Mitochondrion 33 (2017) 84–101

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

The mitochondrial BK_{Ca} channel cardiac interactome reveals BK_{Ca} association with the mitochondrial import receptor subunit Tom22, and the adenine nucleotide translocator

Jin Zhang [a,c], Min Li [a], Zhu Zhang [a], Ronghui Zhu [a], Riccardo Olcese [a,b,d,e,1], Enrico Stefani [a,b,d,e,1], Ligia Toro [a,c,d,e,*]

[a] Department of Anesthesiology, Division of Molecular Medicine, University of California, Los Angeles, Los Angeles, CA, USA
[b] Department of Physiology, University of California, Los Angeles, Los Angeles, CA, USA
[c] Department of Molecular & Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA, USA
[d] Brain Research Institute, University of California, Los Angeles, Los Angeles, CA, USA
[e] Cardiovascular Research Laboratory, University of California, Los Angeles, Los Angeles, CA, USA

ARTICLE INFO

Article history:
Received 26 July 2016
Received in revised form 29 August 2016
Accepted 30 August 2016
Available online 31 August 2016

Keywords:
Proteomics
BK channel
MaxiK channel
Potassium ion channel
Mitochondria
Heart
Tom22
Adenine nucleotide translocator
Macromolecular complex

ABSTRACT

Mitochondrial BK_{Ca} channel, $mitoBK_{Ca}$, regulates mitochondria function in the heart but information on its protein partnerships in cardiac mitochondria is missing. A directed proteomic approach discovered the novel interaction of BK_{Ca} with Tom22, a component of the mitochondrion outer membrane import system, and the adenine nucleotide translocator (ANT). The expressed protein partners co-immunoprecipitated and co-segregated into mitochondrial fractions in HEK293T cells. The BK_{Ca} 50 amino acid splice insert, DEC, facilitated BK_{Ca} interaction with ANT. Further, BK_{Ca} transmembrane domain was required for the association with both Tom22 and ANT. The results serve as a working framework to understand $mitoBK_{Ca}$ import and functional relationships.

1. Introduction

Mitochondrial BK_{Ca} channels ($mitoBK_{Ca}$) are safeguards of cardiac function as their activation protects the heart from ischemic insult, a property that is absent in the knockout animal (Xu et al., 2002; Singh et al., 2013; Soltysinska et al., 2014). One of the proposed mechanisms by which $mitoBK_{Ca}$ activation favors cardiac health after ischemic insult is via improved mitochondrial Ca^{2+} handling and regulation of the mitochondrial permeability transition pore (mPTP) (Singh et al., 2013). Consistent with this idea, in other systems, $mitoBK_{Ca}$ pharmacological inhibition produces cytochrome *c* release, an event associated with the opening of the mPTP and cell death; while Bax (proapoptotic Bcl-2 associated protein X), a mPTP activator, inhibits channel activity (Cheng et al., 2011). In agreement with its protective role, the activation of cardiac $mitoBK_{Ca}$ improves basal mitochondrial energetic performance (Aon et al., 2010), whereas silencing BK_{Ca} expression reduces cardiac oxidative phosphorylation (Soltysinska et al., 2014).

To exert its function in mitochondria, $mitoBK_{Ca}$ must be first transported into this organelle and likely associate with partner proteins, as it does in other regions of the cell (Toro et al., 2013). Supporting this view, a two-hybrid system approach discovered that the regulatory β1 subunit of BK_{Ca} channels directly binds to a mitochondrial protein, cytochrome *c* oxidase subunit I (Ohya et al., 2005).

We have previously demonstrated that the $mitoBK_{Ca}$ pore-forming α subunit is encoded by the same gene that encodes the plasma membrane BK_{Ca} channel, *Kcnma1*, and that a 50 amino acid C-terminal splice insert (named DEC) favors BK_{Ca} channel targeting into mitochondria of adult cardiomyocytes (Singh et al., 2013). Thus, the overall structure of the $mitoBK_{Ca}$ channel is equivalent to that of the well-studied plasma membrane tetrameric channel, with each α-subunit composed of 7 transmembrane segments (S0-S6), a long intracellular C-terminus and

Abbreviations: ANT, adenine nucleotide translocator; BK_{Ca}, large conductance voltage- and Ca^{2+}-dependent K^+ channel; BK_{Ca}-DEC, BK_{Ca} containing DEC splice insert at the C-terminus; co-IP, co-immunoprecipitation; DEC, a 50 amino acid C-terminal BK_{Ca} splice insert; GST, glutathione *S*-transferase; LC/MS/MS, liquid chromatography, mass spectrometry in tandem; mAb, monoclonal antibody; $mitoBK_{Ca}$, mitochondrial BK_{Ca}; mPTP, mitochondrial permeability transition pore; pAb, polyclonal antibody; Tom22, mitochondrial import receptor subunit Tom22.

