New Methods in Cardiovascular Biology

A Novel Mitochondrial $K_{\text{ATP}}$ Channel Assay

Andrew P. Wojtovich, David M. Williams, Marcin K. Karcz, Coeli M.B. Lopes, Daniel A. Gray, Keith W. Nehrke, Paul S. Brookes

Rationale: The mitochondrial ATP sensitive potassium channel ($mK_{\text{ATP}}$) is implicated in cardioprotection by ischemic preconditioning (IPC), but the molecular identity of the channel remains controversial. The validity of current methods to assay $mK_{\text{ATP}}$ activity is disputed.

Objective: We sought to develop novel methods to assay $mK_{\text{ATP}}$ activity and its regulation.

Methods and Results: Using a thallium ($\text{TI}^+$)-sensitive fluorophore, we developed a novel $\text{TI}^+$ flux based assay for $mK_{\text{ATP}}$ activity, and used this assay probe several aspects of $mK_{\text{ATP}}$ function. The following key observations were made. (1) Time-dependent run down of $mK_{\text{ATP}}$ activity was reversed by phosphatidylinositol-4,5-bisphosphate (PIP$_2$). (2) Dose responses of $mK_{\text{ATP}}$ to nucleotides revealed a UDP EC$_{50}$ of $\approx$20 $\mu$mol/L and an ATP IC$_{50}$ of $\approx$5 $\mu$mol/L. (3) The antidepressant fluoxetine (Prozac) inhibited $mK_{\text{ATP}}$ (IC$_{50}$=2.4 $\mu$mol/L). Fluoxetine also blocked cardioprotection triggered by IPC, but did not block protection triggered by a $mK_{\text{ATP}}$-independent stimulus. The related antidepressant zimelidine was without effect on either $mK_{\text{ATP}}$ or IPC.

Conclusions: The $\text{TI}^+$ flux $mK_{\text{ATP}}$ assay was validated by correlation with a classical $mK_{\text{ATP}}$ channel osmotic swelling assay ($R^2$=0.855). The pharmacological profile of $mK_{\text{ATP}}$ (response to ATP, UDP, PIP$_2$, and fluoxetine) is consistent with that of an inward rectifying $K^+$ channel ($K_{\text{IR}}$) and is somewhat closer to that of the $K_{\text{IR}}$6.2 than the $K_{\text{IR}}$.6.1 isofrm. The effect of fluoxetine on $mK_{\text{ATP}}$-dependent cardioprotection has implications for the growing use of antidepressants in patients who may benefit from preconditioning. (Circ Res. 2010;106:1190-1196.)

Key Words: mitochondrial ATP-sensitive potassium channel ■ ischemia/reperfusion ■ ischemic preconditioning ■ fluoxetine

The mitochondrial ATP-sensitive potassium channel ($mK_{\text{ATP}}$) is thought to be essential for cardioprotection recruited by ischemic preconditioning (IPC), but despite intense research the molecular identity of this channel remains unclear. The simplest thesis is that $mK_{\text{ATP}}$ channels are derivative of surface $K_{\text{ATP}}$ channels, and thus composed of inward rectifying $K^+$ channels ($K_{\text{IR}}$) and sulfonylurea receptors (SURs). The cardiomyocyte surface $K_{\text{ATP}}$ channel is comprised of $K_{\text{IR}}$.6.2 and SUR2A isoforms, but efforts to conclusively assign these proteins to the cardiac $mK_{\text{ATP}}$ have been unsuccessful to date.

Neither $K_{\text{IR}}$.6 nor SUR genes contain mitochondrial target sequences, and $K_{\text{IR}}$6/SUR proteins are not found in mitochondrial proteome databases or prediction engines. Furthermore, immune-based methods to detect $K_{\text{IR}}$/SUR subunits in mitochondria are plagued by issues of antibody specificity and mitochondrial purity/contamination. Several of the key pharmacological reagents used to study $mK_{\text{ATP}}$ channels (eg, the agonist diazoxide and antagonist 5-hydroxycarboxylic acid) are also known to exhibit off-target effects. Targeted gene deletion in mice to identify the $mK_{\text{ATP}}$ channel involved in IPC has proven futile, because of the confounding cardiovascular effects of knocking out $K_{\text{IR}}$6 and SUR genes ($Kcnj8$, $Kcnj11$, Abcc8, and Abcc9) on surface $K_{\text{ATP}}$ channel function. In general, $K_{\text{IR}}$ and SUR knockouts exhibit profound defects in glucose/insulin handling, which impacts the response to IPC.

