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The mitochondrial ROMK channel is a molecular component of Mitok$_{atp}$

D. Brian Foster[1,†], **Alice S. Ho**[1,2,†], **Jasma Rucker**[1], **Anders O. Garlid**[3], **Ling Chen**[1], **Agnieszka Sidor**, **Keith D. Garlid**[3], and **Brian O'Rourke**[1,*]

[1]Division of Cardiology, The Johns Hopkins University School of Medicine, Baltimore, MD

[2]Department of Biomedical Engineering, The Johns Hopkins University School of Medicine, Baltimore, MD

[3]Dept of Biology, Portland State University, Portland, OR

Abstract

Rationale—Activation of the mitochondrial ATP-sensitive potassium channel (mitoK$_{ATP}$) 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$_{ATP}$.

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 β. The high-affinity ROMK toxin, tertiapin Q, inhibits mitoK$_{ATP}$ 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$_{ATP}$ channel.

Keywords

ATP-sensitive potassium channel; mitochondria; apoptosis; preconditioning; ischemia; cytoprotection; renal outer medullary potassium channel

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

Address correspondence to: Dr. Brian O'Rourke, Johns Hopkins University, Bernard Laboratory of Fundamental Research in Preventive Cardiology, Division of Cardiology, Department of Medicine, 720 Rutland Ave, 1060 Ross Bldg., Baltimore, MD 21205-2195, Tel.: 410-614-0034, Fax.: 410-502-5055, bor@jhmi.edu.
†Authors contributed equally to this work.

DISCLOSURES
None

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_{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_{ATP} current[7, 8]. Thus, mitoK_{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_{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 *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_{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_{ATP}.

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_{ATP} activity[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[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[21], ROMK/KCNJ1 had the highest ranking of all the inward rectifier K^+ 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 β (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 MFKHLRKWVVTRFFGHSRQRARL 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^+ fluxes in isolated mitochondria, a cardiac-derived cell line (H9C2), and primary cultures of neonatal rat ventricular myocytes (NRVMs), employing a high affinity K^+ channel toxin, pharmacological tools, and molecular methods.

The classical mitoK_{ATP} assay, developed by Garlid and coworkers, employs the 90° light scattering property of isolated mitochondria as a readout of mitochondrial matrix volume[26]. It is based on the principle that the activation of K^+ uptake into mitochondria is accompanied by the movement of osmotically-obligated water, counterbalanced by a K^+/H^+ exchanger[27]. The initial rate of mitochondrial swelling is inhibited by ATP, and this inhibition is reversed by the action of K^+ channel opener compounds (e.g., cromakalim, diazoxide, etc.)[13]. Conversely, mitoK_{ATP} is inhibited by sulfonylureas or by 5-hydroxydecanoate[28]. The expected effects of ATP and diazoxide (30μmol/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[29, 30], abrogates the effect of diazoxide (Figure 3A) with subnanomolar potency (IC_{50}= 25 pmol/L; Figure 3B). The native K_{ATP} sensitivity to Tertiapin Q was also investigated in H9C2 cells and in NRVMs by measuring the initial rates of thallium (Tl^+) uptake (a surrogate for K^+) 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^+ 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^+ channels and is partially selective for ROMK over GIRK1[30] and KCa[31] channels. GIRK channels have not been reported in mitochondria (and we found no mass spectrometric evidence for

them) and although K_{Ca} channels are thought to be present in the mitochondrial inner membrane[32], our experiments were performed in the absence of Ca^{2+}.

The Tertiapin Q experiments provided some indication that ROMK may be a component of the native mitoK_{ATP}; 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 Tl^+ 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 Tl^+ 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 Tl^+ uptake was similar to that observed for control cells treated with the mitoK_{ATP} inhibitor 5-hydroxydecanoate (5-HD; Figure 4A and 4B). In addition, Tl^+ 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 Tl^+ 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 (100μmol/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 K_{ATP} 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 K_{ATP} 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 K_{ATP} channel, however, has remained elusive, and dominant negative suppression[33] or genetic knockout of Kir6.x channels has been ineffective in suppressing mitoK_{ATP} responses[34, 35]. Moreover, antibody-based identification of mitochondrial channel has been confounded by non-specific binding of the available reagents[18].