* Corresponding author at: Department of Anesthesiology, UCLA School of Medicine, BH-509A CHS, Box 957115, Los Angeles, CA 90095-7115, USA.
E-mail address: ltoro@ucla.edu (L. Toro).
[1] Co-senior authors.

http://dx.doi.org/10.1016/j.mito.2016.08.017
1567-7249/

an extracellular N-terminus (Meera et al., 1997). However, the protein partners of mitoBK$_{Ca}$ are unknown.

Efforts to identify BK$_{Ca}$ channel partners at a large scale have been few and restricted to cochlea and brain preparations (Kathiresan et al., 2009; Gorini et al., 2010; Sokolowski et al., 2011; Singh et al., 2016). In this work, we have examined the interactome of mitoBK$_{Ca}$ in adult heart (isolated cardiomyocytes and whole ventricle), using a directed proteomic approach aided by co-immunoprecipitation with BK$_{Ca}$ antibodies and pull-down with recombinant DEC sequence. Two putative protein partners were selected to validate and further examine the regions in BK$_{Ca}$ involved in the associations: i) Tom22 from the mitochondrial import system, and ii) the adenine nucleotide translocator (ANT), which is linked to oxidative phosphorylation and the regulation of mPTP.

2. Materials and methods

2.1. Animals

Sprague-Dawley male rats (3 months old) were used. Protocols received institutional approval.

2.2. Antibodies

The following antibodies were used: anti-BK$_{Ca}$ monoclonal antibody (mAb) (75-022, UC Davis/NIH NeuroMab facility), anti-BK$_{Ca}$ polyclonal (p) Ab (APC-021, Alomone Labs), anti-c-Myc mAb (M4439, Sigma), anti-c-Myc polyclonal pAb (C3956, Sigma), anti-HA mAb (H3663, Sigma), anti-HA pAb (H6908, Sigma), anti-DDK mAb (TA50011, OriGene), goat anti-rabbit IgG Alexa Fluor® 680 conjugate (A21109, Invitrogen), and IRDye® 800CW goat anti-mouse IgG (926-32210, Odyssey).

2.3. Clones

Full length human BK$_{Ca}$ α-subunit starting from Met1 (BK$_{Ca}$) with or without DEC splice insert (EKKWFTDEPD NAYPRNIQIK PMSTHMANQI NQYKSTSSLI PPIREVEDEC) at the C-terminus were used in all experiments except for Figs. 4, 5 and 7 (see below). Compared to NCBI Accession No. U11058.2 (Wallner et al., 1995), which starts from Met3, constructs starting from Met1 have additional 96 amino acids at the N-terminus that includes a 3xHemagglutinin (HA)-tag upstream Met3 (MANGGGGGGG SSGGGGGGGG SSLRMSSNIH ANHLSLDASS SSSSSSSSSS SSSSSSSSSS VHEPK*MYPYD VPDYAGYPYD VPDYAGSYPY DVPDYA*; 3xHA tag sequences are in italics). BK$_{Ca}$-DEC has the C-terminal DEC sequence inserted downstream amino acids RDKQN as found in the heart (Singh et al., 2013). Clones starting from Met3 (Accession No. U11058.2) were either untagged or tagged at the N-terminus with c-Myc epitope (Meera et al., 1997) (Figs. 4 and 7) or with HA epitope (Fig. 5). Clones used in deletion constructs experiments (Figs. 4 and 7) were: BK$_{Ca}$ (1-1113); C-terminal deletion constructs 1-343, 1-441 and 1-711; and N-terminal deletion constructs 322-1113 and 679-1113; numbers correspond to NCBI Accession No. U11058.2. All BK$_{Ca}$ constructs were in pcDNA3. Tom22 construct was the human translocase of outer mitochondrial membrane 22 homolog (Accession No. NM_020243.4) with C-terminal c-Myc and DDK tags in pCMV6. ANT construct was the human mitochondrial adenine nucleotide translocator (Accession No. NM_001151.2) with a C-terminal c-Myc-DDK tag in pCMV6. Tom 22 and ANT clones were purchased from OriGene Technologies (Rockville, MD).

