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A novel mitochondrial K_{ATP} channel assay

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Abstract

Rationale—The mitochondrial ATP sensitive potassium channel (mK_{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_{ATP} activity is disputed.

Objective—We sought to develop novel methods to assay mK_{ATP} activity and its regulation.

Methods & Results—Using a thallium (Tl^+) sensitive fluorophore, we developed a novel Tl^+ flux based assay for mK_{ATP} activity, and used this assay probe several aspects of mK_{ATP} function. The following key observations were made: (i) Time-dependent run-down of mK_{ATP} activity was reversed by phosphatidylinositol-4,5-bisphosphate (PIP_2). (ii) Dose responses of mK_{ATP} to nucleotides revealed a UDP EC_{50} of ~20 μmol/L and an ATP IC_{50} of ~5 μmol/L. (iii) The antidepressant fluoxetine (Prozac™) inhibited mK_{ATP} (IC_{50} 2.4 μmol/L). Fluoxetine also blocked cardioprotection triggered by IPC, but did not block protection triggered by a mK_{ATP} independent stimulus. The related antidepressant zimelidine was without effect on either mK_{ATP} or IPC.

Conclusions—The Tl^+ flux mK_{ATP} assay was validated by correlation with a classical mK_{ATP} channel osmotic swelling assay (R^2 0.855). The pharmacologic profile of mK_{ATP} (response to ATP, UDP, PIP_2, and fluoxetine) is consistent with that of an inward rectifying K^+ channel (K_{IR}) and is somewhat closer to that of the $K_{IR}6.2$ than the $K_{IR}6.1$ isoform. The effect of fluoxetine on mK_{ATP}-dependent cardioprotection has implications for the growing use of antidepressants in patients who may benefit from preconditioning.

Keywords

mitochondrial ATP sensitive potassium channel; ischemia; reperfusion; ischemic preconditioning; fluoxetine

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DISCLOSURES None

INTRODUCTION

The mitochondrial ATP sensitive potassium channel (mK_{ATP}) is thought to be essential for cardioprotection recruited by ischemic preconditioning (IPC),[1, 2] but despite intense research the molecular identity of this channel remains unclear. The simplest thesis is that mK_{ATP} channels are derivative of surface K_{ATP} channels, and thus composed of inward rectifying K^+ channels (K_{IR}) and sulfonylurea receptors (SUR). The cardiomyocyte surface K_{ATP} channel is comprised of $K_{IR}6.2$ and SUR2A isoforms,[3] but efforts to conclusively assign these proteins to the cardiac mK_{ATP} have been unsuccessful to date.

Neither Kir6 nor SUR genes contain mitochondrial target sequences, and Kir6/SUR proteins are not found in mitochondrial proteome databases or prediction engines.[4, 5] Furthermore, immune-based methods to detect K_{IR}/SUR subunits in mitochondria are plagued by issues of antibody specificity[6] and mitochondrial purity/contamination. Several of the key pharmacologic reagents used to study mK_{ATP} channels (e.g. the agonist diazoxide (DZX) and antagonist 5-hydroxydecanoate (5-HD)) are also known to exhibit off-target effects.[7, 8] Targeted gene deletion in mice to identify the mK_{ATP} channel involved in IPC has proven futile, due to the confounding cardiovascular effects of knocking out $K_{IR}6$ and SUR genes (*Kcnj8*, *Kcnj11*, *Abcc8*, and *Abcc9*) on surface K_{ATP} channel function. In general, K_{IR} and SUR knockouts exhibit profound defects in glucose/insulin handling,[9–12] which impacts the response to IPC.[13]

A recent study using custom-made antibodies and SUR knockout mice identified shortform splice variants of SUR2 in mitochondria.[14] Furthermore, recent pharmacological evidence suggests that complex II of the respiratory chain (succinate dehydrogenase) may be a regulatory component of the mK_{ATP} channel.[15, 16] However, both these findings leave the identity of the K^+ channel-forming subunit of mK_{ATP} unknown. In this regard, mK_{ATP} is similar to other mitochondrial ion channels which exist at a phenomenological level but have not been molecularly identified (e.g., the mitochondrial Ca^{2+} uniporter).

