The Mitochondrial ROMK Channel is a Molecular Component of MitoK\textsubscript{ATP}

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ABSTRACT

**Rationale.** Activation of the mitochondrial ATP-sensitive potassium channel (mitoK$_{\text{ATP}}$) has been implicated in the mechanism of cardiac ischemic preconditioning, yet its molecular composition is unknown.

**Objective.** To use an unbiased proteomic analysis of the mitochondrial inner membrane to identify the mitochondrial K$^+$ channel underlying mitoK$_{\text{ATP}}$.

**Methods and Results.** Mass spectrometric analysis was used to identify KCNJ1(ROMK) in purified bovine heart mitochondrial inner membrane and confirmed that ROMK mRNA is present in neonatal rat ventricular myocytes and adult hearts. ROMK2, a short form of the channel, is shown to contain an N-terminal mitochondrial targeting signal and a full length epitope-tagged ROMK2 colocalizes with mitochondrial ATP synthase $\beta$. The high-affinity ROMK toxin, tertiapin Q, inhibits mitoK$_{\text{ATP}}$ activity in isolated mitochondria and in digitonin-permeabilized cells. Moreover, shRNA-mediated knockdown of ROMK inhibits the ATP-sensitive, diazoxide activated, component of mitochondrial thallium uptake. Finally, the heart-derived cell line, H9C2, is protected from cell death stimuli by stable ROMK2 overexpression, while knockdown of the native ROMK exacerbates cell death.

**Conclusions.** The findings support ROMK as the pore-forming subunit of the cytoprotective mitoK$_{\text{ATP}}$ channel.

**Keywords:**
ATP-sensitive potassium channel, mitochondria, apoptosis, preconditioning, ischemia, cytoprotection, renal outer medullary potassium channel

**Non-standard Abbreviations:**
MitoK$_{\text{ATP}}$  Mitochondrial ATP-sensitive potassium channel
MitoROMK  Mitochondrial renal outer medullary potassium channel
SUR  Sulfonylurea receptor
tBHP  tert-butyl hydroperoxide
TPNQ  Tertiapin-Q
INTRODUCTION

The heart possesses an innate ability to protect itself against ischemic injury through a mechanism known as preconditioning\(^1\), whereby one or more cycles of brief ischemia and reperfusion trigger resistance to a subsequent prolonged ischemia. This infarct-sparing effect lasts for several hours (early preconditioning) and also re-emerges as a “second window” of protection that is present 24-72 hours after the initial preconditioning event\(^2\). Potassium channels were implicated as critical mediators of endogenous cardioprotection when it was observed that the effects of ischemic preconditioning were inhibited by K\(^+\) channel blockers\(^3\) and could be mimicked by openers of ATP-sensitive potassium (K\(_{\text{ATP}}\)) channels. The latter effect was independent of changes in the cardiac action potential\(^4,5\). Moreover, the K\(^+\) channel opener diazoxide, an effective cardioprotective agent, is much more potent at enhancing K\(^+\) flux at the mitochondrial, rather than the sarcolemmal, membrane\(^6\) and it protects cells against injury while having little impact on surface membrane K\(_{\text{ATP}}\) current\(^7,8\). Thus, mitoK\(_{\text{ATP}}\), originally described through single-channel recordings of ATP-sensitive K\(^+\) currents in giant liver mitoplasts\(^9\), was linked to ischemic preconditioning\(^10\), ischemic postconditioning\(^11,12\), and cytoprotection in general. Since then, numerous methods have been used to study mitoK\(_{\text{ATP}}\) in cells, mitochondria and tissues\(^7,13-15\), but the low copy number of the channels in the mitochondrial membrane\(^16\), its fleeting activity \textit{in vitro}\(^17\), and the confounding nonspecificity of available pharmacological agents\(^10\) and antibodies\(^18\), has hampered efforts to identify the channel at a molecular level, fostering persistent skepticism among some investigators as to the nature, and even existence, of mitoK\(_{\text{ATP}}\)\(^19,20\).

Here, we employ an unbiased proteomic approach to identify KCNJ1(ROMK) in the mitochondrial inner membrane and demonstrate that ROMK channels localize to mitochondria, mediating ATP-sensitive K\(^+\) flux and conferring protection against cell death stimuli, consistent with mitoROMK being the pore-forming subunit of mitoK\(_{\text{ATP}}\).

METHODS

Methods are described in detail in the Online Supplement available at http://circres.ahajournals.org.

RESULTS

A large scale proteomic analysis of enriched mitochondrial inner membrane fractions from highly purified bovine mitochondria was undertaken to identify low abundance proteins that were underrepresented in previous mitochondrial studies. More than 20 million spectra were collected by two-dimensional liquid chromatography mass spectrometry (2DLC-MS/MS) and 964 proteins were identified with high confidence. The proteomic data were compared with previously published compendia of mitochondrial proteins\(^21,22\). A total of 687 proteins matched either the Mitocarta database\(^21\) or the mitochondrial annotation of the Uniprot KnowledgeBase. However, we identified 186 additional proteins that were likely to be mitochondrial (high Maestro scores\(^22\)), for which there was no previous mass spectral evidence in the heart. Two overlapping peptides, LCLLIR and GGKLCLLIR (Figure 1A), uniquely matched the predicted protein sequence of the bovine KCNJ1 gene product, Kir1.1 (the Renal Outer Medullary Kidney channel, ROMK). The identification was validated statistically (P\(>95\%\); Peptide Prophet\(^23\)) and matching spectra had overlapping contiguous b- and y-ion series (Figure 1A). The ROMK channel is highly expressed in the kidney\(^24\), where the channel mediates K\(^+\) recycling in the thick ascending limb and K\(^+\) secretion in the cortical collecting duct of the nephron. Although expression levels were low in non-renal tissues, we confirmed by reverse transcriptase PCR (RT-PCR) that ROMK isoforms are present (Figure 1B) in neonatal rat ventricular myocytes (NRVM; ROMK1, 2, 6) and adult rat hearts (ROMK1, 2), as well as in brain (ROMK1, 2, 3, 6) and liver (ROMK1, 2) - all of which have...
been reported to have mitoK\textsubscript{ATP} activity\textsuperscript{24}. Of the isoforms found in the heart, the only difference at the protein level is that ROMK1 has an extra 19 amino acids at the N-terminus as compared to ROMK2 or ROMK6\textsuperscript{25}.

Intriguingly, bioinformatic analysis of the bovine ROMK sequence with mitochondrial localization algorithms indicated that trafficking to the mitochondrion was highly likely, yielding probabilities of 99.5\%, 89.9\%, and 99\% with the Mitoprot II, Target P, and Mitopred algorithms, respectively. In a recent genome-wide ranking of mitochondrial localization likelihood in mouse and humans\textsuperscript{21}, ROMK/KCNJ1 had the highest ranking of all the inward rectifier K\textsuperscript{+} channel (Kir) genes. Likewise, among Kir protein sequences in the UniprotKB rat database, ROMK2 (accession no. P35560-2) had the highest mitochondrial localization probability (99\%) according to Mitopred; consequently, we focused on ROMK2 as the prime candidate. These predictions were confirmed experimentally. A ROMK2 construct containing a c-terminal tag (V5 epitope) was heterologously expressed in H9C2 cells, a rat embryonic heart-derived cell line. Cells transfected with ROMK2-V5 were fixed and subjected to immunofluorescence labeling with a V5-specific antibody and imaged using a dual color super-resolution stimulated-emission depletion fluorescence microscope (Leica TCS STED). ROMK2-V5 fluorescence (Alexa 488 secondary Ab) was highly correlated with the mitochondrial marker, ATP synthase \(\beta\) (Pacific Orange 568 secondary Ab), indicating subcellular localization of the channel in mitochondria. Similarly, specific mitochondrial enrichment of ROMK was demonstrated in Chinese Hamster Ovary (CHO) cells transiently transfected with a ROMK2-eGFP fusion protein; GFP signal increased in intensity with stepwise purification of mitochondrial membranes by differential centrifugation in concert with a mitochondrial marker (VDAC), varying inversely with a plasma membrane marker (connexin 37; Figure 2B). We also tested the prediction (from MitoProt II) that the first 24 amino acids of ROMK2 constituted a mitochondrial targeting sequence sufficient to impart mitochondrial targeting. The sequence MFKHLRKVVTRFFGHSRQRARL was fused to the N-terminus of eGFP, transiently transfected into neonatal rat ventricular myocytes, and imaged in living cells (Figure 2C; two-photon laser scanning fluorescence microscopy). The eGFP signal predominantly co-localized with the mitochondrial membrane potential probe tetramethylrhodamine methyl ester (TMRM), confirming that the ROMK2 N-terminal signal confers mitochondrial protein targeting.

