ATP to the *cis* (cytosol) and *trans* (matrix) compartments and incubating for ~1 min. The ATP was then removed by perfusing ATP-free solution, first in the *trans* then the *cis* compartment. This procedure resulted in the reversal of run-down; however, the authors noted that this method of recovering mK_{ATP} activity was sensitive to the precise sequence of events. For instance, the perfusion of the ATP-free solution from the *cis* then the *trans* compartments did not result in activity restoration.

The isolated mitochondrial system is not suited to test Mg/ATP-mediated reversal of run-down, since it is not possible to selectively change the components in the matrix (*trans*) versus the buffer (*cis*). Despite this limitation, we were intrigued to find out if Mg/ATP could re-activate the channel in mitochondria. Mitochondria were prepared according to the normal procedure and after 5 hrs. on ice (for channel run-down) they were incubated with 1 mM Mg/ATP for 2 min. at 37° C. Because the channel itself is inhibited by ATP, the mitochondria were then centrifuged and the supernatant containing the inhibitory ATP was removed. The mitochondria were then subjected to the Tl^+ flux assay.

The transient incubation with Mg/ATP did not recover the lost activity. However, it was possible that this lack of re-activation could be due to a small amount of inhibitory ATP carried over into the assay. Thus, we also tested the ability of the channel opener AA5 (which normally over-rides ATP inhibition in fresh mitochondria) to open the channel. AA5 was without effect, thereby suggesting that in isolated mitochondria Mg/ATP cannot re-activate the mK_{ATP}.

Notably, 5 hr. aged mitochondria that were exposed to a 2 min. incubation (without Mg/ATP), followed by centrifugation, were still capable of having their mK_{ATP} channel re-activated by PIP_2. This indicates that the incubation and centrifugation steps were not responsible for lack of ability to re-activate. These differences between Mg/ATP re-activation in isolated membrane vs. mitochondrial systems are likely attributable to the inability to replicate the exact sequence of events (critical to the recovery of activity) outlined in [10].