A major obstacle in studying the mK_{ATP} channel has been the availability of a reliable assay. Most studies to date have utilized 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.[17, 18] This assay has been criticized as irreproducible by some laboratories,[19] with the precise timing of mitochondrial isolation appearing to be a critical factor.[20]

Studying the literature on surface K_{ATP} channels, two key biochemical properties that appeared to have been overlooked in the mK_{ATP} channel field were the permeability of surface K_{ATP} channels for the heavy metal thallium (Tl^+),[21] and the modulation of channel run-down by phospholipids such as phosphatidylinositol-4,5-bisphosphate (PIP_2).[22, 23] Herein, we developed a novel Tl^+ fluorescence based assay for mK_{ATP} channel activity, and used this assay to show that the channel is subject to run-down that is reversed by PIP_2. It is anticipated that both these discoveries will advance the study of this channel. Furthermore, the antidepressant fluoxetine (FLX), which is known to modulate K_{IR} channels,[24, 25] was found herein to block the mK_{ATP} channel and to block IPC, but FLX did not block mK_{ATP}-independent cardioprotection. The implications of these data for clinical use of FLX in cardiovascular disease patients are discussed.

METHODS

Full experimental details are in the online supplement. Cardiac mitochondria were rapidly isolated from male Sprague-Dawley rat hearts by differential centrifugation in sucrose-based buffer as previously described.[20] Protein was determined by the Folin-phenol method.[26]

Within 1.5 hr of mitochondrial isolation the activity of mK_{ATP} was monitored by the osmotic swelling assay as previously described.[20]

A novel fluorescence-based Tl^+ flux assay for mK_{ATP} activity was also developed. The ionic radii of Tl^+ (0.154 nm) and K^+ (0.144 nm) are similar,[27] and thus Tl^+ is widely used as an analog to study membrane K^+ transport.[21, 27–30] The assay made use of the fluorescent indicator BTC-AM, which is better known as a ratiometric Ca^{2+} 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^+ assay buffer at 37°C. Tested compounds were present from the beginning of the assay, and baseline fluorescence was recorded for 10 s. prior to addition of $TlSO_4$ (2 mmol/L final) via a syringe port. Fluorescence was monitored in a Varian Cary Eclipse spectrofluorometer (λ_{ex}=488nm, λ_{em}525nm) and normalized to baseline. Full details including the concentrations and preparation methods for all reagents used in the assay, are in the online supplement.

Isolated rat heart perfusions (Langendorff) were performed as previously described.[16] Following 20 min. equilibration, hearts were divided into 7 groups: (i) IR alone, comprising 20 min. vehicle (water or DMSO) infusion, 30 s. wash-out, 25 min. global ischemia, 120 min. reperfusion; (ii) FLX + IR, comprising 20 min. FLX infusion (5 μmol/L), 30 s. wash-out, then IR; (iii) IPC + IR, comprising 3 × 5 min. ischemia interspersed with 5 min. reperfusion, then IR; (iv) FLX + IPC + IR, comprising 5 min. FLX infusion (5 μmol/L), plus FLX infused throughout the 3 reperfusion phases of IPC (i.e. 20 min. total FLX delivery), 30 s. wash-out, then IR; (v) Zimelidine + IPC + IR. As above, replacing FLX with zimelidine (5 μmol/L). (vi) FCCP + IR, comprising 20 min. FCCP infusion (30 nmol/L [31, 32]), then IR; vii) FCCP + FLX + IR, comprising 20 min. infusion of both FCCP (30 nmol/L) and FLX (5 μmol/L), then IR. Following reperfusion hearts were stained with tetrazolium chloride (TTC), imaged, and infarct size measured as previously described.[16]