Next, we determined whether ROMK plays a role in modulating mitochondrial K\textsuperscript{+} fluxes in isolated mitochondria, a cardiac-derived cell line (H9C2), and primary cultures of neonatal rat ventricular myocytes (NRVMs), employing a high affinity K\textsuperscript{+} channel toxin, pharmacological tools, and molecular methods.

The classical mitoK\textsubscript{ATP} assay, developed by Garlid and coworkers, employs the 90\(^\circ\) light scattering property of isolated mitochondria as a readout of mitochondrial matrix volume\textsuperscript{26}. It is based on the principle that the activation of K\textsuperscript{+} uptake into mitochondria is accompanied by the movement of osmotically-obligated water, counterbalanced by a K\textsuperscript{+}/H\textsuperscript{+} exchanger\textsuperscript{27}. The initial rate of mitochondrial swelling is inhibited by ATP, and this inhibition is reversed by the action of K\textsuperscript{+} channel opener compounds (e.g., cromakalim, diazoxide, etc.)\textsuperscript{13}. Conversely, mitoK\textsubscript{ATP} is inhibited by sulfonyleureas or by 5-hydroxydecanoate\textsuperscript{28}. The expected effects of ATP and diazoxide (30 \(\mu\)mol/L; in the presence of ATP) on mitochondrial swelling in isolated rat heart mitochondria were observed (Figure 3A). Notably, Tertiapin Q, a stable variant of a peptide bee venom toxin that is a high affinity pore-binding blocker of surface membrane ROMK channels\textsuperscript{29,30}, abrogates the effect of diazoxide (Figure 3A) with subnanomolar potency (IC\textsubscript{50} = 25 pmol/L; Figure 3B). The native K\textsubscript{ATP} sensitivity to Tertiapin Q was also investigated in H9C2 cells and in NRVMs by measuring the initial rates of thallium (Tl\textsuperscript{+}) uptake (a surrogate for K\textsuperscript{+}) into mitochondria in partially-permeabilized (digitonin-treated) cells using a fluorescent reporter assay (see Online Supplement: Methods). Tertiapin Q (100 nmol/L) reduced the rate of Tl\textsuperscript{+} uptake in both permeabilized H9C2 cells (Figure 3C and 3D) and NRVMs (Figure 3E and 3F). Tertiapin Q is highly selective for ROMK over Kir2.1 type inward rectifier K\textsuperscript{+} channels and is partially selective for ROMK.
over GIRK1 and KCa channels. GIRK channels have not been reported in mitochondria (and we found no mass spectrometric evidence for them) and although KCa channels are thought to be present in the mitochondrial inner membrane, our experiments were performed in the absence of Ca2+.

The Tertiapin Q experiments provided some indication that ROMK may be a component of the native mitoKATP; nevertheless, we sought molecular evidence that ROMK channels participate in mitochondrial K+ uptake directly. Therefore, we established a stable H9C2 cell line expressing two short hairpin RNA (shRNA) constructs targeted to the core exon of the ROMK channel and determined the effect of ROMK knockdown on the initial rates of Ti+ uptake into mitochondria in partially-permeabilized (digitonin-treated) cells. The amount of ROMK expression was reduced by 72% on the mRNA level, as confirmed by quantitative real-time PCR, compared to a negative control scrambled shRNA (0.021ng ROMK/ng 18s in control vs. 0.005ng ROMK/ng 18s in knockdown cells). Knockdown of the native ROMK protein was also confirmed by western blot (see Online Supplement Figure III). Knocking down endogenous ROMK expression resulted in a 70% decrease in the Ti+ uptake rate in H9C2 cells, measured in the absence of ATP, compared to the scrambled shRNA negative controls (Figure 4A and 4B). The degree of suppression of Ti+ uptake was similar to that observed for control cells treated with the mitoKATP inhibitor 5-hydroxydecanoate (5-HD; Figure 4A and 4B). In addition, Ti+ uptake was inhibited by 1 mmol/L ATP and this inhibition was reversed by 10 µM diazoxide in control cells (Figure 4C and 4D) or by the K+ ionophore valinomycin (see Online Supplement Figures I and II). However, 10 µM diazoxide did not reverse the ATP inhibition of Ti+ flux when the native ROMK was knocked down by stable shRNA expression, indicating that ROMK is a necessary component of the ATP-sensitive, diazoxide-activated, mitochondrial K+ channel.

The potential cytoprotective role of ROMK against oxidative stress-induced cell injury was examined by increasing or decreasing the level of ROMK expression in H9C2 cell lines and treating them with tert-butyl hydroperoxide (tBHP). Flow cytometry was used to count the number of apoptotic (Annexin-V positive) and necrotic (propidium iodide (PI) positive: indicates loss of surface membrane integrity) cells after incubation with tBHP. After 22 hours of tBHP (100 µmol/L) treatment, survival was markedly enhanced in the stable ROMK2-V5-overexpressing cell line compared to control cells, clearly evident as an increased population of living cells in the lower left quadrant of the fluorescence dot plot (Figure 5A; right panel). Summary data from five paired experiments in which cells were treated with either 50 or 100 µmol/L tBHP shows the protective effect of ROMK overexpression; the dose dependent decrease in live cells and increase in necrotic and apoptotic cells with tBHP treatment was blunted by ROMK (Figure 5B). Conversely, H9C2 cells in which the native ROMK was knocked down by stable expression of shRNA displayed increased sensitivity to tBHP-induced necrosis and apoptosis compared to a cell line expressing a negative control scrambled shRNA (Figures 5C and 5D).

DISCUSSION

Canonical KATP channels serve to couple membrane K+ conductance to the metabolic state of the cell. They typically consist of four K+-selective pore-forming subunits from the Kir6.x family of inward rectifiers and four auxiliary regulatory subunits from the sulfonylurea receptor family (SUR1/2). The precise Kir and SUR isoform composition of KATP channels varies by tissue, consistent with unique roles in the regulation of processes ranging from insulin secretion, to action potential modulation, to vascular tone. The composition of the mitochondrial KATP channel, however, has remained elusive, and dominant negative suppression or genetic knockout of Kir6.x channels has been ineffective in suppressing mitoKATP responses. Moreover, antibody-based identification of mitochondrial channel has been confounded by non-specific binding of the available reagents.

Apart from the abundant voltage-dependent anion channel (VDAC) of the mitochondrial outer membrane, proteomic efforts, thus far, have failed to unequivocally identify any ion channels in the inner...
membrane. The present approach differed from previous studies primarily by increasing the amount of mitochondrial starting material and increasing the degree of fractionation at the level of the membrane, in order to decrease the complexity of the peptide mixture analyzed by 2DLC-MS/MS. This allowed us to find more low abundance proteins, including ROMK, which was the only K⁺ channel to be identified with high confidence. From the peptide evidence in hand, it is not currently possible to determine which of the ROMK protein isoforms is present, since the peptides were from the common exon expressed in all ROMK channels²⁵. The data do not preclude the possibility that other K⁺ channels may be present, however, since a negative result by mass spectrometry cannot be taken as evidence of absence.