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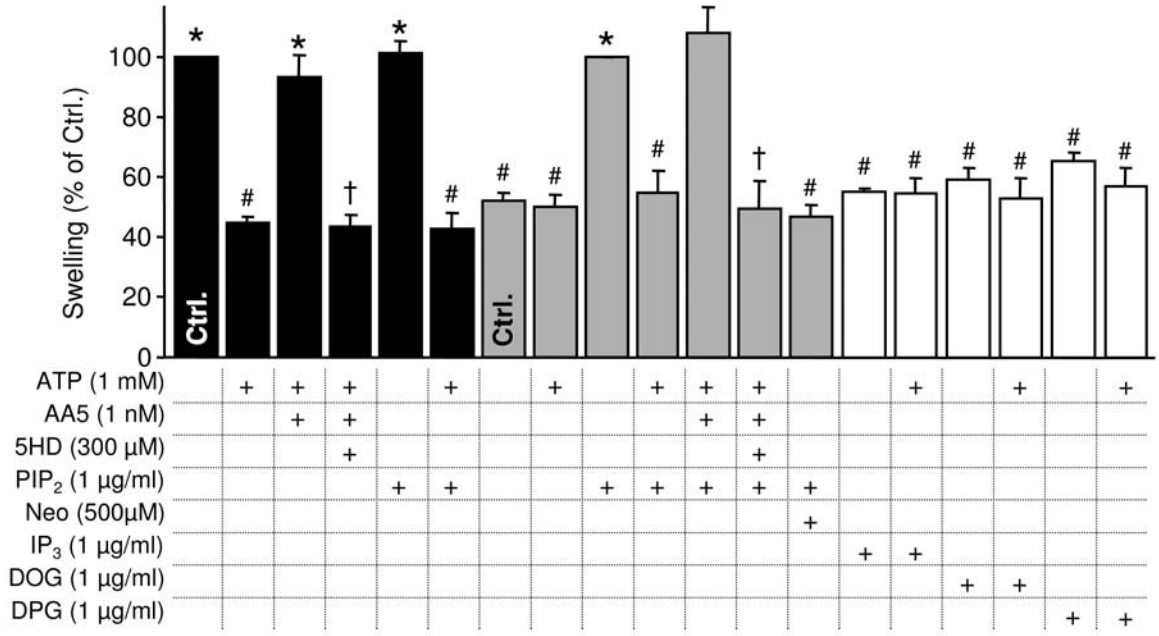
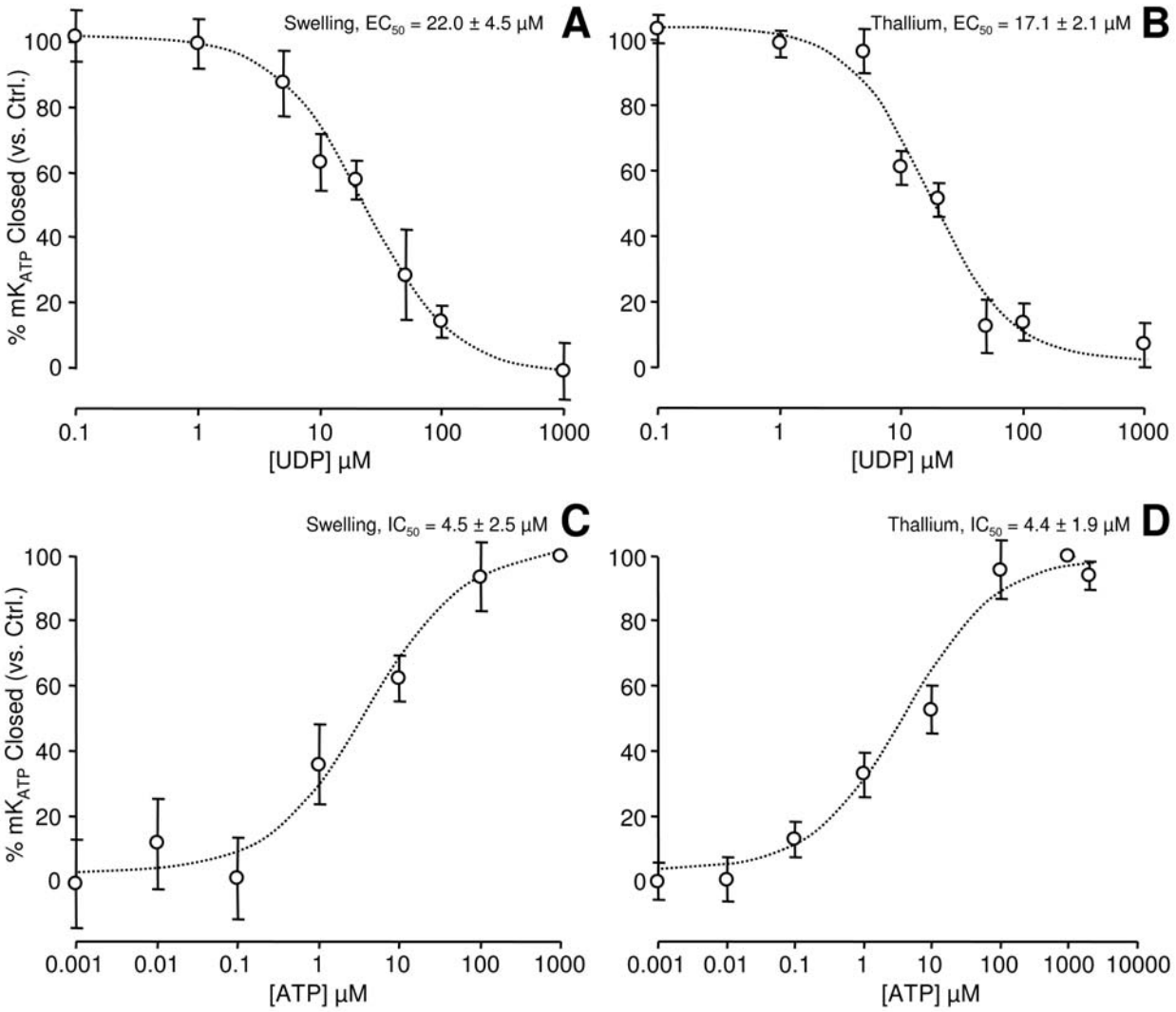
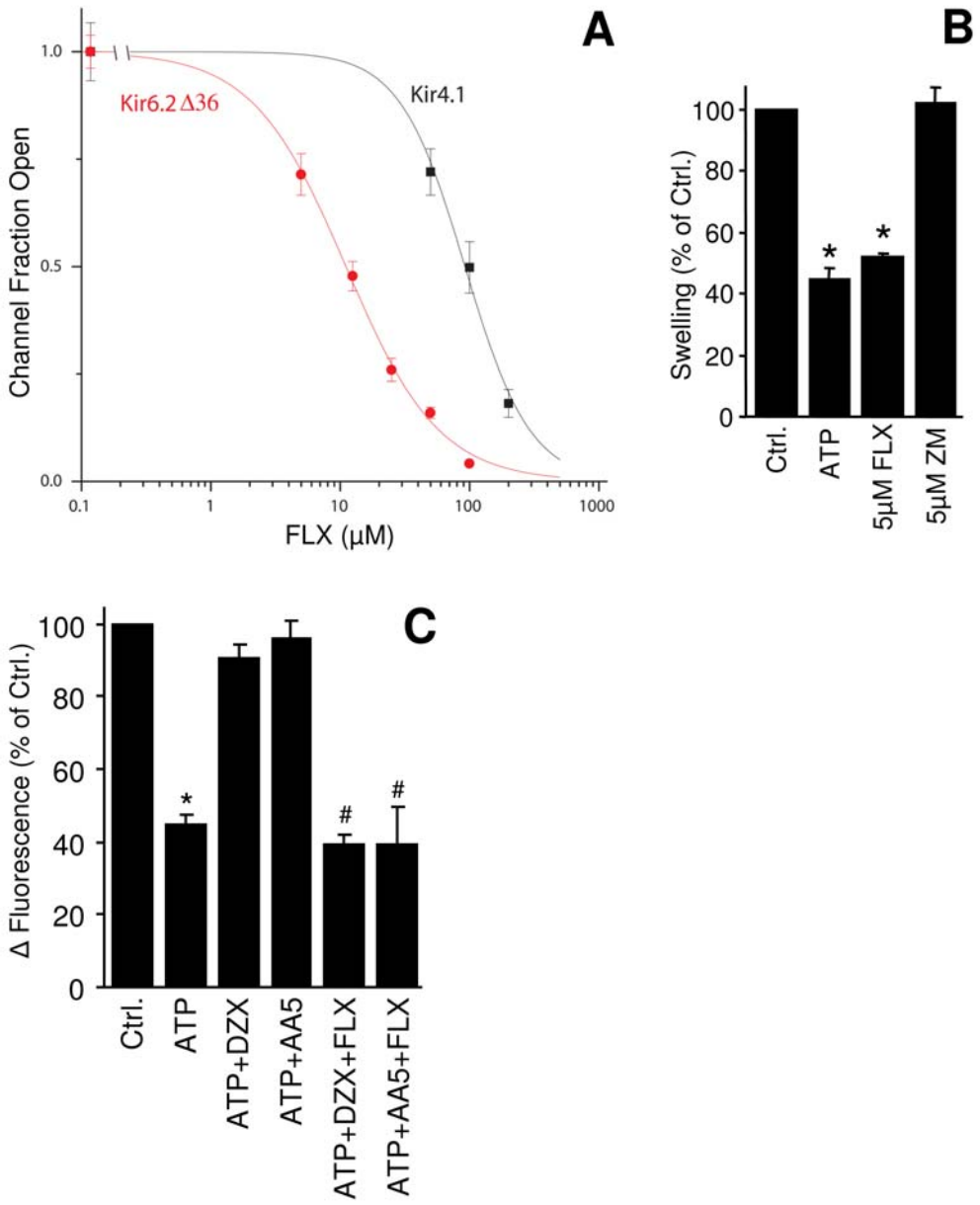