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 channels[25]. 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 mitoK_{ATP}. 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 K_{ATP} channels, it is both activated and inhibited by ATP[24, 36]. Channel activity of excised membrane patches containing ROMK channels rapidly runs down when Mg^{2+} is present. This effect can be reversed by applying MgATP or the catalytic subunit of protein kinase A, suggesting phosphorylation-dependent activation[36]. The ROMK channel, and particularly ROMK2, is inhibited by millimolar concentrations of MgATP ($K_{1/2}$=2.3 mmol/L) [36]. This inhibition is independent of ATP hydrolysis - moreover, mutations in the Walker site of ROMK2 shift the MgATP inhibition curve[36]. Like other K_{ATP} channels, ROMK can be activated by PIP_2[37], a feature that has also been documented for mitoK_{ATP}[38], and it is regulated by pH[39] and protein kinase C[40]. In addition, it has been reported that ROMK2 may be intrinsically sensitive to the K_{ATP} inhibitor glibenclamide (which also inhibits mitoK_{ATP} [28]) even in the absence of SUR coexpression[41]. Finally, ROMK can interact with members of the ATP binding cassette family of proteins, including CFTR[42] and SUR[43]. For example, when coexpressed in Xenopus oocytes, ROMK2, but not ROMK1 or ROMK3, was shown to physically associate with SUR2B to form glibenclamide-sensitive channels[44].

Circumstantial evidence aside, the fact that Tertiapin Q, a high affinity ROMK toxin[30], could inhibit diazoxide-induced K^+-specific swelling in isolated mitochondria, as well as ATP-sensitive mitochondrial Tl^+ uptake in partially-permeabilized cells, provides the first evidence that ROMK is a component of native mitoK_{ATP}. Moreover, 5-HD inhibits, and diazoxide activates, a similar component of the Tl^+ influx pathway that is suppressed by ROMK knockdown, providing additional support for the conclusion that ROMK is a component of mitoK_{ATP}.

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 localization[45]. 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 mitoK_{ATP} channels[46, 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 mitoK_{ATP} 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 mitoK_{ATP} or mitoK_{Ca}, are cytoprotective. Identification of mitoROMK provides, for the first time, a molecular target for mechanistic and therapeutic investigation of this important cell survival pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations

MitoK_{ATP}	Mitochondrial ATP-sensitive potassium channel
MitoROMK	Mitochondrial renal outer medullary potassium channel
SUR	Sulfonylurea receptor
tBHP	tert-butyl hydroperoxide
TPNQ	Tertiapin-Q

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Novelty and Significance

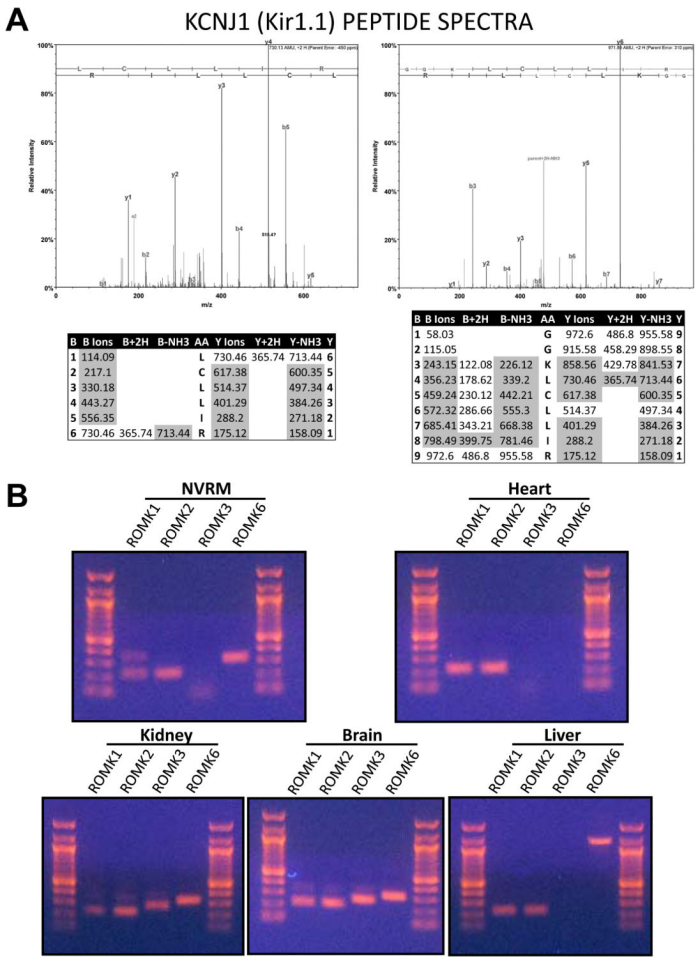
What is Known?

- Mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$) have been implicated in the mechanism of cardiac preconditioning.
- The molecular composition of mitoK$_{ATP}$ 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$_{ATP}$ opening and also inhibits mitochondrial ATP-sensitive thallium (Tl^+; a surrogate for K^+) uptake.
- Genetic knockdown of ROMK also suppresses mitochondrial ATP-sensitive Tl^+ 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$_{ATP}$ channel.