Confirmation of the ability of the ROMK2 leader sequence to target eGFP to the mitochondria, and localization of the full length ROMK2 to mitochondria were enough to motivate further investigation of mitoROMK properties; however, there were also several known characteristics of the ROMK channel that heightened our interest in ROMK as a candidate for mitoK₁ATP. In addition to its having the highest probably of mitochondrial targeting (>95%), as determined by multiple intracellular targeting algorithms, ROMK contains a Walker A type ATP binding motif on its C-terminus and, similar to K₁ATP channels, it is both activated and inhibited by ATP²⁴, ³⁶. Channel activity of excised membrane patches containing ROMK channels rapidly runs down when Mg²⁺ is present. This effect can be reversed by applying MgATP or the catalytic subunit of protein kinase A, suggesting phosphorylation-dependent activation³⁶. The ROMK channel, and particularly ROMK2, is inhibited by millimolar concentrations of MgATP (K₁/²=2.3 mmol/L)³⁶. This inhibition is independent of ATP hydrolysis - moreover, mutations in the Walker site of ROMK2 shift the MgATP inhibition curve³⁶. Like other K₁ATP channels, ROMK can be activated by PIP₂³⁷, a feature that has also been documented for mitoK₁ATP³⁸, and it is regulated by pH³⁹ and protein kinase C⁴⁰. In addition, it has been reported that ROMK2 may be intrinsically sensitive to the K₁ATP inhibitor glibenclamide (which also inhibits mitoK₁ATP³⁶) even in the absence of SUR coexpression⁴¹. Finally, ROMK can interact with members of the ATP binding cassette family of proteins, including CFTR⁴² and SUR⁴³. For example, when coexpressed in Xenopus oocytes, ROMK2, but not ROMK1 or ROMK3, was shown to physically associate with SUR2B to form glibenclamide-sensitive channels⁴⁴.

Circumstantial evidence aside, the fact that Tertiapin Q, a high affinity ROMK toxin³⁰, could inhibit diazoxide-induced K⁺-specific swelling in isolated mitochondria, as well as ATP-sensitive mitochondrial T₁⁺ uptake in partially-permeabilized cells, provides the first evidence that ROMK is a component of native mitoK₁ATP. Moreover, 5-HD inhibits, and diazoxide activates, a similar component of the T₁⁺ influx pathway that is suppressed by ROMK knockdown, providing additional support for the conclusion that ROMK is a component of mitoK₁ATP.

Future studies will be required to determine potential binding partners for native or heterologously expressed ROMK channels in the heart; however, a recent study found a short (55kDa) splice variant of SUR that displays mitochondrial localization⁴⁵. We did not identify any SUR peptides in our proteomic dataset, but SUR coexpression or concomitant knockdown strategies are possible. Similarly, recent studies have implicated connexin 43 in the modulation of mitoK₁ATP channels³⁶, ⁴⁷: the molecular identification of mitoROMK and the assay techniques developed herein enable a detailed investigation of the proteins and regulatory interactions required for cell protection. Because it is well accepted that ROMK channels can be readily targeted to the surface membrane to fulfill their primary functional role in the kidney, additional work will also be necessary to define how ROMK channels are targeted to different sites in different tissues including the heart, brain, liver or skeletal muscle - tissues where mitoK₁ATP has been documented.

A limitation of the present study is that the necessary tools required for exploring the role of mitoROMK in the intact animal, a prerequisite for assessing its impact on ischemic pre- or postconditioning, are not yet at hand. Also, while the K⁺ selectivity and conductance of ROMK channels...
localized to the surface membrane are well characterized, more work will be required to determine if the channel expressed in mitochondria possesses similar biophysical properties.

Perhaps most importantly, the results demonstrate that mitoROMK confers protection against cell death, and even provides a basal level of protection against oxidative stress in the absence of preconditioning stimuli. In this respect, the data confirm the general idea that mitochondrial K⁺ channels, such as mitoK̅ATP or mitoK̅Ca, are cytoprotective. Identification of mitoROMK provides, for the first time, a molecular target for mechanistic and therapeutic investigation of this important cell survival pathway.

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**DISCLOSURES**

None

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REFERENCES


FIGURE LEGENDS

**Figure 1.** Identification of KCNJ1/Kir1.1/ROMK in heart mitochondria and its expression in non-renal tissues. A) Acquired MS/MS spectra were searched against a custom database of bovine protein reference sequences (NCBI), using the Mascot search algorithm (Matrix Sciences). Data were uploaded into Scaffold (Proteome Software) for statistical validation. 6 spectra matched to 2 overlapping peptides with Peptide ID probabilities of 95%. The cumulative Protein ID probability was >99%. Manual inspection of the spectral matches confirmed that all major peaks were assigned and that b- and y-ion matches formed contiguous and overlapping series. B) Expression of ROMK isoforms in NRVM and adult rat heart by RT-PCR. ROMK1, ROMK2 and ROMK6 were detected in NRVM. In adult rat heart, ROMK1 and ROMK2 were detected. ROMK3 and ROMK6 were undetectable in the heart. ROMK isoforms 1, 2, 3, and 6 were detected in kidney and similar isoforms were found in brain. ROMK expression in liver resembled that of heart, as only ROMK1 and ROMK2 were detected. The high molecular weight ROMK6 PCR product in the liver sample likely reflects residual genomic DNA contamination.

**Figure 2.** Targeting of ROMK2 to mitochondria.
A) The ROMK2 isoform, fused with a V5 tag on its C-terminus, was transiently expressed in H9C2 cells. Cells were fixed, permeabilized and incubated with antibodies against V5 (rabbit) and ATP synthase β, a mitochondrial marker. ROMK-V5 was subsequently stained with anti-rabbit secondary antibody conjugated to Alexa 488 (left panel), and anti-mouse secondary conjugated Pacific Orange 458 (middle panel). The right hand panel depicts the merged images. B) Subcellular fractions of CHO cells transiently expressing ROMK2-eGFP were labelled with connexin 37, VDAC, and eGFP antibodies as markers of plasma membrane, mitochondrial membrane, and ROMK2, respectively. Western blot shows co-enrichment of ROMK-eGFP and the mitochondria marker. All lanes were loaded with equal total protein amounts. C) The predicted N-terminal mitochondrial targeting sequence of ROMK2 was fused to eGFP and imaged in living NRVM (left panel). It colocalized with tetramethylrhodamine methyl ester (TMRM), a mitochondrial membrane potential probe. The right hand panel depicts the merged images.

**Figure 3.** Inhibition of mitoK<sub>ATP</sub> activity with a ROMK toxin. A) Swelling of isolated rat heart mitochondria in the presence of K<sup>+</sup> and P<sub>i</sub> measured spectrophotometrically as a change in 90<sup>0</sup> light scattering at 520nm excitation. The matrix volume increase is inhibited by ATP while the K<sup>+</sup> channel opener diazoxide reverses the inhibition. Tertiapin Q (TPNQ), a stable variant of a bee venom toxin that inhibits of ROMK channels, potently suppressed mitoK<sub>ATP</sub>. B) Concentration-response curve for TPNQ inhibition of mitoK<sub>ATP</sub> in isolated rat heart mitochondria. C) Inhibition of thallium uptake by 100 nmol/L TPNQ in permeabilized H9C2 cells (Fluozin-2 indicator used in this experiment). D) Summary of TPNQ’s effect on initial thallium uptake rate in H9C2 cells (n=3 experiments) E) Inhibition of thallium uptake by 100 nmol/L TPNQ in permeabilized NRVM monolayers (Fluozin-2 indicator). F) Summary of TPNQ’s effect on initial thallium uptake rate in NRVMs (n=3). * denotes statistically significant differences between control and TPNQ treated samples (p<0.05).

**Figure 4.** Suppression of mitochondrial thallium uptake by ROMK knockdown. A) H9C2 cells were permeabilized with digitonin and mitochondrial thallium uptake (2 mmol/L; ATP-free), was determined as the initial rate of change in fluorescence intensity, normalized to the baseline K<sup>+</sup> and Tl<sup>+</sup> free fluorescence, of a mitochondrial matrix-localized thallium-binding reporter, BTC-AM (F/F<sub>0</sub>). Three cell populations were examined: i) a stable cell line expressing scrambled control shRNA, ii) the same control cells treated with 500 µM 5-HD, and iii) a stable cell line expressing ROMK shRNA. B) Summary of initial thallium uptake rates for each group (n=3 experiments). * denotes statistically significant differences between control and TPNQ treated samples (p<0.05).
differences between control and TPNQ treated samples (p<0.05).  C) Thallium uptake in the presence of (i) 1 mmol/L ATP and (ii) 1 mmol/L ATP with 10µM diazoxide in permeabilized H9C2 cells pre-loaded with Fluozin-2 indicator (Left panel: control cells; Right panel: ROMK knockdown cells).  D) Summary of initial thallium uptake rates for each group (n=3 for each group other than control cells with ATP [n=4] and knockdown cells with ATP [n=5]). Statistically significant differences were determined by 2-way ANOVA followed by Tukey’s test (p<0.05): * denotes a significant difference compared to scrambled control cells alone; † difference compared to scrambled control cells with ATP.