A recent study using custom-made antibodies and SUR knockout mice identified short-form splice variants of SUR2 in mitochondria. Furthermore, recent pharmacological evidence suggests that complex II of the respiratory chain (succinate dehydrogenase) may be a regulatory component of the $mK_{\text{ATP}}$ channel. However, both these findings leave the identity of the $K^+$ channel-forming subunit of $mK_{\text{ATP}}$ unknown. In this regard, $mK_{\text{ATP}}$ is similar to other mitochondrial ion channels which exist at a phenomenological level but have not been molecularly identified (eg, the mitochondrial $Ca^{2+}$ uniporter).

A major obstacle in studying the $mK_{\text{ATP}}$ channel has been the availability of a reliable assay. Most studies to date have used an isolated mitochondrial rapid swelling assay, in which...
K⁺ uptake into mitochondria is followed by osmotically-obliged water, leading to mild swelling that is assayed as light scattering in a spectrophotometer. This assay has been criticized as irreproducible by some laboratories, with the precise timing of mitochondrial isolation appearing to be a critical factor.

Studying the literature on surface Kₐtp channels, two key biochemical properties that appeared to have been overlooked in the mKₐtp channel field were the permeability of surface Kₐtp channels for the heavy metal thallium (Tl⁺), and the modulation of channel run-down by phospholipids such as phosphatidylinositol-4,5-bisphosphate (PIP₂). Herein, we developed a novel Tl⁺ fluorescence based assay for mKₐtp channel activity, and used this assay to show that the channel is subject to run-down that is reversed by PIP₂. It is anticipated that both these discoveries will advance the study of this channel. Furthermore, the antidepressant fluoxetine (FLX), which is known to modulate KIR channels, was found herein to block the mKₐtp channel and to block IPC, both FCCP (30 nmol/L) and FLX (5 μmol/L), then IR. Following reperfusion, hearts were stained with tetrazolium chloride, and imaged, and infarct size measured as previously described.

In all experiments, each “N” was an independent heart perfusion or mitochondrial isolation from a single animal on one day. Statistical differences between groups were determined using ANOVA, with significance defined as *P<0.05.*

**Results**

In seeking to develop an assay for mKₐtp channel activity that does not measure secondary effects such as water uptake (as is the case for the osmotic swelling assay), we discerned that the heavy metal thallium (Tl⁺) is widely used as a surrogate substrate to study K⁺ channel function. A fluorescent probe that responds to Tl⁺ is commercially available (FluxOR, Invitrogen, Carlsbad, Calif), but careful analysis of the literature underlying this reagent revealed that the active component was BTC-AM, a more economical reagent. Thus, isolated mitochondria were loaded with BTC-AM as the basis for a Tl⁺ uptake assay of K⁺ channel activity.

Figure 1A shows the addition of Tl⁺ to BTC-AM loaded mitochondria resulted in increased fluorescence because of rapid Tl⁺ influx and the establishment of a new steady-state. The fluorescence increase was largely inhibited by ATP, consistent with Tl⁺ transport by a Kₐtp channel. Furthermore, the effect of ATP could be overridden by the mKₐtp channel opener atpenin A5 (AA5), and this effect was in-turn blocked by the mKₐtp antagonist 5-hydroxydecanone. These data are quantified in Figure 1B, which also shows the effects of mKₐtp reagents diazoxide (agonist) and glyburide (antagonist). The ionophores valinomycin and nonactin, both of which transport Tl⁺, resulted in maximal Tl⁺ flux into mitochondria, and the mitochondrial uncoupler FCCP inhibited Tl⁺ uptake indicating a requirement for membrane potential. It was hypothesized that the steady state is likely attributable to balancing of Tl⁺ influx by its efflux through the K⁺/H⁺ exchanger (KHE). However, attempts to modulate KHE activity with the inhibitors N,N'-dicyclohexylcarbodiimide and quinine were inconclusive (data not shown). Validation of the Tl⁺ assay for mKₐtp channel activity was also performed by a direct comparison with results from mKₐtp osmotic swelling assays run in parallel under a variety of open/closed conditions. Figure 1C shows that the 2 assays correlated well ($r^2=0.855$).