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 mK_{ATP} 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.[21, 27–30] A fluorescent probe that responds to [Tl^+] is commercially available (FluxOR™, Invitrogen, Carlsbad CA), but careful analysis of the literature underlying this reagent revealed that the active component was BTC-AM, a more economical reagent.[28–30] 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 due to 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 K_{ATP} channel. Furthermore, the effect of ATP could be overridden by the mK_{ATP} channel opener AA5, and this effect was in-turn blocked by the mK_{ATP} antagonist 5-HD. These data are quantified in Figure 1B, which also shows the effects of mK_{ATP} reagents DZX (agonist) and glyburide (antagonist). The ionophores valinomycin and nonactin, both of which transport Tl^+,[33] 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 due to balancing of Tl^+ influx by its efflux through the K^+/H^+ exchanger (KHE). However, attempts to modulate KHE activity with the inhibitors DCCD and quinine were inconclusive (data not shown). Validation of the Tl^+ assay for mK_{ATP} channel activity was also performed by a direct comparison with results from mK_{ATP} 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 K_{IR} channels is their tendency to "run-down" over time, a phenomenon attributed to loss of the phospholipid PIP_2 from a binding site on the channel.[34] The mK_{ATP} channel (which is constitutively open in isolated mitochondria) also loses activity following mitochondrial isolation, which may underlie the reported poor reproducibility of mK_{ATP} channel activity measurements.[19] Upon investigating the relationship between these phenomena, it was found that incubation of mitochondria on ice for 5 hrs. resulted in complete loss of mK_{ATP} channel activity, and that PIP_2 addition restored channel activity (Figure 2). Furthermore, the full pharmacologic profile of mK_{ATP} channel activity (i.e. inhibition by ATP, activation by AA5, and re-inhibition by 5HD) was recovered in PIP_2-treated aged mitochondria. The same concentrations of the PIP_2 breakdown products inositol triphosphate (IP_3), 1,2-dioctanoyl glycerol (DOG), or 1,2-dipalmitoyl glycerol (DPG) did not affect mK_{ATP} activity. The polyvalent cation neomycin, which is known to inhibit K_{IR} channel activity by sequestering PIP_2,[35] was able to reverse the mK_{ATP} channel-restorative effects of PIP_2. Identical results were obtained with the osmotic swelling mK_{ATP} channel assay (Figure S1). Overall these data suggest that the mK_{ATP} channel contains a PIP_2 sensitive subunit, possibly a K_{IR} channel. Consistent with this, both the Tl^+ and swelling assays revealed that mK_{ATP} sensitivity to the nucleotides UDP and ATP (Figure S2) was closer to that of the $K_{IR}6.2$ than the $K_{IR}6.1$ isoform.[36–39]

Several classes of K_{IR} channel are known to be inhibited by fluoxetine (Prozac™), an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class.[24, 25] As shown in Figure S3, $K_{IR}6$ channels (components of K_{ATP} channels) are an order of magnitude more sensitive to FLX than $K_{IR}4$ channels, a well-known FLX target.[40] Thus, we investigated the possibility that FLX might block mK_{ATP} channels. As shown in Figure 3, FLX blocked mK_{ATP} channel activity with an IC_{50} of 2.3 μmol/L, while a related SSRI zimelidine (ZM) did not. Identical results were obtained with the osmotic swelling mK_{ATP} assay (Figure S3). Furthermore, FLX blocked AA5- or DZX-mediated opening of mK_{ATP} (Figure S3).