Figure 5. ROMK2 overexpression protects against necrotic/apoptotic cell death induced by oxidative stress - ROMK knockdown exacerbates cell death.  A) Representative flow cytometry fluorescence dot plots for Annexin-V and Propidium Iodide (PI) labelling in control and ROMK2 overexpressing H9C2 cells subjected to 100µM tertbutyl hydroperoxide (tBHP) treatment for 22 hours. The three populations selected to quantify live, apoptotic (Annexin positive), and dead (PI positive) cells are illustrated by the dotted lines.  B) Summary of the percentages of live, apoptotic, and dead cells from paired experiments of control and ROMK2-overexpressing cells (ROMK2-OE) subjected to no tBHP, 50µM and 100µM tBHP. Statistically significant differences between groups were determined by 2-way ANOVA followed by Tukey’s test (p<0.05): # live control cells vs. tBHP treatment; † apoptotic control cells vs. tBHP treatment; * live control cells vs. live ROMK2-OE cells at 100µM tBHP; ‡ apoptotic control cells vs. apoptotic ROMK2-OE at 50µM tBHP; ¥ - apoptotic control cells vs. apoptotic ROMK2-OE at 100µM tBHP.  C) Representative flow cytometry fluorescence dot plots for Annexin-V and PI labeling in scrambled shRNA control and ROMK knockdown cell lines subjected to 50µM tBHP incubation for 22 hours.  D) Summary of the percentages of live, apoptotic, and dead cells from paired experiments of scrambled control and ROMK knockdown cells (ROMK-KD) subjected to no tBHP, 50µM and 100µM tBHP. Statistical significance (p<0.05): # live control cells vs. tBHP treatment; † apoptotic control cells vs. tBHP treatment; € dead control cells vs. tBHP treatment; * live ROMK-KD vs. tBHP treatment; § apoptotic ROMK-KD cells vs. tBHP treatment; ‡ apoptotic control cells vs. apoptotic ROMK-KD at 100µM tBHP.
Novelty and Significance

What is Known?

- Mitochondrial ATP-sensitive potassium channels (mitoK<sub>ATP</sub>) have been implicated in the mechanism of cardiac preconditioning.

- The molecular composition of mitoK<sub>ATP</sub> has not been determined, limiting advancement in understanding its role in cytoprotection.

What New Information Does This Article Contribute?

- ROMK/KCNJ1, an ATP-sensitive potassium channel, was identified in the inner mitochondrial membrane using a proteomic approach.

- An isoform of ROMK contains a mitochondrial targeting motif at its N-terminus.

- Inhibition of ROMK with the honey bee venom toxin, tertiapin-Q, inhibits the classical mitochondrial swelling response attributed to mitoK<sub>ATP</sub> opening and also inhibits mitochondrial ATP-sensitive thallium (Tl<sup>+</sup>; a surrogate for K<sup>+</sup>) uptake.

- Genetic knockdown of ROMK also suppresses mitochondrial ATP-sensitive Tl<sup>+</sup> uptake.

- Overexpression of ROMK protects H9C2 cells from oxidant-induced cell death, while knockdown of ROMK renders them susceptible to cell death.

- A resident mitochondrial isoform of ROMK is the likely pore forming subunit of the long-sought cardioprotective mitoK<sub>ATP</sub> channel.

Several lines of evidence support the existence of a mitochondrial ATP-sensitive K<sup>+</sup> channel (mitoK<sub>ATP</sub>), which has been linked to the mechanism of cardiac preconditioning through the actions of pharmacological agents that preferentially interact with the mitochondrial, as opposed to the sarcolemmal, channel. The lack of a molecular identification of mitoK<sub>ATP</sub> and nonspecific actions of the compounds employed, has restricted progress in understanding mitoK<sub>ATP</sub>-mediated cytoprotection. Here, we report that an isoform of a renal outer medullary K<sup>+</sup> channel, ROMK, is present in the mitochondrial inner membrane and mediates mitochondrial ATP-sensitive K<sup>+</sup> flux. MitoK<sub>ATP</sub> activity in isolated mitochondria or permeabilized cells was potently inhibited by the ROMK toxin, tertiapin-Q, and genetic knockdown of ROMK also suppressed mitochondrial Tl<sup>+</sup> uptake. Finally, ROMK overexpression protected cells from oxidant-induced cell death and knockdown of the native channel exacerbated death. Establishing ROMK as a pore-forming subunit of mitoK<sub>ATP</sub> overcomes a significant barrier in the field of cardioprotection, and opens the door for future studies to examine the mechanism of mitoK<sub>ATP</sub> action, find binding partners of the channel, and develop more potent and specific modulators of cardioprotection.
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| 4 | 356.23 | L  | 730.46 | 365.74 | 713.44 |
| 5 | 459.24 | C  | 617.38 | 600.35 |
| 6 | 572.32 | L  | 514.37 | 497.34 |
| 7 | 685.41 | I  | 288.2  | 271.18 |
| 8 | 798.49 | I  | 288.2  | 271.18 |
| 9 | 972.6  | R  | 175.12 | 158.09 |
Figure 2

A

ROMK2-V5  ATP synthase β  Merged

B

<table>
<thead>
<tr>
<th>Lysate</th>
<th>Post-Nuclear Sup</th>
<th>Mito (Crude)</th>
<th>Mito (Washed)</th>
<th>Mito (Washed x2)</th>
</tr>
</thead>
</table>

C

ROMK2(N- Term)-eGFP  TMRM  Merged
Figure 3

A

B

C

D

E

F
The Mitochondrial ROMK Channel is a Molecular Component of MitoK<sub>ATP</sub>
D. Brian Foster, Alice S. Ho, Jasma Rucker, Anders O. Garlid, Ling Chen, Agnieszka Sidor, Keith D. Garlid and Brian O'Rourke

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Enrichment and Separation
At Mitochondrial, Submitochondrial, Protein and Peptide Levels

Isolation of Bovine Heart Mitochondria

High-purity bovine mitochondria, suitable for proteomic analysis, were prepared as reported in Foster et al.1. Briefly crude mitochondria were obtained essentially as originally described by Smith2, specifically “Procedure 2” therein. Briefly, bovine hearts were obtained from a local abattoir and packed on ice. In the lab the ventricles were excised, defatted and rinsed. Ventricles (approx. 800g) were rinsed, cubed, minced and allowed to stir for 15 min in isolation buffer (10 mM HEPES, 200 mM mannitol, 50 mM sucrose, 1 mM EGTA; pH 7.2) containing trypsin, en lieu of Nagarse used by Smith2. The pH of the buffer was periodically readjusted to > 7 with NaOH. Trypsin was removed by squeezing the mince through 2 layers of cheesecloth. The mince was diluted into isolation medium containing pepstatin, leupeptin and PMSF, and homogenized briefly in a heavy-duty blender. The pH of the solution was readjusted to >7 prior to centrifugation of the homogenate at 1000 x g for 10 minutes in a Sorvall GS3 rotor. The supernatant was carefully decanted subsequently centrifuged at 8000 x g for 10 minutes. The supernatant was discarded and the pellet, containing crude mitochondria was kept for density-gradient centrifugation.