A general property of KIR channels is their tendency to “run-down” over time, a phenomenon attributed to loss of

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A5</td>
<td>atpenin A5</td>
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<tr>
<td>BTC-AM</td>
<td>benzothiazole coumarin acetoxyxymethyl ester</td>
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<tr>
<td>FLX</td>
<td>fluoxetine</td>
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<tr>
<td>FCCP</td>
<td>carbonyl cyanide-p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>IPC</td>
<td>ischemic preconditioning</td>
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<tr>
<td>IR</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>Kᵢr</td>
<td>inward rectifying potassium channel</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
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<tr>
<td>SUR</td>
<td>sulfonylurea receptor</td>
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<td>ZM</td>
<td>zimelidine</td>
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**Methods**

Full experimental details are in the Online Data Supplement, available at [http://circres.ahajournals.org](http://circres.ahajournals.org). Cardiac mitochondria were rapidly isolated from male Sprague-Dawley rat hearts by differential centrifugation in sucrose-based buffer as described previously. Protein was determined by the Folin-phenol method. Within 1.5 hour of mitochondrial isolation the activity of mKₐtp was monitored by the osmotic swelling assay as described previously. A novel fluorescence-based Tl⁺ flux assay for mKₐtp activity was also developed. The ionic radii of Tl⁺ (0.154 nm) and K⁺ (0.144 nm) are similar, and thus Tl⁺ is widely used as an analog to study K⁺ channel transport. The assay made use of the fluorescent indicator BTC-AM (benzothiazole coumarin acetoxyxymethyl ester), which is better known as a ratiometric Ca²⁺ sensor, but is also sensitive to Tl⁺ with a distinct spectral response preventing signal overlap between these sensitivities. Mitochondria were loaded with BTC-AM during the isolation procedure and stored on ice until use. In the assay, 0.3 mg BTC-AM loaded mitochondria were added to a rapidly stirred cuvet containing 2 mL of chloride-free Tl⁺ buffer at 37°C. Tested compounds were present from the beginning of the assay, and baseline fluorescence was recorded for 10 seconds before addition of TISO₄ (2 mmol/L final) via a syringe port. Fluorescence was monitored in a Varian Cary Eclipse spectrofluorometer ($λ_{ex}=488$ nm, $λ_{em}=525$ nm) and normalized to baseline. Full details including the concentrations and preparation methods for all reagents used in the assay, are in the Online Data Supplement.

Isolated rat heart perfusions (Langendorff) were performed as previously described. Following 20 minutes of equilibration, hearts were divided into 7 groups: (1) ischemia/reperfusion (IR) alone, comprising 20 minutes of vehicle (water or DMSO) infusion, 30 seconds of washout, 25 minutes of global ischemia, 120 minutes of reperfusion; (2) FLX plus IR, comprising 20 minutes of FLX infusion (5 μmol/L), 30 seconds of washout, then IR; (3) IPC plus IR, comprising 3×5 minutes of ischemia interspersed with 5 minutes of reperfusion, then IR; (4) FLX plus IPC plus IR, comprising 5 minutes of FLX infusion (5 μmol/L), plus FLX infused throughout the 3 reperfusion phases of IPC (ie, 20 minutes; total FLX delivery), 30 seconds of washout, then IR; (5) zimelidine (ZM) plus IPC plus IR, as above, replacing FLX with ZM (5 μmol/L); (6) FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) plus IR, comprising 20 minutes of FCCP infusion (30 mmol/L); then IR; (7) FCCP plus FLX plus IR, comprising 20 minutes of infusion of both FCCP (30 mmol/L) and FLX (5 μmol/L), then IR. Following run-in parallel under a variety of open/closed conditions. Figure 1C shows that the 2 assays correlated well ($r^2=0.855$).