Given the importance of the mK_{ATP} channel in IPC, we hypothesized that FLX may block IPC. Figure 3 shows that 5 μmol/L FLX completely blocked IPC-mediated cardioprotection in a rat perfused heart model of IR injury, while ZM was without effect. Notably FLX did not enhance baseline IR injury in this model, indicating that blockage of IPC was not due to an equal-but-opposite injurious effect, canceling out cardioprotection. Furthermore, FLX had no effect on cardioprotection mediated by FCCP (Figure S4), which occurs independent of the mK_{ATP} channel.[31, 32]

DISCUSSION

The major findings of this study are as follows: (i) Development of a novel Tl^+ flux based assay for the mK_{ATP} channel; (ii) Time-dependent loss of mK_{ATP} channel activity is a genuine run-down phenomenon and is reversed by PIP_2; (iii) FLX blocks both mK_{ATP} channel activity and IPC-mediated cardioprotection. This is the first demonstration of the modulation of a mitochondrial ion channel by PIP_2, and the first identification of a mitochondrial ion channel target for FLX. Collectively, the data support the concept that

mK_{ATP} contains a bona fide K_{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.[41]

Work on the mK_{ATP} channel to date has relied on a variety of assays, many of which measure downstream effects of mitochondrial K^+ uptake such as changes in respiration,[42] matrix alkalinization,[42] flavoprotein fluorescence,[43] and swelling induced light scatter.[42] Such methods are limited by the ability of other mitochondrial phenomena (e.g. electron transport chain activity, volume changes, membrane potential) to interfere with the measured parameters. Direct measurement of mitochondrial K^+ fluxes using the potassium-binding fluorescent indicator (PBFI) is difficult because its K_d for K^+ of ~8 mmol/L[42] would result in saturation at typical intramitochondrial K^+ levels (~150 mmol/L).[44] Thus herein we chose to exploit another property of K^+ channels, their ability to transport Tl^+ as a surrogate for K^+.[21, 27] While Tl^+ acetate has previously been used in swelling-based studies on mK_{ATP}[45], this study is the first application of a Tl^+ sensitive probe, BTC-AM,[28–30] to study mK_{ATP}.

The kinetics of the Tl^+ based mK_{ATP} channel assay are superior to those of the swelling based assay.[16] Following Tl^+ addition maximal fluorescence is attained within 2–4 s., compared to a time-lag of 20–30 s. for maximal signal intensity in the osmotic swelling assay. Unfortunately the high flux rate of Tl^+ through K^+ channels (~2x K^+ flux [21]), 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.[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.[20] Previous work showed that the purified mK_{ATP} channel runs-down in an electrophysiology setting and can be re-activated by very high concentrations of UDP.[48] However, the cause of channel activity loss in intact mitochondria was unknown, and could easily be due 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.[49]

K_{ATP} channels were the first channels identified to depend on phosphoinositides such as PIP_2,[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, [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+},[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 (i.e. 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 (e.g., PI_3K), phospholipases (e.g., PLC), and other components of the IP_3/DAG signaling pathway in regulating mK_{ATP} is also unknown, but the involvement of such signaling components in IPC[52, 53] suggests a potential novel pharmacological target (i.e., mitochondrial PIP_2 turnover) to modulate preconditioning.

The Tl^+ assay was also used to probe the response of mK_{ATP} to nucleotides (Figure S2). The mK_{ATP} EC_{50} for UDP (~20 μmol/L) was closer to that of K_{IR}6.2 (~200 μmol/L) than K_{IR}6.1 (~4 mmol/L), and the mK_{ATP} IC_{50} for ATP (~4.5 μmol/L) was also closer to that of K_{IR}6.2 (~15 μmol/L) than K_{IR} 6.1 (~350 μmol/L). While these data agree with previous studies on

mK_{ATP},[48] 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. [6, 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}. Fluoxetine has previously been shown to inhibit K_{IR} channels, while related SSRIs (e.g. zimelidine) had no effect.[24, 25] Our data (Figure S3) suggest that $K_{IR}6$ channels may be the most sensitive to FLX of all K_{IR} isoforms,[24, 25, 40, 63] and in agreement with this the mK_{ATP} exhibits a strikingly low FLX IC_{50} of 2.4 μmol/L (Figure 3). Physiological concentrations of FLX are in the range of 1 – 20 μmol/L.[63] The fact that FLX is a lipophilic cation (LogP 4.8),[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.[1, 16, 65] The lack of effect of another SSRI, zimelidine, 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,[31, 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 current 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 US, antidepressants are the most commonly prescribed class of medication,[66] with FLX alone prescribed >23 million times in 2008.[67] Although SSRIs are known to negatively impact the outcome of cardiac surgery,[41] they are widely prescribed to patients with acute coronary syndrome.[68] Notably, while IPC elicits solid protection in animal models of IR injury, its application in humans is limited by confounding effects such as age,[69] gender,[70] diabetes,[13] and other medications.[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 lelvels[72] and to induce cardioprotection,[73] suggesting that some of the protective effects of lithium previously attributed to GSK-3β inhibition[73] 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.