Isolation of highly enriched density-purified mitochondria was inspired by Taylor et al.3, who used a method based on protocol of Storrie and Madden4. Our variation on the method entailed two rounds of density centrifugation. Briefly, crude mitochondria were resuspended in sucrose buffer (10 mM Tris-HCl pH 7.5, 250 mM sucrose), and layered onto a discontinuous gradient consisting of (from top to bottom) 6% Percoll, 17 % and 35% Histodenz (Sigma), each made up in sucrose buffer. The sample was centrifuged at 50,000 x g for 30 minutes. The layer at the 17%/35% interface was collected and mixed with 12 mL of 35% Histodenz. The mitochondria were placed at the bottom of centrifuge tubes. The 6% Percoll and 17.5% layers were successively layered on top of the mitochondrial layer. Floating density centrifugation was conducted at 50,000 x g for 30 minutes. Again the layer at the 17%/35% interface was collected. Mitochondria were diluted with isolation medium and centrifuged at 10000 x g to remove the Histodenz. This was repeated to remove trace Histodenz. Subfractionation of the density-purified mitochondria into submitochondrial compartments was performed immediately.

Assessment of Mitochondrial Purity

The purity of the bovine mitochondrial preparation has been assessed previously by Foster et al.1, particularly in Figure 3A therein. Briefly, equal amounts of protein from each stage of mitochondrial purification (i.e. homogenate, post-nuclear supernatant, crude mitochondria and gradient pure mitochondria) were probed by immunoblotting with antibodies to Na+/K+ ATPase (Santa Cruz), SERCA2a (Thermofisher), cardiac Troponin I (Spectral Diagnostics) and VDAC (Molecular Probes). High-purity mitochondria showed no signal for Na/K ATPase or SERCA2a after 1 min exposure of the blots developed using Supersignal West-Pico Reagent from Thermofisher. Longer exposures (5-10 min) revealed faint signals. The level of contamination in our bovine mitochondrial preparations is often lower than we find in preparations from other species, such
as the rat, where residual contamination can be detected even after further mitochondrial subfractionation. The higher purity in the bovine preparations is likely to stem from the incubation of the heart mince with trypsin for 15 minutes, which is harsher than our other protocols and may lead to degradation of contaminants and weakening of mitochondrial contacts with typical contaminating membranes such as the sarcoplasmic reticulum.

**Preparation of Enriched Submitochondrial Compartments**

Notwithstanding that our study sought to identify novel channels of the mitochondrial inner membranes, multiple submitochondrial compartments were analyzed. Low abundance proteins tend to be systematically underrepresented in proteomic datasets, since high-abundance peptides dominate the duty cycle of mass spectrometers in data-dependent data acquisition mode\(^5\)\(^,\)\(^6\). Since mitochondrial subfractionation yields enriched, though not absolutely pure subcompartments, analyzing all mitochondrial subcompartments served to maximize chances of rare IMM protein discovery.

**Outer and Inner Membrane-enriched fractions**

Mitochondrial subfractions were prepared essentially as described by Maisterrena et al. for pig heart mitochondria\(^7\) with variations. Briefly, gradient pure mitochondria (approx 500 mg) were subjected to hypotonic swelling in approximately 1.5 L of 20 mM KH\(_2\)PO\(_4\) for 40 min, at which point they were centrifuged at 8000 x g to pellet swollen mitoplasts. The supernatant was decanted and kept for isolation of proteins from the intermembrane space (next paragraph). The swollen mitoplasts were resuspended in 250 mM sucrose homogenized with a dounce homogenizer to dislodge the outer mitochondrial membrane. The solution was layered onto a discontinuous sucrose gradient consisting of layers containing of 37.7%/51.7% and 61.5% (w/v). Sucrose gradients were centrifuged at 77,000 x g for 90 min in a SW 32 rotor. Light membranes containing outer membrane markers, were collected from the top of the 37.7% layer and was called the outer mitochondrial membrane (OMM)-enriched fraction. Mitoplasts were collected from the 37.7%/51.7% interface, diluted in 250 mM sucrose, and homogenized in a dounce homogenizer. The suspension was centrifuged at 100,000 x g for 30 min to pellet membranes. The supernatant (containing matrix proteins) was decanted. The pellet was resuspended in 10 mM Tris pH 8.0, 250 mM Tris-HCl, sonicated at 20 W for 1 min on ice, and then layered onto a second sucrose density gradient. Again the sample at the 37.7%/51.7% interface was collected, and is hereafter called the IMM-enriched fraction. The OMM and IMM fractions were stored as frozen pellets at -80°C until required

**Matrix- and Intermembrane Space-Enriched Fractions.**

The supernatant arising from the hypotonic swelling and centrifugation of the mitoplast described in the previous paragraph was collected. Ammonium sulfate was added gradually until the solution was 80% saturated. The solution was stirred for 30 min at 4°C. The solution was centrifuged at 10,000 x g for 20 min a Sorvall GS3. Though there was no compact pellet, a flocculent layer formed on the surface of the supernatant and a thin whitish translucent film adhered to one side of the centrifuge bottle. The bulk of the supernatant was discarded. The flocculent layer and film were recovered using residual 80% ammonium sulfate solution and
concentrated by centrifuging in a 50 mL conical tube at 15,000 x g in a Fiberlite PTi rotor for 15 min.

The matrix-enriched fraction used for proteomic analysis was obtained simply by subjecting mitochondria (approx. 50mg) to hypo-osmotic swelling in a small volume (approx. 4mL) and sonicating the sample at 18-20 W on ice, four times for one minute each. Membranes were removed by centrifugation at 100,000 x g for 30 min. This fraction, though matrix-enriched, would also be expected to contain soluble proteins of the IMS and soluble, weakly-bound membrane associated proteins.

**Separation of Macromolecular complexes by Sucrose Density Gradient Centrifugation**

Proteins of the IMM, OMM- and Matrix-enriched fractions were fractionated essentially as described previously1,8. Membranes were solubilized at a concentration of 5mg/mL in 1% lauryl maltoside for 30 min. Insoluble aggregates (minimal) were removed by centrifugation at 100,000 x g for 30min. Soluble protein was then separated on a discontinuous sucrose gradient (10-35% w/v) by centrifugation in a SW 41 rotor at 32,000rpm (approx. 128,000 x g at r<sub>ave</sub>) for 18 hours at 4°C. Each layer (1.5mL) was collected by making a hole with a 25-gauge needle in the bottom of the polyallomer tube and collecting drop-wise into 2mL microcentrifuge tubes. Each sucrose fraction was dialyzed overnight against 50 mM ammonium bicarbonate, 0.1% lauryl maltoside (3 X 1L) in preparation tryptic digestion.

**Separation of Peptides Derived from Sucrose Fractions**

**By Strong Cation Exchange Chromatography**

Dialyzed gradient fractions were digested with trypsin (protein/trypsin approx. 100/1) at 37°C overnight. Ammonium bicarbonate was evaporated in a vacuum centrifuge. Peptides were re-dissolved in de-ionized water and residual ammonium bicarbonate was removed again by evaporation. The combined peptide mixture was fractionated by strong cation exchange (SCX) chromatography on an 1100 HPLC system (Agilent) using a PolySulfoethyl A column (2.1x100mm, 5µm, 300Å, PolyLC, Columbia, U.S.A.). Sample was dissolved in 4mL of SCX loading buffer (25% v/v acetonitrile, 10mM KH<sub>2</sub>PO<sub>4</sub>, pH 2.8), pH was adjusted to 2.8 by adding 1 N phosphoric acid. The whole sample was loaded and washed isocratically for 30 min at 250µL/min. Peptides were eluted with a gradient of 0-350mM KCl (25% v/v acetonitrile, 10mM KH<sub>2</sub>PO<sub>4</sub>, pH 2.8) over 40min at a flow rate of 250µL/min. The absorbance at 214 nm was monitored and 8-10 SCX fractions were collected along the gradient.