A general property of KIR channels is their tendency to “run-down” over time, a phenomenon attributed to loss of**
The major findings of this study are as follows: (1) development of a novel Tl\(^+\) flux based assay for the mK\(_{\text{ATP}}\) channel; (2) time-dependent loss of mK\(_{\text{ATP}}\) channel activity is a genuine run-down phenomenon and is reversed by PIP\(_2\); and phospholipid PIP\(_2\) from a binding site on the channel.\(^{34}\) The mK\(_{\text{ATP}}\) channel (which is constitutively open in isolated mitochondria) also loses activity following mitochondrial isolation, which may underlie the reported poor reproducibility of mK\(_{\text{ATP}}\) channel activity measurements.\(^{19}\) On investigating the relationship between these phenomena, it was found that incubation of mitochondria on ice for 5 hours resulted in complete loss of mK\(_{\text{ATP}}\) channel activity, and that PIP\(_2\) addition restored channel activity (Figure 2). Furthermore, the full pharmacological profile of mK\(_{\text{ATP}}\) channel activity (ie, inhibition by ATP, activation by AA5, and reinhibition by 5HD) was recovered in PIP\(_2\)-treated aged mitochondria. The same concentrations of the PIP\(_2\) breakdown products inositol triphosphate, 1,2-dioctanoyl glycerol, or 1,2-dipalmitoyl glycerol did not affect mK\(_{\text{ATP}}\) activity. The polyvalent cation neomycin, which is known to inhibit K\(_{\text{IR}}\) channel activity by sequestering PIP\(_2\),\(^{35}\) was able to reverse the mK\(_{\text{ATP}}\) channel-restorative effects of PIP\(_2\). Identical results were obtained with the osmotic swelling mK\(_{\text{ATP}}\) channel assay (Online Figure I). Overall these data suggest that the mK\(_{\text{ATP}}\) channel contains a PIP\(_2\) sensitive subunit, possibly a K\(_{\text{IR}}\) channel. Consistent with this, both the Tl\(^+\) and swelling assays revealed that mK\(_{\text{ATP}}\) sensitivity to the nucleotides UDP and ATP (Online Figure II) was closer to that of the K\(_{\text{IR}}6.2\) than the K\(_{\text{IR}}9.1\) isoform.\(^{36-39}\)

Several classes of K\(_{\text{IR}}\) channel are known to be inhibited by FLX, an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class.\(^{24,25}\) As shown in Online Figure III, K\(_{\text{IR}}6\) channels (components of K\(_{\text{ATP}}\) channels) are an order of magnitude more sensitive to FLX than K\(_{\text{IR}}4\) channels, a well-known FLX target.\(^{40}\) Thus, we investigated the possibility that FLX might block mK\(_{\text{ATP}}\) channels. As shown in Figure 3, FLX blocked mK\(_{\text{ATP}}\) channel activity with an IC\(_{50}\) of 2.4 \(\mu\)mol/L, whereas a related SSRI ZM did not. Identical results were obtained with the osmotic swelling mK\(_{\text{ATP}}\) assay (Online Figure III). Furthermore, FLX blocked AA5- or diazoxide-mediated opening of mK\(_{\text{ATP}}\) (Online Figure III).

Given the importance of the mK\(_{\text{ATP}}\) channel in IPC, we hypothesized that FLX may block IPC. Figure 3 shows that 5 \(\mu\)mol/L FLX completely blocked IPC-mediated cardioprotection in a rat perfused heart model of IR injury, whereas ZM was without effect. Notably FLX did not enhance baseline IR injury in this model, indicating that blockage of IPC was not attributable to an equal-but-opposite injurious effect, canceling out cardioprotection. Furthermore, FLX had no effect on cardioprotection mediated by FCCP (Online Figure IV), which occurs independent of the mK\(_{\text{ATP}}\) channel.\(^{31,32}\)

**Discussion**

**Figure 1. BTC-AM Tl\(^+\) fluorescence assay for mK\(_{\text{ATP}}\) activity in isolated heart mitochondria.**

A. Representative traces of BTC-AM Tl\(^+\) fluorescence in isolated mitochondria. Fluorescence \((\lambda_{\text{ex}} 488 \text{ nm}, \lambda_{\text{em}} 525 \text{ nm})\) was normalized to the 10 seconds of baseline before the injection of TISO\(_4\) (arrow). Where indicated, 1 mmol/L ATP (thick, black), 1 mmol/L AA5+ATP (thin, gray), or 300 \(\mu\)mol/L 5HD+AA5+ATP (thick gray) were present from the beginning of incubations. B. Magnitude of the change in BTC-AM fluorescence following Tl\(^+\) addition, relative to control. Change in fluorescence was determined by subtracting the average baseline fluorescence from the stabilized average fluorescence at 30 to 60 seconds. Delta fluorescence in controls was 26.4±3.3 arbitrary units. Experimental conditions are listed below the x axis. Data are means±SEM; N=4. #P<0.05 vs control, *P<0.05 vs ATP, †P<0.05 vs the effect of ATP+AA5 or ATP+diazoxide (DZX).