Novelty and Significance (Wojtovich *et al.*)

What is known?

- The mitochondrial ATP-sensitive potassium channel (mK_{ATP}) mediates protection from cardiac ischemia reperfusion injury by ischemic preconditioning.
- The molecular identity of the mK_{ATP} remains controversial and the validity of current methods to assay mK_{ATP} activity is disputed.

- Another limitation to the investigation of this channel is the rapid (~ 1 hr.) loss of channel activity following mitochondrial isolation.

What new information does this article contribute?

- We describe a novel thallium flux assay for measurement of mK_{ATP} activity.
- The rapid loss of mK_{ATP} activity after isolation is shown to be due to classical channel run-down, and is recovered by the phospholipid PIP_2.
- Both the mK_{ATP} channel and ischemic preconditioning are inhibited by fluoxetine (Prozac™).

Summary of Novelty and Significance

Cardiac ischemia/reperfusion (IR) injury is an important worldwide morbidity factor. Strategies to protect the heart from IR injury (such as during heart attack) are limited, but one promising avenue is ischemic preconditioning (IPC). The mitochondrial ATPsensitive K_+ channel (mK_{ATP}) has been suggested to mediate the protection afforded by IPC; however, the molecular identity of this channel is unknown, and its assay is also technically challenging, thus hindering drug-development efforts. Using a Tl_+-sensitive fluorophore, a novel assay was developed herein to measure mK_{ATP} activity. Using this assay, we show that loss of mK_{ATP} channel activity over time is reversed by the lipid PIP_2. These findings should greatly facilitate mK_{ATP} research, hopefully leading to a molecular identity. Furthermore, this is the first report of a PIP_2 sensitive phenomenon in mitochondria; it may possibly relate to the mechanism of channel regulation in IPC itself. Finally, we found that the antidepressant fluoxetine (Prozac™) inhibited mK_{ATP} and also blocked the protective effects of IPC. Given the widespread use of fluoxetine in cardiac patients, this may have important implications for the potential application ofIPC in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FLX	Fluoxetine
ZM	Zimelidine
BTC-AM	Benzothiazole coumarin acetyoxymethyl ester
PIP_2	phosphatidylinositol-4,5-bisphosphate
5-HD	5-hydroxydecanoate
AA5	Atpenin A5
Glyb	Glyburide

DCCD	N,N′-Dicyclohexylcarbodiimide
DZX	Diazoxide
IPC	Ischemic preconditioning
Kir	Inward rectifying potassium channel
SUR	sulfonylurea receptor
Neo	Neomycin
DOG	1,2-dioctanoyl glycerol
IP_3	Inositol triphosphate
DPG	1,2-dipalmitoyl glycerol
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

