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Molecular identity of cardiac mitochondrial chloride intracellular channel proteins

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ABSTRACT

Emerging evidences demonstrate significance of chloride channels in cardiac function and cardioprotection from ischemia–reperfusion (IR) injury. Unlike mitochondrial potassium channels sensitive to calcium (BK_{Ca}) and ATP (K_{ATP}), molecular identity of majority of cardiac mitochondrial chloride channels located at the inner membrane is not known. In this study, we report the presence of unique dimorphic chloride intracellular channel (CLIC) proteins namely CLIC1, CLIC4 and CLIC5 as abundant CLICs in the rodent heart. Further, CLIC4, CLIC5, and an ortholog present in *Drosophila* (*Dm*CLIC) localize to adult cardiac mitochondria. We found that CLIC4 is enriched in the outer mitochondrial membrane, whereas CLIC5 is present in the inner mitochondrial membrane. Also, CLIC5 plays a direct role in regulating mitochondrial reactive oxygen species (ROS) generation. Our study highlights that CLIC5 is localized to the cardiac mitochondria and directly modulates mitochondrial function.

1. Introduction

Al Awqati and his colleagues using R(+)-methylindazone, R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy] acetic acid (IAA-94) (Landry et al., 1987) isolated the first chloride intracellular ion channel (CLIC) proteins (p64) from bovine kidney cortex microsomal membrane fractions and tracheal apical epithelium (Landry et al., 1989; Redhead et al., 1992). So far six paralogs of CLIC are identified in mammals (CLIC1–CLIC6) (Ashley, 2003; Berryman and Bretscher, 2000; Duncan et al., 1997; Edwards, 1999; Friedli et al., 2003; Heiss and Poustka, 1997; Littler et al., 2010; Nishizawa et al., 2000; Qian et al., 1999; Tulk and Edwards, 1998), four in plants (*At*DHAR1–*At*DHAR4) (Elter et al., 2007; Littler et al., 2010) and three in invertebrates (EXC-4, EXL-1 and *Dm*CLIC) (Berry et al., 2003; Littler et al., 2008). CLICs exist in both soluble and integral membrane forms and exhibit trans-redox regulated channel activity (Singh, 2010; Singh and Ashley, 2006) in artificial bilayers or upon overexpression in mammalian cells (Tulk et al., 2002; Valenzuela et al., 2013; Warton et al., 2002). They exhibit differential tissue specific distribution and as the name specifies are localized to the intracellular organelles (Singh, 2010) specifically nuclear membrane (Ulmasov et al., 2007; Valenzuela et al., 1997), secretory vesicles of hippocampal neurons (Chuang et al., 1999), caveolae (Edwards and Kahl, 2010), trans-Golgi network (Edwards and Kahl, 2010), endoplasmic reticulum (Duncan et al., 1997) and mitochondria (Arnould et al., 2003; Edwards and Kahl, 2010; Fernandez-Salas et al., 1999).

CLICs are multifunctional proteins playing a role in membrane trafficking, cytoskeletal function (Berryman et al., 2004), apoptosis (Fernandez-Salas et al., 2002; Suh et al., 2004), cell cycle control (Valenzuela et al., 2000), tubulogenesis (Berry et al., 2003), VEGF-mediated angiogenesis of endothelial cells (Tung et al., 2009), modulation of ryanodine receptors (Board et al., 2004; Takano et al., 2012) and cell differentiation (Suh et al., 2007). CLICs are also implicated in modulating cardiovascular physiology, specifically, CLIC4 regulates vascular endothelial growth factor (VEGF)-mediated tubulogenesis in mammalian endothelial cells (Ulmasov et al., 2009). Recently, it was reported that inhibition of CLIC4 attenuates the development of pulmonary hypertension in chronically hypoxic mice (Wojciak-Stothard et al., 2014). Additionally, pharmacological pretreatment of heart using IAA-94

Abbreviations: CLIC, chloride intracellular channel; DIDS, 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid; ETC, electron transport chain; IAA-94, R(+)-methylindazone, R(+)-[(6,7-dichloro-2-cyclopentyl-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)-oxy]acetic acid; IMAC, inner membrane anion channel; IMM, Inner mitochondrial membrane; IPC, ischemic preconditioning; IR, Ischemia–reperfusion; MI, myocardial infarction; mPTP, mitochondrial permeability transition pore; MTS, mitochondrial targeting sequence; NPPB, 5-nitro-2-(3-phenylpropyl-amino) benzoic acid; OMM, outer mitochondrial membrane; PBS, phosphate buffer saline; ROS, reactive oxygen species; VDAC, voltage dependent anion channel.