C. Correlation between 2 assays for mK\(_{\text{ATP}}\) channel activity. Experiments using a variety of conditions that modulate the mK\(_{\text{ATP}}\) were performed using either the osmotic swelling assay and the BTC-AM-Tl\(^+\) assay. Both sets of results were expressed as a percentage of their respective controls. Linear regression curve fit revealed an \(r^2\) of 0.855. AA5 indicates Atpenin A5; 5HD, 5-hydroxydecanoate; Gly, Glyburide; DZX, Diazoxide; Val, valinomycin; Non, nonactin; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

**Figure 1 (Continued).**
(3) FLX blocks both mK\textsubscript{ATP} channel activity and IPC-mediated cardioprotection. This is the first demonstration of the modulation of a mitochondrial ion channel by PIP\textsubscript{2}, and the first identification of a mitochondrial ion channel target for FLX. Collectively, the data support the concept that mK\textsubscript{ATP} contains a bona fide K\textsubscript{IR} channel. The effects of FLX on IPC may elucidate some of the reported negative impact of SSRI use on the outcome of cardiac surgery in humans.\(^4\) The initial RPP (mm Hg \cdot min\(^{-1}\)) in each protocol. Data are split across 2 experiments. Data were measured in the absence of ATP. Curve fit using the Hill equation revealed the FLX IC\textsubscript{50} to be 2.4 \pm 0.22 \mu M. Data are means \pm SEM; N \approx 4. A, mK\textsubscript{ATP} activity was measured by the BTC-AM-Tl\textsuperscript{+} assay. Where indicated, FLX or ZM were present. Data are means \pm SEM; N \approx 4. * P<0.05 vs control. Figure 3. Modulation of mK\textsubscript{ATP} activity and IPC-mediated cardioprotection by fluoxetine. A, FLX dose–response of mK\textsubscript{ATP} activity. Activity was measured using the BTC-AM-Tl\textsuperscript{+} assay. Data were plotted as % mK\textsubscript{ATP} inhibition, with 100% inhibition defined as the condition in the presence of 1 mmol/L ATP, and 0% closed (ie, open) being the baseline (Ctrl.) condition without ATP. FLX experiments were measured in the absence of ATP. Curve fit using the Hill equation revealed the FLX IC\textsubscript{50} to be 2.4 \pm 0.22 \mu M. Data are means \pm SEM; N \approx 4. B, mK\textsubscript{ATP} activity was measured by the BTC-AM-Tl\textsuperscript{+} assay. Where indicated, FLX or ZM were present. Data are means \pm SEM; N \approx 4. * P<0.05 vs control. C, Effect of FLX or ZM on IR injury and IPC. Hearts were subjected to Langendorff perfusion as detailed in Methods. Infarct size/area-at-risk was quantified as % of initial) in hearts subjected to infarction. Data are means \pm SEM; N \approx 4. * P<0.05 vs IR. D, Rate pressure product (RPP) (expressed as % of initial) in hearts subjected to each protocol. Data are split across 2 graphs for clarity (IR data shown in each). FLX indicates Fluoxetine; ZM, Zimelidine.
The kinetics of the Tl\(^{+}\) based mK\(_{ATP}\) channel assay are superior to those of the swelling based assay.\(^\text{16}\) Following Tl\(^{+}\) addition maximal fluorescence is attained within 2 to 4 seconds, compared to a time lag of 20 to 30 seconds for maximal signal intensity in the osmotic swelling assay. Unfortunately, the high flux rate of Tl\(^{+}\) through K\(^{+}\) channels (\(\sim 2 \times 10^{-5} \text{ K}^{-1} \text{ flux s}^{-1}\)), coupled with the relatively slow mixing time in the fluorescence cuvet, does not permit precise channel kinetics to be determined in this apparatus. Current best estimates for mK\(_{ATP}\) channel conductivity range from 10 to 300 pS.\(^\text{15,46,47}\)