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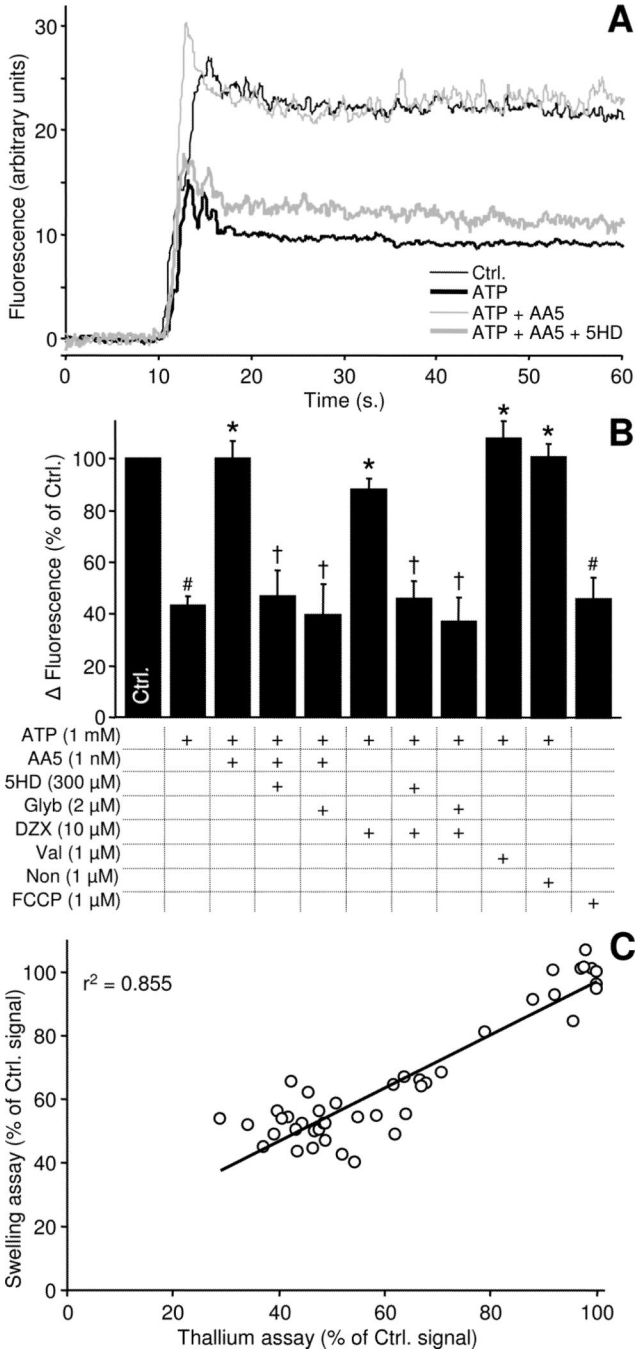

Figure 1. BTC-AM Tl^+ fluorescence assay for mK_{ATP} activity in isolated heart mitochondria

(A): Representative traces of BTC-AM Tl^+ fluorescence in isolated mitochondria. Fluorescence (λ_{ex} 488 nm, λ_{em} 525 nm) was normalized to the 10 s. of baseline prior to the injection of $TlSO_4$ (arrow). Where indicated, 1 mmol/L ATP (thick, black), 1 nmol/L AA5 plus ATP (thin, grey), or 300 μmol/L 5HD plus AA5 plus ATP (thick grey) 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 30–60 s. Delta fluorescence in controls was 26.4 ± 3.3 arbitrary units. Experimental conditions are listed below the *x*-axis. Data are mean ± SEM, N≥4. # P<0.05 versus control,

* P<0.05 versus ATP, † P<0.05 versus the effect of ATP+AA5 or ATP+DZX. **(C):** Correlation between two assays for mK_{ATP} channel activity. Experiments using a variety of conditions that modulate the mK_{ATP} were performed using either the osmotic swelling assay and the BTC-AM-Tl^+ assay. Both sets of results were expressed as a percent of their respective controls. Linear regression curve fit revealed an r^2 of 0.855.