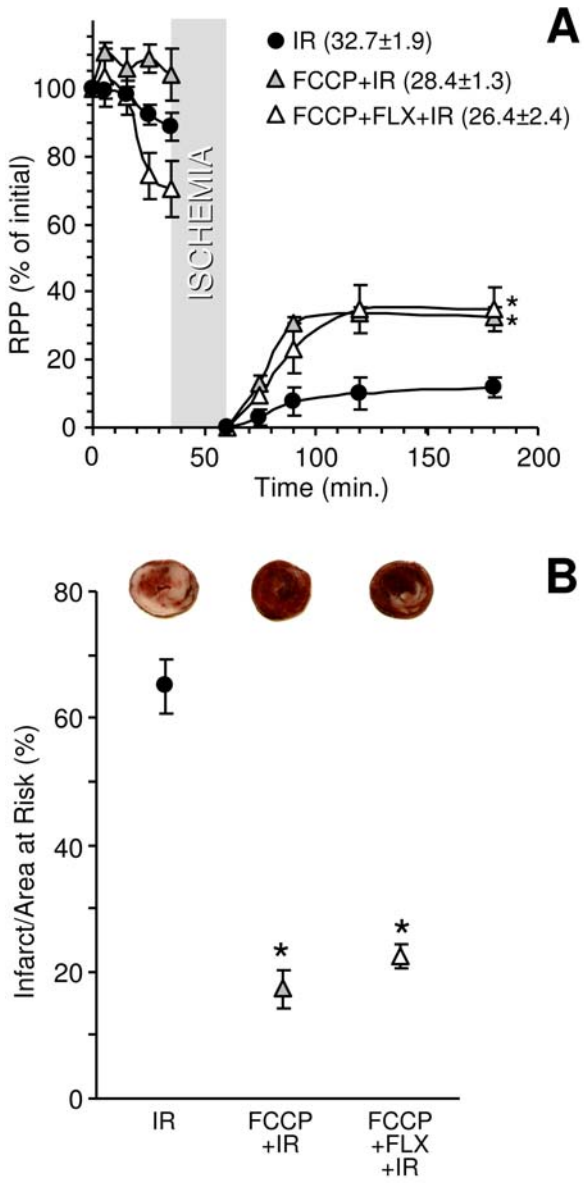
Online Figure I: PIP_2 modulation of mK_{ATP} channel activity using the swelling assay. (A): mK_{ATP} activity was monitored using the swelling assay in fresh mitochondria (black bars), or mitochondria 5 hrs. post isolation (gray and white bars) as in Figure 2 (main manuscript). Fresh mitochondria data were normalized to control (ΔOD_{520} 0.022±0.001) while 5 hr. mitochondria were normalized to control + PIP_2 (the 3rd gray bar, ΔOD_{520} 0.020±0.001). Experimental conditions are listed below the *x*-axis. Data are means ± SEM, N≥4. Fresh mitochondria: # P<0.05 versus control, * P<0.05 versus ATP, † P<0.05 versus ATP+AA5. 5 hr. mitochondria: # P<0.05 versus control+PIP_2, * P<0.05 versus ATP, † P<0.05 versus ATP+AA5, like symbols are not significantly different.