Another barrier to investigating the mK\(_{ATP}\) channel has been the rapid loss of channel activity over time in isolated mitochondrial preparations.\(^\text{20}\) Previous work showed that the purified mK\(_{ATP}\) channel runs-down in an electrophysiology setting and can be reactivated by very high concentrations of UDP.\(^\text{48}\) However, the cause of channel activity loss in intact mitochondria was unknown, and could easily be attributable to proteolytic degradation. The finding herein that time-dependent mK\(_{ATP}\) channel inactivation in intact mitochondria can be reversed by PIP\(_2\) indicates this is a genuine run-down phenomenon, which is a common property of K\(_{IR}\) channels.\(^\text{49}\)

K\(_{ATP}\) channels were the first channels identified to depend on phosphoinositides such as PIP\(_2\).\(^\text{22,23}\) and this is the first study to identify a mitochondrial ion channel that responds to PIP\(_2\). Such regulation of mK\(_{ATP}\) channel activity by PIP\(_2\) may have implications for the function of this channel in IPC. PIP\(_2\) has been found in mitochondrial membranes,\(^\text{50}\) but its endogenous source in mitochondria is unknown. Notably, the run-down of a planar lipid bilayer reconstituted mK\(_{ATP}\) was reversed by ATP/Mg\(^{2+}\)\(^,\text{51}\) suggesting the mitochondrial high energy phosphate pool may be important in maintaining membrane lipid phosphorylation status. However, this phenomenon required very specific experimental conditions (ie, addition and removal of ATP/Mg\(^{2+}\) to and from different sides of the membrane in a particular order), and attempts to reproduce this in isolated mitochondria were unsuccessful (data not shown). The role of lipid kinases (eg, PI\(_{3}\)K), phospholipases (eg, phospholipase C), and other components of the inositol triphosphate/diacylglycerol signaling pathway in regulating mK\(_{ATP}\) is also unknown, but the involvement of such signaling components in IPC\(^\text{52,53}\) suggests a potential novel pharmacological target (ie, mitochondrial PIP\(_2\) turnover) to modulate preconditioning.

The Tl\(^{+}\) assay was also used to probe the response of mK\(_{ATP}\) to nucleotides (Online Figure II). The mK\(_{ATP}\) EC\(_{50}\) for UDP (\(\sim 20 \mu\text{mol/L}\)) was closer to that of K\(_{IR}\) 6.2 (\(\sim 200 \mu\text{mol/L}\)) than K\(_{IR}\) 6.1 (\(\sim 4 \mu\text{mol/L}\)), and the mK\(_{ATP}\) IC\(_{50}\) for ATP (\(\sim 4.5 \mu\text{mol/L}\)) was also closer to that of K\(_{IR}\) 6.2 (\(\sim 15 \mu\text{mol/L}\)) than K\(_{IR}\) 6.1 (\(\sim 350 \mu\text{mol/L}\)). Although these data agree with previous studies on mK\(_{ATP}\),\(^\text{46}\) a variety of labeling, electrophysiological and genetic studies across multiple species and tissues have suggested the presence of either K\(_{IR}\) 6.1, K\(_{IR}\) 6.2, both, or neither in mitochondria.\(^\text{10,54–62}\) An overall consensus is that mK\(_{ATP}\) likely contains a K\(_{IR}\) channel, but the definitive assignment of a particular K\(_{IR}\) isoform is not yet possible.

The discovery that mK\(_{ATP}\) activity is blocked by FLX is also consistent with the consensus that mK\(_{ATP}\) contains a K\(_{IR}\)-

FLX has previously been shown to inhibit K\(_{IR}\) channels, whereas related SSRIs (eg, ZM) had no effect.\(^\text{24,25}\) Our data (Online Figure III) suggest that K\(_{IR}\) 6 channels may be the most sensitive to FLX of all K\(_{IR}\) isoforms and in agreement with this the mK\(_{ATP}\) exhibits a strikingly low FLX IC\(_{50}\) of 2.4 \(\mu\text{mol/L}\). (Figure 3). Physiological concentrations of FLX are in the range of 1 to 20 \(\mu\text{mol/L}\).\(^\text{63}\) The fact that FLX is a lipophilic cation (LogP 4.8),\(^\text{64}\) coupled with the highly membranous nature of mitochondria, may serve to concentrate FLX in the organelle. In a mitochondria-rich tissue such as myocardium, the mitochondrion may be a primary target for FLX.