**By Reversed-Phase Liquid Chromatography**

The HPLC was driven by an Eksigent nano-2DLC pump. The trap contained C18 (75µm) fused silica fritted with Kasil 1624 and hand packed to 3cm with YMC 5-10 µM irregular C18. The column was C18 75µm column hand packed with YMC ODS-AQ 3.5µm particle size, 120A pore size. The solvent system consisted of 0.1% Formic Acid (Buffer A) and 0.1% Formic Acid /90% Acetonitrile (Buffer B). Samples were injected at buffer composition of 1%B from the autosampler into the nanoflowpath at 8.5%B (flow rate: 300 nL/min). Peptides were eluted by ramping up to 30% B in 15 minutes, then to 60% B by 18 minutes. Buffer B was then quickly ramped to 100% B by 22 minutes and held for 2 minutes before returning to 100 % A ending at 30 minutes.
Proteomic Analysis

Mass Spectrometry
Eluted peptides were injected by electrospray (2.4kV) into an LTQ ion trap mass spectrometer (ThermoFinnigan). Precursor ion scans ranged from 350-1800 m/z. The top eight ions picked for MS/MS scans. Collision Energy was set to 30, (Q=0.250; Activation Time=30) Dynamic Exclusion settings: Repeat count: 2; Exclusion duration: 120 seconds

Database Searching
Mascot and X!Tandem searches conducted against a custom FASTA database of all bovine sequences in NCBI as of 06/11/2008, assuming trypsin as the digestion enzyme. The parent ion mass tolerance was set to 1.5 Da, and fragment mass tolerance was set to 0.8 Da. Oxidation of methionine was specified in Mascot and X!Tandem as a variable modification.

Criteria for Protein Identification
Scaffold (version Scaffold-01_07_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at >80.0% probability as specified by the Peptide Prophet algorithm. Protein probabilities were assigned by the Protein Prophet algorithm. Protein identifications were considered for the final list if they scored at >50% protein probability stemming from identification of a high quality peptide. False positive protein identification was minimized by manually evaluating all proteins identified by a single peptide hit.

Manual Evaluation of Single Peptide-Spectrum Matches
Manual curation was carried out essentially as describe in Cammarato et al. in the method supplement. In particular, proteins identified on the basis of a single peptide were scrutinized to ensure: 1) that the MS² spectra yielded sequential b- and y-ions, 2) that the b- and y-series overlapped, and 3) that spectra were consistent with currently accepted peptide fragmentation biases.

Bioinformatic Analysis
Prediction of Mitochondrial Localization
Fully curated protein sequences of K⁺-channel isoforms (147 total) were retrieved from UniProtKB. Rat sequences were used, though human sequences were substituted if a rat entry was unavailable in UniProtKB at the time of analysis. The mitochondrial localization probability for the K⁺-channel sequences assessed using MitoPred http://bioapps.rit.albany.edu/MITOPRED/.
Consultation of the Maestro database, which provides a genome-wide ranking of human and mouse genes according to the likelihood of mitochondrial localization reveals that the KCNJ1 gene was the highest ranking among inward rectifying potassium channels

Molecular/Cell Biology
*Isolation of Neonatal Rat Ventricular Myocytes*

NRVMs were enzymatically dissociated from the ventricles of 2-day-old rats with trypsin. Freshly isolated NRVMs were resuspended in M-199 culture medium supplemented with 10% FBS, glucose, and vitamin B12. Two preplating steps were performed to enrich cardiac myocyte content in the culture. The final cell suspension was collected and plated at the desired density for the downstream experiment.

*Detection of ROMK Transcripts by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)*

Total RNA was isolated from freshly isolated adult rat heart using the RNeasy Fibrous Tissue Mini Kit (Qiagen) according to manufacturer’s instructions. Rat kidney, brain, and liver total RNA were extracted with RNeasy Mini Kit (Qiagen) according to manufacturer’s instructions. Isolated Neonatal rat ventricular myocytes were plated into a T75 flask, grown for 2 days into a beating monolayer, then treated with RNAProtect Cell Reagent (Qiagen) to collect and preserve the total RNA content prior to extraction using the RNeasy Mini Kit.

RT-PCR was performed with One step RT-PCR kit (Qiagen) according to manufacturer’s instruction, with the specific primers listed below for detecting different ROMK Isoforms. 2µL of 1:100 diluted RT-PCR products were subsequently subjected to another “nested” step with isoform specific primers below to detect the presence of low copy ROMK transcripts using Taq Polymerase (Invitrogen). Resulting PCR product from the nested step were then ran on a 1% agarose gel for visualization (product size shown in the chart below).

<table>
<thead>
<tr>
<th>ROMK Isoform</th>
<th>RT / Nested</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROMK1</td>
<td>RT</td>
<td>GAACGGAGTGTGTTCA</td>
<td>GTCTAGAGATCTTGGCTA</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nested</td>
<td>GAGTGTTCCAGAGTGT</td>
<td>GTACCTCCATTCCAGGTCCA</td>
<td>220</td>
</tr>
<tr>
<td>ROMK2</td>
<td>RT</td>
<td>GGTCATCCATTTACCCAGC</td>
<td>GTCTAGAGATCTTGGCTA</td>
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</tr>
<tr>
<td></td>
<td>Nested</td>
<td>TTACCCCAAGCATTCCATGA</td>
<td>GTACCTCCATTCCAGGTCCA</td>
<td>222</td>
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<tr>
<td>ROMK3</td>
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<tr>
<td></td>
<td>Nested</td>
<td>GGCACTACAGACAATGTTG</td>
<td>GTCTAGAGATCTTGGCTA</td>
<td>271</td>
</tr>
<tr>
<td>ROMK6</td>
<td>RT</td>
<td>GAAGCGGAGTGTGTTCA</td>
<td>GTCTAGAGATCTTGGCTA</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Nested</td>
<td>ACGGAAGTCATCTGCTCA</td>
<td>GTACCTCCATTCCAGGTCCA</td>
<td>317</td>
</tr>
</tbody>
</table>

*ROMK Fusion Constructs*

A plasmid containing the gene encoding for KCNJ1 was obtained from the I.M.A.G.E. consortium via Open Biosystems (Clone ID 30915211). A stretch of nucleotide encoding the first 24 amino acids of the ROMK2 isoform was amplified by PCR and subcloned into the pDONR/zeo vector, then subsequently cloned into the N-terminus of an eGFP fusion expression vector using the Gateway system (Invitrogen). The eGFP fusion expression vector was based on the pEGFP-N1 vector (Clontech), converted into a Gateway destination vector using the Gateway Vector Conversion System kit (Invitrogen).

ROMK2 was amplified by PCR from the original KCNJ1 plasmid by PCR and subsequently cloned into the pENTR/D-TOPO vector (Invitrogen). EF1-α promoter was amplified by PCR from the pEF1alpha-IRES Vector (Clontech), and subcloned into the pENTR5’-TOPO according to manufacturer’s instructions (Invitrogen). Gateway LR-plus subcloning was performed to combine
the EF1-α promoter upstream of ROMK2 and into the pLenti6/R4R2/V5-DEST destination vector (Invitrogen). Gateway LR subcloning was also performed between the ROMK2-pENTR/D-TOPO and the converted pEGFP-N1/DEST vector to obtain a CMV-ROMK2-eGFP construct.

**Mitochondrial Enrichment from CHO-K1 Cells**

CHO-K1 cells were transiently transfected with ROMK2/pEGFP-N1 DEST to overexpress ROMK2 tagged with a eGFP on its C-terminus. After 24 hours, mitochondria were isolated from the cells using a protocol reported by Gottlieb and Adachi15. Briefly, 2x10⁸ cells were centrifuged and re-suspended in 3 ml of isolation buffer (225mM mannitol, 75mM sucrose, 1mM EGTA @4°C), then transferred to pre-cooled cavitation chamber of a nitrogen bomb. The cell suspension was subjected to a pressure of 450psi for 15 minutes with stirring, after which it was instantaneously released into atmospheric pressure. Cell homogenate was centrifuged at 1300g to remove nuclear material. The supernatant was then centrifuged at 7500g to collect for crude mitochondria pellet. This pellet was washed twice at 7500g with the isolation buffer, and finally re-suspended in suspension buffer (isolation buffer without EGTA). Samples were subjected to immunoblot analysis as described shortly hereafter.