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abrogated the cardioprotective effects of ischemic pre-conditioning (IPC) (Batthish et al., 2002; Diaz et al., 1999), and also cyclosporine A-mediated cardioprotection against ischemia–reperfusion injury (IR) (Diaz et al., 2013). Although these studies suggest the prominence of chloride (Cl) channel-mediated cell volume regulation in cardioprotection, the molecular identity of CLIC channels in cardiac mitochondria has not been deciphered. As CLIC4 (also known as mtCLIC4) localizes to the mitochondria of both keratinocytes and L929 cells (Arnould et al., 2003; Fernandez-Salas et al., 1999; Suh et al., 2007), and IAA-94-sensitive CLIC-like currents were observed in cardiac mitoplast (Misak et al., 2013), we set on to determine the presence and localization of CLICs in cardiac cells as well as isolated cardiac mitochondria.

We demonstrate that CLIC1, CLIC4 and CLIC5 are the most abundant CLIC transcripts present in the heart. CLIC4 and CLIC5 but not CLIC1 localize to adult cardiac mitochondria. CLIC localization in the mitochondria was also observed in cardiac tubes of *Drosophila melanogaster*. Further, we show that CLIC4 is enriched in the outer mitochondrial membrane (OMM) whereas CLIC5 localizes to inner mitochondrial membrane (IMM). Also, cardiac mitochondria from CLIC5 knockout (KO) mice showed increased reactive oxygen species (ROS) generation, thus implicating a direct role of CLIC5 in modulating mitochondrial ROS generation.

2. Methods

All experiments were conducted in accordance with guidelines and approved by Drexel University IACUC committee. Neonatal cardiomyocytes were prepared from postnatal day 3 rat pups. A complete description of the protocols can be found in data supplement.

2.1. Animals

Two months old *Rattus norvegicus* (Sprague–Dawley) and CD1 background mice were purchased from Charles River (PA). The *clic1*$^{-/-}$ and *clic4*$^{-/-}$ are generated in CD1 background and are characterized in previous studies (Chalothorn et al., 2009; Edwards et al., 2014). *clic5*$^{-/-}$ (CLIC5^{jbg}) mice generated in C3H/HeJ background was obtained from Jackson laboratories (Gagnon et al., 2006) and bred in the laboratory. Wild type *D. melanogaster* were purchased from Bloomington, Indiana University. *clic*^{109} mutant (Berryman et al., 2010) and wild type flies were cultured on Jazz mix fly media purchased from Fisher Scientific.

2.2. Real time PCR analysis

RNA was prepared from the hearts of 2 month old Sprague–Dawley rats using TRIZOL reagent (Life technologies) according to the manufacturer's instructions. DNAaseI digestion was carried out for 30 min at 37 °C prior to cDNA synthesis (Qiagen kit). Gene expression of different CLIC using its specific primer (Supplementary Table I) was analyzed by SYBR green based quantitative real time PCR (iQ cycler, Applied Biosystems) in accordance with MIQE guidelines (Bustin et al., 2009).

2.3. Isolation of neonatal, adult cardiomyocytes and purified mitochondria

Hearts were surgically excised from postnatal day 3 (p3) pups and placed in a dissociation buffer (mmol/L, 16 NaCl, 20 HEPES, 0.8 Na_2HPO_4, 5.4 glucose, 5.4 KCl, 0.8 $MgSO_4$, pH 7.35 containing 0.25% trypsin). Ventricles were minced in dissociation buffer and the digestion was carried out at 37 °C. Dissociated neonatal cardiomyocytes were seeded on 0.1% (*w*/*v*) poly-D-lysine coated coverslip in DMEM containing 10% (*v*/*v*) FBS and penicillin (100 I.U.)/streptomycin (100 μg/mL), cultured in humidified 5% (*v*/*v*) CO_2 incubators at 37 °C. Adult cardiomyocytes and mitochondria were isolated from the left ventricle and used immediately as described earlier (Singh et al., 2013, 2012).