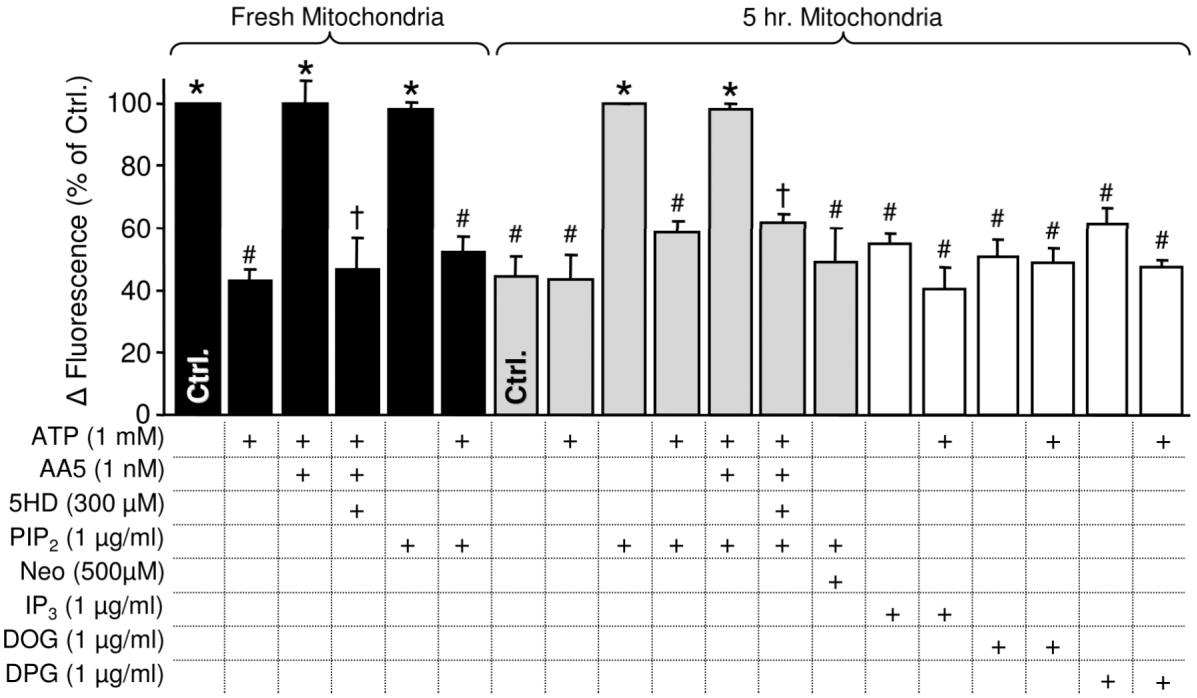

Figure 2. PIP_2 modulation of mK_{ATP} channel activity using the swelling and BTC-AM-Tl^+ assays

mK_{ATP} activity was monitored using the BTC-AM-Tl^+ assay in fresh mitochondria (black bars), or mitochondria 5 hrs. post isolation (gray and white bars). Fresh mitochondria data were normalized to control (Δ fluorescence 26.4±3.3) while 5 hr. mitochondria were normalized to control + PIP_2 (3rd gray bar, Δ fluorescence 29.3±6.4). Experimental conditions are listed below the *x*-axis. Data in both panels 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.