Several lines of evidence support the existence of a mitochondrial ATP-sensitive K^+ channel (mitoK$_{ATP}$), 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$_{ATP,}$ and nonspecific actions of the compounds employed, has restricted progress in understanding mitoK$_{ATP}$-mediated cytoprotection. Here, we report that an isoform of a renal outer medullary K^+ channel, ROMK, is present in the mitochondrial inner membrane and mediates mitochondrial ATP-sensitive K^+ flux. MitoK$_{ATP}$ 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^+ 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$_{ATP}$ overcomes a significant barrier in the field of cardioprotection, and opens the door for future studies to examine the mechanism of mitoK$_{ATP}$ action, find binding partners of the channel, and develop more potent and specific modulators of cardioprotection.



B	B Ions	B+2H	B-NH3	AA	Y Ions	Y+2H	Y-NH3	Y
1	114.09			L	730.46	365.74	713.44	6
2	217.1			C	617.38		600.35	5
3	330.18			L	514.37		497.34	4
4	443.27			L	401.29		384.26	3
5	556.35			I	288.2		271.18	2
6	730.46	365.74	713.44	R	175.12		158.09	1

B	B Ions	B+2H	B-NH3	AA	Y Ions	Y+2H	Y-NH3	Y
1	58.03			G	972.6	486.8	955.58	9
2	115.05			G	915.58	458.29	898.55	8
3	243.15	122.08	226.12	K	858.56	429.78	841.53	7
4	356.23	178.62	339.2	L	730.46	365.74	713.44	6
5	459.24	230.12	442.21	C	617.38		600.35	5
6	572.32	286.66	555.3	L	514.37		497.34	4
7	685.41	343.21	668.38	L	401.29		384.26	3
8	798.49	399.75	781.46	I	288.2		271.18	2
9	972.6	486.8	955.58	R	175.12		158.09	1

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.

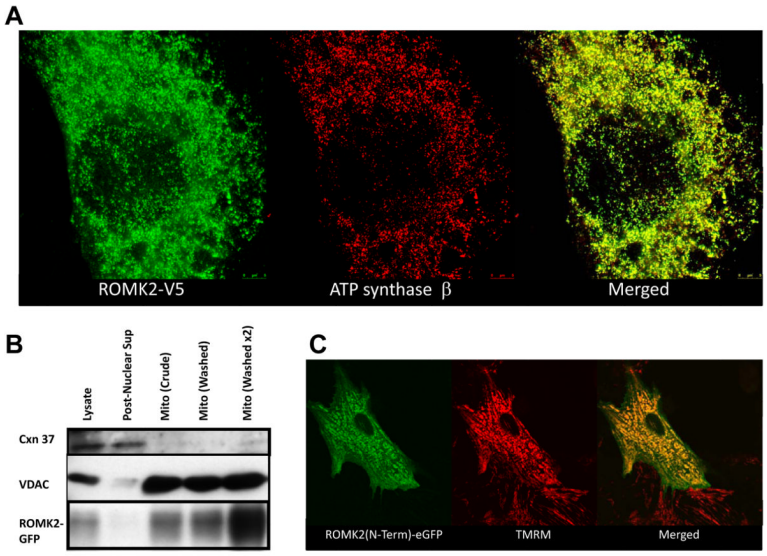


Figure 2. Targeting of ROMK2 to mitochondria

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.