2.4. Measurement of ROS generation

Cardiac mitochondria was isolated from 3 month old wild type (CD1 and C3H/HeJ), *clic1*$^{-/-}$, *clic4*$^{-/-}$ and *clic5*$^{-/-}$ mice, respectively, according to protocols described earlier (Singh et al., 2012). ROS generation by cardiac mitochondria was detected by amplex red using a fluorescence spectrophotometer (Hitachi F-2710) (Singh et al., 2012). Briefly, 5 μg horseradish peroxidase (Sigma-Aldrich) was added to the ROS buffer [mmol/L, 20 Tris–HCl, 250 sucrose, 1 EGTA-Na_4, 1 EDTA-Na_2, pH 7.4 at 37 °C] and the baseline fluorescence was measured (excitation at 560 nm and emission at 590 nm). After 1 min, 10 μmol/L amplex red (Life technologies) was added followed by 25 μg of mitochondria. 3 mmol/L succinate (Sigma-Aldrich) was added to activate the mitochondria. Fluorescence was monitored continuously for 60 min at 5 s resolution and the rate of ROS production was calculated. Total ROS produced was measured by calculating area under curve using an equation (Nykamp, 2015):

$$\text{Total area} = \lim_{\Delta x \to 0} \sum_{i=1}^{N} fi(x)dx.$$

2.5. Bioinformatics analysis

Several algorithms including predotar (Small et al., 2004), mitoprot (Claros and Vincens, 1996), targetP (Emanuelsson et al., 2000), Yloc (Briesemeister et al., 2010) and cello (Yu et al., 2006) were used to predict the localization of CLIC proteins in the mitochondria based on the mitochondrial targeting sequence. Algorithms were used on CLIC1 (NP_001002807.1), CLIC2 (NP_001009651.1), CLIC3 (NP_001013098.2), CLIC4 (NP_114006.1), CLIC5 (NP_446055.1), CLIC6 (NP_788267.1) and *Dm*CLIC (NP_572928.1). VDAC2 (NP_001275134.1), a well-established mitochondrial marker, was used as a positive control in our *in silico* analysis.

2.6. Data analysis

Data are presented as mean ± SEM, n ≥ 3. Student's two-tailed *t* test or one-way ANOVA was used to determine statistical significance. A *p* value of <0.05 was considered statistically significant.

3. Results

IAA-94 abrogated the protective effects of IPC as it increased myocardial infarction (MI) due to IR injury *in vitro* (Diaz et al., 1999), and also prevented cyclosporine A mediated cardioprotection (Diaz et al., 2013). Moreover, IAA-94-sensitive currents were observed in cardiac mitoplast (Misak et al., 2013) but the molecular identity of cardiac mitochondrial CLICs is not yet elucidated. Therefore, we first set onto examine the identity of CLICs in cardiac mitochondria.

As there are six paralogs of CLIC in humans and rats, we tested the abundance of each CLICs in cardiac tissue. Real time qPCR analysis revealed the relative abundance of CLIC4 (89.6 ± 10.4%), CLIC5 (66.7 ± 14.0%), CLIC1 (63.2 ± 3.8%), CLIC2 (35.6 ± 10.6%), CLIC3 (4.5 ± 0.7%) and CLIC6 (0.4 ± 0.2%) (Fig. 1A, B) in the rat heart. Relative abundance was normalized to GAPDH (Fig. 1A) transcript levels. Negative controls without reverse transcriptase (−RT) showed no detectable signals (Fig. 1B). Expression of CLIC1, CLIC4 and CLIC5 proteins was corroborated using CLIC-specific antibodies (Supplementary Fig. 1) on Western blot of whole rat heart lysates (Fig. 1C). Calculated molecular weights of CLIC1, CLIC4 and CLIC5 are ~26.98, 28.63, and 28.23 kDa, respectively. However, on 4–20% SDS gel, CLIC1 migrated at ~30 kDa, CLIC4 at ~28 kDa and CLIC5 at ~30 and ~50 kDa. Two distinct bands were observed for CLIC5 in the heart lysate which could either be attributed to a dimer or presence of another isoform (NP_001107558.1). Additional lower molecular weight bands were also observed for CLIC4 in kidney