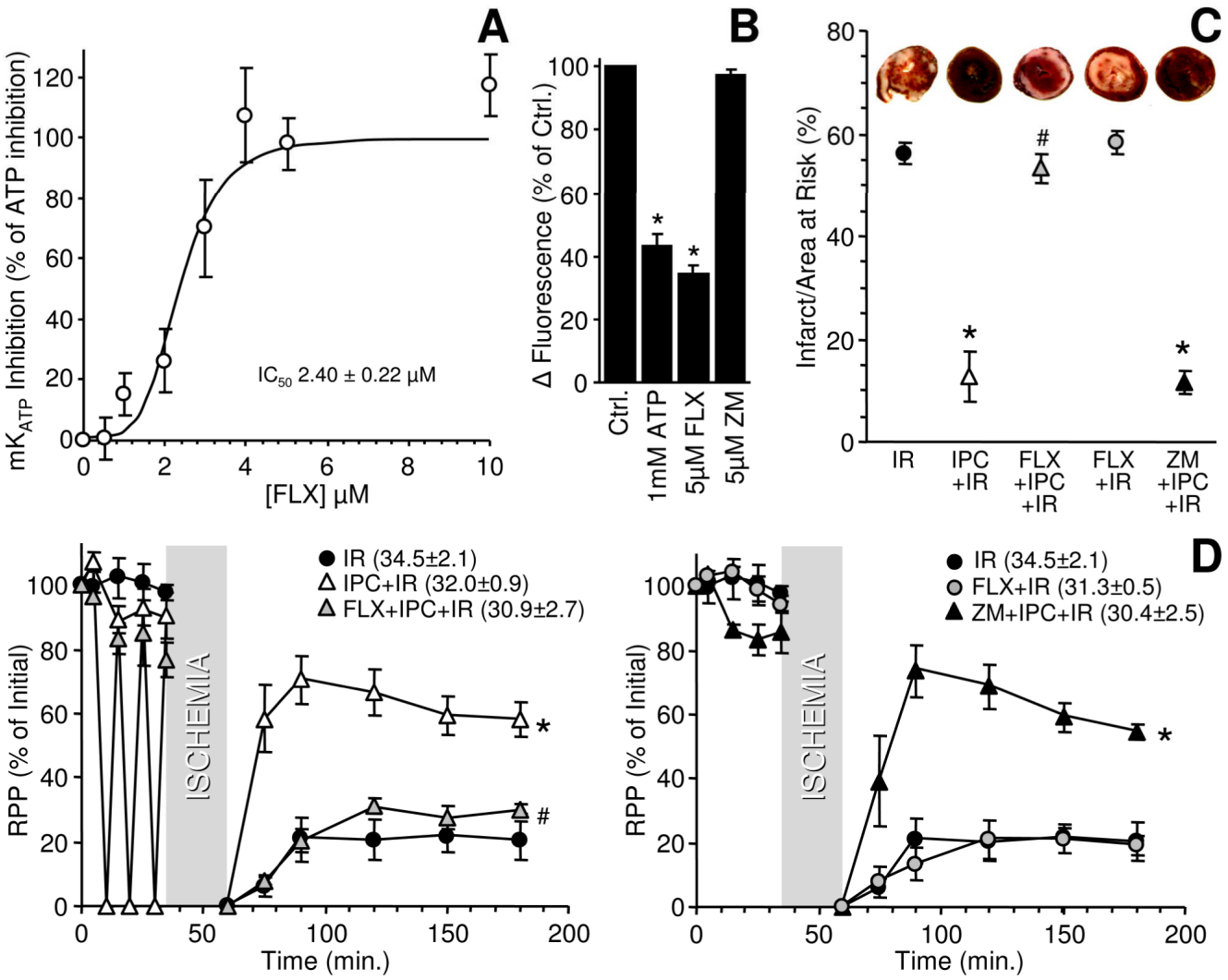

Figure 3. Modulation of mK_{ATP} activity and IPC-mediated cardioprotection by fluoxetine

(A): FLX dose-response of mK_{ATP} activity. Activity was measured using the BTC-AM-Tl^+ assay. Data were plotted as % mK_{ATP} inhibition, with 100% inhibition defined as the condition in the presence of 1 mmol/L ATP, and 0% closed (i.e. 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_{50} to be 2.39 ± 0.22 μmol/L. Data are means ± SEM, N≥4. **(B):** mK_{ATP} activity was measured by the BTC-AM-Tl^+ assay. Where indicated, FLX or ZM were present. Data are means ± SEM, N≥4. *P<0.05 versus control. **(C):** Effect of FLX or ZM on IR injury and IPC. Hearts were subjected to Langendorff perfusion as detailed in the methods. Infarct size / area at risk was quantified from TTC staining, with representative stained hearts shown above each condition. Data are means ± SEM, N≥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 two panels for clarity (IR data shown in both panels), and are means ± SEM, N≥4. *P<0.05 vs. IR, #P<0.05 vs. IPC+IR. The initial RPP (mmHg·min^{-1}, $\times 10^3$) for each group is listed in the legend.