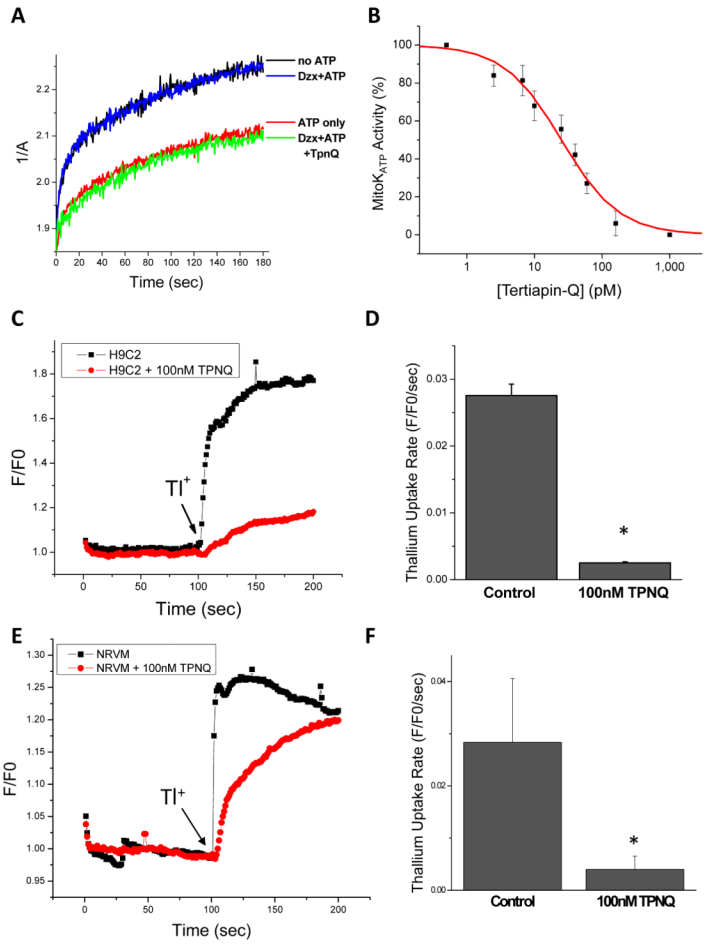


Figure 3. Inhibition of mitoK$_{ATP}$ activity with a ROMK toxin

A) Swelling of isolated rat heart mitochondria in the presence of K^+ and P_i measured spectrophotometrically as a change in 90° light scattering at 520nm excitation. The matrix volume increase is inhibited by ATP while the K^+ 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$_{ATP}$. **B)** Concentration-response curve for TPNQ inhibition of mitoK$_{ATP}$ 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 ($p<0.05$).

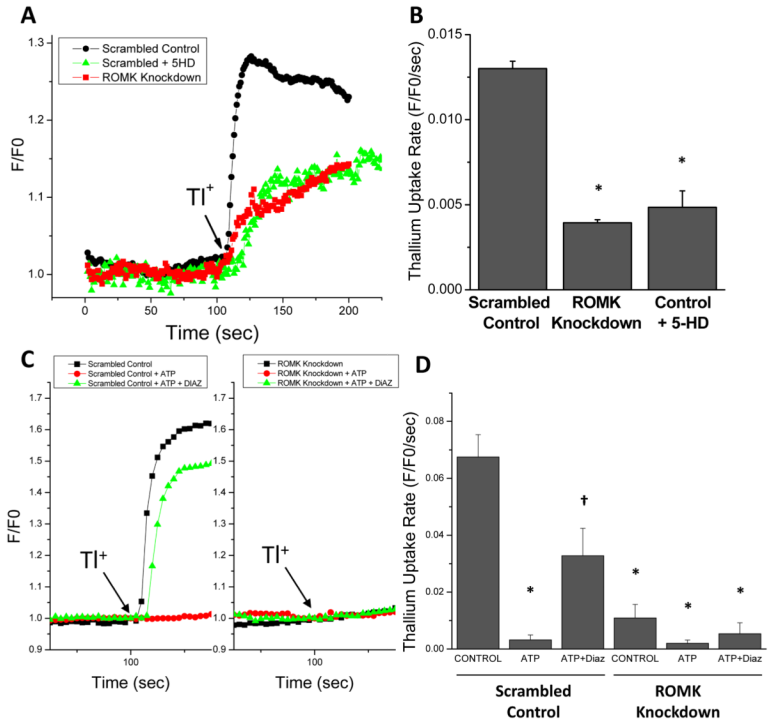


Figure 4. Suppression of mitochondrial thallium uptake by ROMK knockdown

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^+- and Tl^+- free fluorescence, of a mitochondrial matrix-localized thallium-binding reporter, BTC-AM (F/F_0). 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 ($p<0.05$). **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.

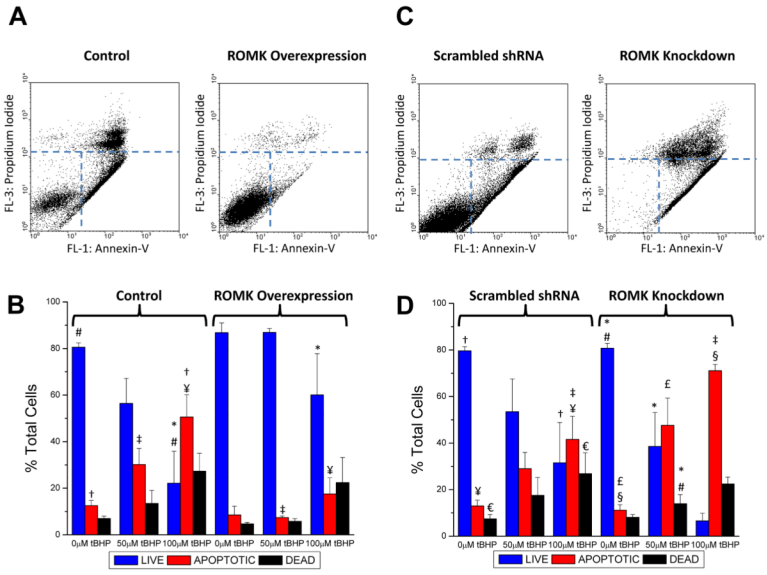


Figure 5. ROMK2 overexpression protects against necrotic/apoptotic cell death induced by oxidative stress - ROMK knockdown exacerbates cell death

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.