The discovery that FLX can block IPC-mediated cardioprotection is both consistent with its effect on mK\(_{ATP}\) activity, and consistent with a critical role for mK\(_{ATP}\) in IPC signaling.\(^\text{1,16,65}\) The lack of effect of another SSRI, ZM, on either IPC or mK\(_{ATP}\) activity suggests that this effect is not mediated via the SSRI mode of action. The observation that FLX also blocks mK\(_{ATP}\) channel opening by the highly specific agonist AA5 also suggests a direct mK\(_{ATP}\) effect. Furthermore, the lack of effect of FLX on FCCP-mediated cardioprotection, which is completely independent of mK\(_{ATP}\) channels,\(^\text{21,32}\) suggests that the protection-blocking effect of FLX is specific to mK\(_{ATP}\) channel-mediated protection, and does not extend to all modes of protection. The present lack of a molecular identity for the mK\(_{ATP}\) does not permit decisive knock-out experiments to verify whether the effects of FLX observed in the intact heart are mediated by mK\(_{ATP}\).

In the United States, antidepressants are the most commonly prescribed class of medication,\(^\text{66}\) with FLX alone prescribed \(>23\) million times in 2008.\(^\text{67}\) Although SSRIs are known to negatively impact the outcome of cardiac surgery,\(^\text{41}\) they are widely prescribed to patients with acute coronary syndrome.\(^\text{68}\) Notably, although IPC elicits solid protection in animal models of IR injury, its application in humans is limited by confounding effects such as age,\(^\text{69}\) gender,\(^\text{70}\) diabetes,\(^\text{13}\) and other medications.\(^\text{71}\) To this list of medications FLX must now be added, with the implication that successful cardioprotection in humans may require FLX withdrawal. Furthermore, the mood enhancer lithium is also known to both increase cardiac PIP\(_2\) levels\(^\text{22}\) and to induce cardioprotection,\(^\text{73}\) suggesting that some of the protective effects of lithium previously attributed to glycogen synthase-3\(\beta\) inhibition\(^\text{75}\) may be mediated via the mK\(_{ATP}\) channel.

In summary, we have developed herein a novel assay for the mK\(_{ATP}\) channel, and used this assay to reveal novel sensitivities of the channel to phosphoinositides and antidepressants. It is anticipated that this assay may find widespread use in the mK\(_{ATP}\) field, leading ultimately to the identification of this important channel.

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Disclosures

None.

References


We describe a novel thallium flux assay for measurement of mKATP activity.

The rapid loss of mKATP activity after isolation is shown to be attributable to classical channel run-down and is recovered by the phospholipid PIP2.

Both the mKATP channel and ischemic preconditioning are inhibited by fluoxetine (Prozac).
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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. 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$_{\text{ATP}}$ 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. Protein concentration was determined by the Folin-phenol method.

mK$_{\text{ATP}}$ swelling assay: The activity of mK$_{\text{ATP}}$ was monitored spectrophotometrically at 520 nm, as the light scatter (absorbance) change due to K$^+$ uptake and swelling, as previously described, within 1.5 hr of mitochondrial isolation. Briefly, mitochondria (0.25 mg/mL) were added rapidly to a stirring cuvette containing mK$_{\text{ATP}}$ swelling buffer (100 mmol/L KCl, 10 mmol/L HEPES, 2 mmol/L succinate, 2 mmol/L MgCl$_2$, 2 mmol/L KH$_2$PO$_4$, 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$_{\text{ATP}}$ 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. 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. 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$_2$HPO$_4$, 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 TlSO₄ stimulus via a syringe port to the stirring cuvette. The change in fluorescence was monitored in a 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 MgSO₄, 25 mmol/L NaHCO₃, 1.2 mmol/L KH₂PO₄, 11 mmol/L glucose, 2.5 mmol/L CaCl₂, gassed with 95% O₂, 5% CO₂) in constant flow mode, essentially as described. 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 for oocyte expression.