2.4. Expression of recombinant fusion proteins in E. coli

Glutathione *S*-Transferase (GST)-DEC and GST in pGEX3 vector were transformed into *E. coli* BL21DE3 (Invitrogen) and cultured at 37 °C using Luria-Bertani (LB) broth (Thermo Fisher Scientific) supplemented with 100 ng/mL ampicillin until $OD_{600} = 0.5$. The expression of GST-DEC and GST was then induced by adding IPTG to a final concentration of 0.2 mM. Cultures were continued in a rotary shaker for 4 h at 37 °C. Bacteria were centrifuged at 1000*g* for 5 min to collect the pellet. The pellet was further lysed using sonication (15 s) at power level 2 in lysis buffer (mM): 50 Tris, pH 7.4, 100 NaCl, 5 $MgCl_2$, 1% Triton X-100, 10% glycerol, supplemented with fresh 2 mM DTT, 200 mM PMSF and 1:500 dilution of protease inhibitor cocktail (11697498001, Roche).

2.5. Isolation of cardiomyocytes from left ventricle.

Sprague-Dawley rats were anesthetized and injected with heparin (200 IU/kg, i.v.). After 15 min hearts were harvested in ice-cold Tyrode's solution (mM): 130 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.6 Na_2HPO_4, 10 glucose, 5 taurine, 10 2,3-butanedione monoxime, and 10 HEPES, pH 7.4, oxygenated with 95% O_2-5% CO_2 (v/v), and mounted on a modified Langendorff apparatus with an 80 cm H_2O constant pressure. After 5–10 min of perfusion with Tyrode's solution at 37 °C, the hearts were next perfused for 15 min with Tyrode's solution containing 372 U/mL Collagenase Type-2 and 1.0 U/mL Protease Type-XIV, and washed for 10–15 min with KB solution (mM): 25 KCl, 10 KH_2PO_4, 5 creatine, 2 $MgSO_4$, 20 glucose, 20 taurine, 100 K-glutamate, 10 aspartic acid, 5 HEPES, 0.5 EGTA, and 1% (wt/v) BSA, pH 7.2 oxygenated with 95% O_2-5% CO_2 (v/v). After washing, the left ventricles were tweezed into pieces in KB solution to release cells. Isolated cardiomyocytes were filtered through a 100 μm strainer, and centrifuged at 1000*g* for 2 min.

2.6. Isolation of "crude" mitochondria and Percoll-purification

i) "Crude" mitochondria. Isolated cardiomyocytes or left ventricles were homogenized with a Potter-Elvehjem homogenizer (20 rapid strokes) on ice and using isolation buffer A (mM): 70 sucrose, 210 mannitol, 50 Tris-HCl, and 1 Na_2-EDTA, pH 7.4. The homogenate was centrifuged at 2400*g* for 5 min at 4 °C. The supernatant was then centrifuged at 17,000*g* for 10 min at 4 °C. The pellet containing "crude" mitochondria was either further purified (ventricle samples) using Percoll gradient or directly resuspended in lysis buffer (isolated cardiomyocyte samples) (see Section 2.7). ii) Percoll purification. "Crude" mitochondria was carefully added onto 3 mL of 30% (v/v) Percoll (Graham, 2001) in buffer B (mM): 250 sucrose, 10 Na-HEPES, 1 Na_2-EDTA, pH 7.4. Samples were centrifuged in a fixed angle rotor at 50,000*g* for 45 min. After ultracentrifugation, three clear layers were observed, and labeled as M1, M2 and M3 (Singh et al., 2012). The M3 fraction, corresponding to the purified mitochondria, was carefully isolated and resuspended in 1 mL of isolation buffer A and centrifuged at 17,000*g* for 10 min. The pellet was washed twice by resuspension with the same buffer and centrifuged again at 17,000*g* for 5 min each. The purified mitochondria were lysed within 2 h after isolation.

2.7. Mitochondria and cardiomyocyte lysates

Mitochondria or isolated cardiomyocyte preparations were incubated at 4 °C (on a rotatory shaker for 1 h) in cell lysis buffer (1 and 3 mL, respectively) (mM): 50 Tris, 150 NaCl, 5 EDTA, 0.1% Nonylphenyl Polyethylene Glycol (NP-40 alternative, Calbiochem), and 0.25% Na-deoxycholate, pH 7.4 supplemented with protease inhibitor cocktail (one tablet/50 mL) and 200 mM PMSF. After lysis, samples were centrifuged at 17,000*g* for 10 min at 4 °C and the supernatant was saved at −80 °C for future use. Protein concentration was measured with Bio-Rad Protein Assay method.

2.8. Pull down assay and SDS-PAGE

Recombinant fusion protein GST-DEC and GST (control) were used. Glutathione Sepharose 4B beads (GE healthcare) were first prewashed with bacteria lysis buffer and then incubated with 3 mg of GST or