**shRNA Constructs and Knockdown Efficiency Validation**

Six validated shRNAs against the core section of KCNJ1 from The RNAi Consortium (TRC) shRNA libraries were obtained from the ChemCORE facility at The Johns Hopkins University (TRCN0000005593, TRCN0000005594, TRCN0000005595, TRCN0000005596, TRCN0000005597, TRCN0000068526). A scrambled shRNA control vector was also obtained from the ChemCORE facility at The Johns Hopkins University. The human U6 promoter, along with the shRNA sequence, was subcloned into the pDONR/zeo vector using Gateway BP subcloning method. The CMV promoter and mCherry fluorophore sequences from the pmCherry-N1 Vector (Clontech) were subcloned into the pENTR5'-TOPO according to manufacturer’s instructions (Invitrogen). The shRNA-pDONR/zeo and CMV-mCherry-pENTR5'-TOPO were combined into the pLenti6/R4R2/V5-DEST destination vector (Invitrogen). The resulting constructs were then screened for ROMK knockdown efficiency through transient co-transfection of the shRNA construct and CMV-ROMK2-eGFP in H9C2 cells. Transient transfection was performed using lipofectamine LTX with Plus Reagent (Invitrogen) according to the optimized protocol reported by Vitiello et al16. Out of the set, TRCN0000005595 and TRCN0000005596 were individually determined to be most effective in knocking down 50% of GFP signal quantified by flow cytometry in the BD FACScan system (BD Biosciences). When combined, these two shRNAs can knock-down 70% of eGFP signal in flow cytometry. These two shRNA-pLenti6/R4R2/V5-DEST vectors were combined for use in all subsequent experiments.

**Lentivirus Production**

The following protocol was used to produce lentivirus from ROMK2- or shRNA- pLenti6/R4R2/V5-DEST vectors. 293FT cells were pre-plated onto eight gelatin coated 150mm-plates the day before transfection to ensure 80% confluence in 24 hours. On the day of transfection, for each plate, 30µg of ViraPower Packaging Mix (containing pLP1, pLP2, and pLP/VSVG) and 10µg of pLenti vector were mixed with 82µl Lipofectamine 2000 in Opti-MEM (Invitrogen) at room temperature for 25 minutes, then added dropwise to 293FT cells in Opti-MEM supplemented...
with 10% fetal bovine serum and 25μM chloroquine. 9 hours later, this media was replaced by Dulbecco’s Modified Eagle Medium (DMEEM) supplemented with 10μM sodium butyrate to facilitate virus production. Lentivirus was collected from the media at 24 and 48 hours after transfection, sterilized by filtration through 0.45μm Durapore Membrane Filter (Millipore) and subsequently concentrated by ultrafiltration using Centricron Plus70 (100K) (Millipore) at 720rcf. Lentivirus concentrate was aliquoted and stored at -80°C. Lentivirus was titered with 293FT cells by assessing fluorescence expression level in limiting dilution with 8mg/ml Polybrene (Sigma). In the case where no fluorescent tags are available, concentration level was assessed by 5mg/ml Blasticidin (Invitrogen) selection of transduced 293FT colonies.

**Establishment of Stable Cell Lines**

H9C2 cells in low passage were transduced with (1) ROMK2-V5 lentivirus (overexpressors); (2) scrambled controlled shRNA lentivirus (negative control); or (3) combined ROMK shRNA lentiviruses (ROMK knockdown), all at MOI of 50. Day 3 after transduction, the cells were subjected to 5mg/ml Blasticidin selection in DMEM supplemented with 10% FBS and Anti-Anti. After 14 days of selection, ROMK levels of these cell lines were assessed by quantitative RT-PCR (detection primer sets are shown in the chart below). Expression levels were quantified using the standard curve absolute quantitation method, normalized to 18s rRNA. For the stable overexpressors, the human primer sets were used to detect ROMK levels, whereas for detecting native expression of ROMK in H9C2 cells, the rat primer sets were used. Stable ROMK2 overexpressors were determined to be expressing at 1.3ng ROMK/ng 18s rRNA, compared to 0.003ng ROMK/ng 18s rRNA in untransduced cells.

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward</th>
<th>Reverse</th>
<th>Probe (6FAM-TAMRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>TGGACATCTGGACAACGGTA</td>
<td>CGGGAGGTCTTTGTGAATGT</td>
<td>TCACAGGCTCTTTGGAGTTGT</td>
</tr>
<tr>
<td>Rat</td>
<td>GCTGGAGGCCAGGTGAT</td>
<td>TTCTGTCTAGTGCCCTGAT</td>
<td>ATATCAATGACCACCCCTTCT</td>
</tr>
</tbody>
</table>

**Detection of ROMK, ROMK-GFP, ROMK-V5 by Immunoblot Analysis**

Immunoblot analysis was conducted using the same methods for each experiment and differed only with respect to the identity and concentration of primary antibodies, as well as the type of chemiluminescent reagent used. Briefly, protein samples were concentrated by quantitative methanol chloroform precipitation and resolubilized in 2% (w/v) sodium dodecyl sulfate for protein determination using the BioRad DC assay, which is based on the method of Lowry et al. Samples were mixed with 2x LDS sample buffer under reducing conditions, heated and 20 μg were loaded onto a 4-12% NuPAGE Bis-Tris precast gel. Electrophoresis was conducted with MOPS running buffer at 150 V for 50 min. Protein was transferred to nitrocellulose membrane at 20 V, for 9 min, using an iBlot apparatus (Invitrogen). Blots were incubated with 5% milk powder (BioRad) in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBS-T) for 1 hour. They were subsequently incubated with primary antibody diluted in according to the manufacturer, in 2.5% milk in TBS-T overnight at 4°C. Rabbit secondary antibody conjugated to horseradish peroxidase (HRP; Amersham) was used at a dilution of 1/20000. HRP activity was developed by chemiluminescence using Supersignal West-Femto Reagent (Thermo Fisher) and visualized by exposure to Amersham Hyperfilm ECL (GE Biosciences) for 20 min. MagicMark protein ladders (Invitrogen) provided molecular weight standards.
Specific Experiments
Detection of Overexpressed ROMK-GFP Enriched from CHO Cells (Figure 2B)
20 µg from each step of purification (Lysate, Post nuclear supernatant, Crude Mitochondria, Washed Mitochondria) subjected to immunoblot analysis as describe above. Blots were probed with primary antibodies against eGFP, Porin, GFP78 BiP, or Connexin 37 (Abcam) diluted according to manufacturers’ instructions. SuperSignal West Pico Substrate Kit (Thermo Scientific) was used to develop the chemiluminescence.

Detection of Overexpressed ROMK and ROMK-V5 in H9C2 Cells (Supplemental Figure)
H9C2 cells (untransduced, transduced with ROMK-V5, ROMK-V5 with negative control shRNA, ROMK-V5 with ROMK-specific shRNA ) were lysed with RIPA buffer (Sigma-Aldrich), concentrated and 20 µg of protein from each samples was analyzed as describe above. Primary antibodies to ROMK (Sigma) and V5-tag (Sigma) were diluted according the manufacturer. SuperSignal West Femto Substrate Kit (Thermo Scientific) was used to develop the chemiluminescence.

Detection of Endogenous ROMK and its Ablation in H9C2 Cells
H9C2 cells, transduced with either a scrambled shRNA or a ROMK-specific knockdown shRNA construct, were lysed, concentrated and 20 µg were analyzed as described above. Primary antibody to ROMK (Sigma) was diluted according to the manufacturer. SuperSignal West Femto Substrate Kit (Thermo Scientific) was used to develop the chemiluminescence.

Colocalization/Imaging Studies
Fixed H9C2 Cell Imaging
H9C2 cells in low passage pre-plated on laminin coated #1.5 glass coverslips (Fisherbrand) were transiently transfected with the ROMK2-pLenti6/R4R2/V5-DEST construct with Lipofectamine LTX and Plus Reagent (Invitrogen). 16 hours after transfection, cells were fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.25% Triton X-100 (Sigma), and blocked with 10% goat serum before primary antibodies were applied overnight at 4°C. Polyclonal anti-V5 antibody (Sigma) was used to probe for ROMK2, and ATP synthase subunit beta monoclonal antibody (Invitrogen) was used to probe for mitochondria. Secondary antibodies were applied separately after washing and blocking with 10% goat serum in-between. Cells were incubated with goat anti-mouse IgG Pacific Orange 458 (Invitrogen; ATP synthase staining) overnight at 4°C, and goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen; V5 staining) for 1 hour at room temperature. Cells were rinsed and mounted in Prolong Gold with antifade and cured for 48 hours prior to imaging using two-color stimulated emission depletion (STED) microscopy (Leica TCS STED CW; 531nm depletion beam wavelength).