**Electrophysiology:** Xenopus oocytes were harvested, dissociated and defolliculated by collagenase type I (Sigma) treatment. 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 MgCl₂, 1.8 CaCl₂, 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 CaCl₂, 1 MgCl₂ and 5 HEPES with pH adjusted to 7.4 with KOH. For Kir6.2 experiments solutions contained (in mmol/L): 110 KCl, 2 CaCl₂, 1 MgCl₂ 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. Ba²⁺-sensitive currents were determined using bath solution containing 5 mmol/L BaCl₂.
**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$_{\text{ATP}}$ channel activity were determined. In Figure 2, the novel Ti$^+$ assay was used, whereas here in Online Figure I the mK$_{\text{ATP}}$ was assayed using the classical mK$_{\text{ATP}}$ osmotic swelling assay.

Online Figure II shows dose responses of mK$_{\text{ATP}}$ 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 (Ti$^+$ and swelling) yielded comparable results.

Online Figure IIIA shows the dose response to FLX of Kir4.1 and Kir6.2Δ36 channels expressed in Xenopus oocytes. Online Figure IIB reproduces the data in Figure 3B of the main manuscript, using the osmotic swelling mK$_{\text{ATP}}$ assay instead of the Ti$^+$ assay. Online Figure IIIC shows the ability of FLX to close the mK$_{\text{ATP}}$ 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$_{\text{ATP}}$.

*Investigation into the modulation of mitochondrial K$^+$/H$^+$ exchanger (KHE) and its effects on the Ti$^+$ 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$_{\text{ATP}}$ (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 Ti$^+$ 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\textsubscript{ATP} channel run down: In a recent study\textsuperscript{10}, the authors recovered mK\textsubscript{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\textsubscript{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\textsuperscript{+} 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\textsubscript{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\textsubscript{ATP} channel re-activated by PIP\textsubscript{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\textsuperscript{10}.

REFERENCES


Online Figure I: PIP$_2$ modulation of mK$_{\text{ATP}}$ channel activity using the swelling assay. (A): mK$_{\text{ATP}}$ 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 ($\Delta$OD$_{520}$ 0.022±0.001) while 5 hr. mitochondria were normalized to control + PIP$_2$ (the 3rd gray bar, $\Delta$OD$_{520}$ 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<sub>ATP</sub> activity using the swelling and BTC-AM-Tl<sup>+</sup> assays. (A): UDP-dependent activation of the mK<sub>ATP</sub> channel determined with the swelling assay. Since the channel is open under baseline conditions, the mK<sub>ATP</sub> was closed with 1 mmol/L ATP and UDP was then added to re-open the channel. Data were plotted as % mK<sub>ATP</sub> 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<sub>50</sub> was found to be 22.0 ± 4.5 µmol/L. (B): UDP-dependent activation of the mK<sub>ATP</sub> channel using the BTC-AM-Tl<sup>+</sup> assay. Conditions were as in panel A and the EC<sub>50</sub> was found to be 17.1 ± 2.1 µmol/L. (C): ATP-dependent inhibition of mK<sub>ATP</sub> using the swelling assay. The IC<sub>50</sub> of ATP was found to be 4.5 ± 2.5 µmol/L. (D): ATP-dependent inhibition of mK<sub>ATP</sub> channel activity using the BTC-AM-Tl<sup>+</sup> assay. Conditions were as in panel C and the IC<sub>50</sub> 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 Kir channel activity by fluoxetine. (A): Dose response to fluoxetine treatment in Xenopus oocytes expressing either Kir4.1 or Kir6.2Δ36 channel subunits. The Kir6.2Δ36 mutant subunit, where the endoplasmic reticulum retention motif was deleted, was used to allow channel expression at the cell surface without the sulphonylurea receptor. Data points represent means ± SEM, N>6. (B): mKATP 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. # P<0.05 versus AA5/DZX+ATP. (C): FLX blocks AA5- and DZX-mediated mKATP 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$_{\text{ATP}}$-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·min$^{-1}$, $\times 10^3$) 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\geq4, *P<0.05 vs. IR.