Online Figure II: Nucleotide modulation of mK_{ATP} activity using the swelling and BTC-AM-Tl^+ assays. (A): UDP-dependent activation of the mK_{ATP} channel determined with the swelling assay. Since the channel is open under baseline conditions, the mK_{ATP} was closed with 1 mmol/L ATP and UDP was then added to re-open the channel. Data were plotted as % mK_{ATP} closed, with 100% closed being defined as the condition in the presence of 1 mmol/L ATP alone, and 0% closed (i.e. open) being the baseline condition without ATP. The EC_{50} was found to be 22.0 ± 4.5 µmol/L. **(B):** UDP-dependent activation of the mK_{ATP} channel using the BTC-AM-Tl^+ assay. Conditions were as in panel A and the EC_{50} was found to be 17.1 ± 2.1 µmol/L. **(C):** ATP-dependent inhibition of mK_{ATP} using the swelling assay. The IC_{50} of ATP was found to be 4.5 ± 2.5 µmol/L. **(D):** ATP-dependent inhibition of mK_{ATP} channel activity using the BTC-AM-Tl^+ assay. Conditions were as in panel C and the IC_{50} was found to be 4.4 ± 1.9 µmol/L. Lines were curve fitted using the Hill equation. Data are means ± SEM, N≥4.



Online Figure III: Modulation of K_{IR} channel activity by fluoxetine. (A): Dose response to fluoxetine treatment in *Xenopus* oocytes expressing either $K_{IR}4.1$ or $K_{IR}6.2\Delta36$ channel subunits. The $K_{IR}6.2\Delta36$ mutant subunit, where the endoplasmic reticulum retention motif was deleted, was used to allow channel expression at the cell surface without the sulphonylurea receptor.[11] Data points represent means ± SEM, N>6. **(B):** mK_{ATP} activity was measured using the swelling assay. Where indicated, FLX or ZM were present. Data are means ± SEM, N=4. * P<0.05 versus control. **(C):** FLX blocks AA5- and DZX-mediated mK_{ATP} opening as determined by the BTC-AM-Tl^+ assay. Where indicated 1 mmol/L ATP, 1 nmol/L AA5, 10 μmol/L DZX, 5 μmol/L FLX were present. Data are means ± SEM, N=4. * P<0.05 versus control. # P<0.05 versus AA5/DZX+ATP.



Online Figure IV: Fluoxetine does not affect mK_{ATP}-independent cardioprotection. (A): The effect of FLX on FCCP-mediated cardioprotection (30 nmol/L) was determined using Langendorff perfusion, as described in the methods. Rate pressure product (RPP, expressed as % of initial) is shown for hearts subjected to each protocol, as in Figure 3 of the main manuscript. Initial values of RPP ($mmHg \cdot min^{-1}$, $x10^3$) are shown in the legend for each symbol. **(B):** Hearts were stained with TTC and the infarct size / area at risk was quantified, with representative stained hearts shown above each condition. Data are means ± SEM, N≥4, *P<0.05 vs. IR.