Live Neonatal Rat Ventricular Myocytes Imaging
Freshly isolated neonatal rat ventricular myocytes (NRVMs) were transfected with the expression construct via electroporation with the AMAXA system (Lonza) by electroporation according to the manufacturer’s instructions. Transfected cells were allowed to recover for 3 days prior to live cell imaging using 2-photon laser scanning fluorescence microscopy (Bio-Rad MP1024; excitation 900nm, emission at 525nm for eGFP, 605nm for tetramethylrhodamine methyl ester). Prior to imaging, cells were incubated with 50nM tetramethylrhodamine methyl ester (TMRM) at 37°C to label polarized mitochondria.
Mitochondrial Swelling Assay for MitoK_{ATP} Activity.

Volume changes secondary to respiration-driven mitochondrial uptake of K+ salts and water were followed by light scattering (1/A)\(^2\). Mitochondria were isolated from hearts of male Sprague-Dawley rats and assayed at 0.1 mg/ml in medium containing K\(^+\) salts of Cl\(^-\) (120 mM), HEPES (10 mM), EGTA (0.1 mM), succinate (10 mM), MgCl\(_2\) (0.5 mM), ATP (200 \(\mu\)M), rotenone (2.5 \(\mu\)M) oligomycin (1 \(\mu\)g/ml), and phosphate (10 mM), pH 7.2. MitoKATP was opened with diazoxide (30 \(\mu\)M) in the presence of TPNQ at concentrations given in Fig. 3B, and steady state values of 1/A were measured at 120 s. 100% “MitoKATP Activity” is the diazoxide-activated activity without TPNQ and 0% is the value without diazoxide or TPNQ.

Thallium Uptake Assay

Stable H9C2 cells were plated on laminin-coated circular glass coverslips such that they will be at 80% confluency on the day of the assay. Prior to the assay, cells were loaded with a fluorescent indicator, either benzothiazole coumarin acetoxyethyl ester (BTC-AM; 20\(\mu\)M) or Flouzin-2 AM (5\(\mu\)M) (Invitrogen). BTC was shown to be effective in measuring mitochondrial K\(_{ATP}\) activity in isolated mitochondria\(^2\), while Flouzin-2 has previously been applied in high throughput screening of small molecule inhibitors for plasma membrane localized ROMK channels\(^2\); both dyes are sensitive to thallium, which is used to substitute for potassium. Dyes were loaded in their acetoxyethyl ester (AM) form mixed with 0.2% (w/v) Pluronic F-127 prior to addition to incubating media. Cells were incubated with BTC-AM for 10 minutes, or in the case of Flouzin-2 AM, incubated for 20 minutes. All incubation steps were carried out at 37° C, followed by two PBS wash steps to remove membrane bound dyes. Just prior to the Ti\(^+\) assay, cells were treated with 150\(\mu\)M digitonin for 30s to permeabilize the plasma membrane, as evidenced by rapid loss of the cytosolic component of dye, leaving only the mitochondrial compartment loaded with the indicator. This protocol has previously been used by Ljubkovic et al.\(^2\) to preferentially localize potassium sensitive indicators to the mitochondria.

Images were collected by exciting at 480nm and recording the fluorescence emission at 525nm using a cooled CCD camera (Cascade II 512, Photometrics). Bath Ti\(^+\) was rapidly switched from 0 to 2mM Ti\(^+\) at 100s by means of a custom-built flow switching device positioned directly over the field of interest to minimize bath exchange times. The chloride-free, 0 Ti\(^+\) assay bath solution contained (in mM): 195 mannitol, 10 HEPES, 2 MgSO\(_4\), 2 Na\(_2\)HPO\(_4\), 2 succinate, 0.6 CaOH\(_2\), 1 EGTA and 1\(\mu\)g/ml oligomycin (pH 7.2) and the 2mM Ti\(^+\) solution was identical except 2mM TiSO\(_4\) was added. Images were collected every second, and the fluorescence intensities of the dyes within the mitochondria were determined offline by ImageJ. F/F0 was calculated for each time point (F0 was the basal fluorescence in the 0 Ti\(^+\) condition) and the initial rates were determined by averaging the first derivatives of F/F0 ratios at 106-125s for BTC-AM loaded cells (Figs. 3C and 3D), and at 102-119s for Flouzin-2 AM loaded cells (Figs. 3E, 3F, and S1).

To study thallium uptake in NRVMs, freshly isolated NRVMs were plated on fibronectin-coated glass coverslips which allowed for the formation of monolayers prior to the assay. Cells were pre-loaded with Flouzin-2 AM. Buffer solutions were the same except that 100\(\mu\)M cyclopiazonic acid was added to specifically inhibit the SERCA pumps. Data collection was performed as described above. Statistical analysis was done with Microcal Origin by 2-way ANOVA with post hoc Tukey test and p<0.05 was the criterion of statistical significance.
**Cell Death Assay**

Stable H9C2 cell lines with various expression levels of ROMK2 were plated on 6 well plates at equal densities. The cells were subjected to 0, 50µM, or 100µM tert-butyl hydroperoxide (tBHP) treatment for 22 hours to induce oxidative stress. After incubation with tBHP, cells were labeled with Vybrant apoptosis assay kit #2 (Invitrogen) for annexin-V-FITC and propidium iodide (PI) according to the manufacturer’s instructions for flow cytometry. Cells were immediately subjected to flow cytometry analysis in the BD FACScan system (BD Biosciences), using FL-1 channel for detecting annexin-V and FL-3 channel for PI. Unlabeled cells were considered live cells, cells positively stained only with annexin-V were considered apoptotic cells, and cells that were labeled with PI were considered to be dead cells. All experiments were performed with the appropriate controls. Statistical analysis was done with Microcal Origin by 2-way ANOVA with post hoc Tukey test and p<0.05 was the criterion of statistical significance.

All experimental protocols complied with the Guiding Principles in the Use and Care of Animals published by the National Institutes of Health and were approved by IACUC at The Johns Hopkins University or at Portland State University (data in Figures 3A and 3B).

**Author Contributions**

Bovine heart proteomic discovery work and bioinformatic analyses were performed by DBF. Molecular cloning, lentivirus production, stable cell line establishment, colocalization studies, thallium assays, and cell death assays were designed, performed, and analyzed by ASH. Mitochondrial light scattering assays were performed and analyzed by AG in the laboratory of KG. All authors took part in writing and editing the manuscript.
References


**SUPPLEMENTAL MATERIAL**

**Online Figure I**

Thallium uptake in the absence of ATP (black) and in the presence of 1mM ATP (red) or 1mM ATP plus 1µM valinomycin (green) in scrambled control H9C2 cells.

**Online Figure II**

Summary of initial thallium uptake rates for each group (n=3 for each group). * denotes statistically significant differences compared to control cells alone (p<0.05).
Online Figure III
Knockdown of endogenous ROMK

Figure III. H9C2 cells stably expressing scrambled shRNA (control), or a combination of 2 ROMK shRNAs (shRNA), were harvested for immunoblot analysis (see methods supplement). Cell lysates (20 μg) were probed with an antibody to ROMK (Sigma Prestige, anti-KCNJ1 Cat #: HPA026962). The control blot showed 5 bands of differing intensity. One band, around 45kDa, consistent with the known molecular weight of either ROMK1 or ROMK2, is absent from the shRNA lane. A second band, around 65 kDa was also knocked down in the shRNA lane, though its origin is unknown.
Online Figure IV
ROMK2-V5 overexpression and its knockdown by ROMK shRNA

Figure IV. Untransfected H9C2 cells, cells stably expressing an epitope-tagged ROMK2-V5 (ROMK2-OE), or cells expressing both ROMK2-V5 and a ROMK shRNA were harvested for immunoblot analysis (see methods supplement). Cell Lysates (20 μg) were probed with an antibody to ROMK (Sigma; leftmost blot), Anti-V5 (center) or a tubulin loading control (right). Both the anti-ROMK antibody and the V5 antibody detected a band between 40 and 50 Kda that increased with ROMK overexpression and decreased with concomitant ROMK shRNA expression, while a scrambled control shRNA had no effect on ROMK expression.