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Voltage-gated calcium channel blockers deregulate macroautophagy in cardiomyocytes

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ABSTRACT

Voltage-gated calcium channel blockers are widely used for the management of cardiovascular diseases, however little is known about their effects on cardiac cells *in vitro*.

We challenged neonatal ventricular cardiomyocytes (CMs) with therapeutic L-type and T-type Ca^{2+} channel blockers (nifedipine and mibefradil, respectively), and measured their effects on cell stress and survival, using fluorescent microscopy, Q-PCR and Western blot. Both nifedipine and mibefradil induced a low-level and partially transient up-regulation of three key mediators of the Unfolded Protein Response (UPR), indicative of endoplasmic (ER) reticulum stress. Furthermore, nifedipine triggered the activation of macroautophagy, as evidenced by increased lipidation of microtubule-associated protein 1 light chain 3 (LC3), decreased levels of polyubiquitin-binding protein p62/SQSTM1 and ubiquitinated protein aggregates, that was followed by cell death. In contrast, mibefradil inhibited CMs constitutive macroautophagy and did not promote cell death. The siRNA-mediated gene silencing approach confirmed the pharmacological findings for T-type channels.

We conclude that L-type and T-type Ca^{2+} channel blockers induce ER stress, which is divergently transduced into macroautophagy induction and inhibition, respectively, with relevance for cell viability. Our work identifies VGCCs as novel regulators of autophagy in the heart muscle and provides new insights into the effects of VGCC blockers on CMs homeostasis, that may underlie both noxious and cardioprotective effects.

1. Introduction

Voltage-gated Ca^{2+} channels (VGCCs) are essential for initiating and regulating cardiac function. During the cardiac action potential, Ca^{2+} influx through L-type channels triggers the sarcoplasmic reticulum Ca^{2+} release that enables the excitation–contraction (E–C) coupling (reviewed by Bodi et al., 2005). Ca^{2+} can also enter cardiac myocytes through low-voltage-activated T-type channels, which are expressed throughout cardiac development until the end of the neonatal period, and can contribute to pacemaker activity as well as E–C coupling to some extent (reviewed by Vassort et al., 2006). Importantly, T-type channels are re-expressed in ventricular myocytes under diverse pathological conditions such as hypertrophy and heart failure, suggesting that they play a role in cardiac disease (Vassort et al., 2006; Ono and Iijima, 2010).

VGCC blockers are widely used for the management of a variety of adult and pediatric cardiovascular diseases. Clinical trials have proved the effectiveness of dihydropyridine and non-dihydropyridine VGCC blockers as anti-anginal and anti-ischemic agents (Chouairi et al., 1995; Leitch et al., 1998; Kobrin et al., 1998). Mibefradil a non-dihydropyridine VGCC blocker displaying a higher affinity for T-type over L-type channels (Cohen et al., 1992; Mishra and Hermsmeyer, 1994; De Paoli et al., 2002) stands out for their beneficial effects in patients with stable angina (Lee et al., 2002). T-type channel blockers have also been shown to display anti-arrhythmic properties (Billman and Hamlin, 1996; Muller et al., 1998), to prevent sudden death (Kinoshita et al., 2009), to ameliorate (Sandmann et al., 2001; Min et al., 2002) or prevent (Horiba

Abbreviations: ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; E–C, excitation–contraction; VGCCs, voltage-gated calcium channels; CMs, cardiac myocytes; UPR, unfolded protein response; MDC, monodansylcadaverine; LR, lysotracker red; GADD153/CHOP, CEBP-homologous protein; GRP78/BIP, 78 kDa glucose-regulated protein; XBP-1, *Xho*I site– binding protein 1; Atg, autophagy related gene; LC3, microtubule-associated protein1 light chain 3; p62, polyubiquitin-binding protein p62/sequestosome-1; Q-PCR, quantitative RT-PCR; siRNA, small interfering RNA; FDA, U.S. Food and Drug Administration.

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et al., 2008) hypertrophy, and to limit infarct size by a possible direct cardioprotective effect (Schulz et al., 1999). The dihydropyridine nifedipine targets L-type channels with high affinity and has long been used as an anti-hypertensive and anti-anginal agent, by acting as an arterial vasodilator (Snider et al., 2008).

We set out to examine the effects of both VGCC blockers on the homeostasis and viability of primary cultures of cardiac myocytes (CMs), because Ca^{2+} fluxes underly pivotal cellular processes such as growth, apoptosis and autophagy (Harr and Distelhorst, 2010). Autophagy, the process of degrading organelles and protein aggregates, is a basal homeostatic process that plays a prominent role in the heart physiology and patophysiology, particularly in cardiomyocytes as quiescent cells that need to sustain high metabolic rates (reviewed by Gottlieb and Mentzer, 2010). Our results show that L-type and T-type channel blockers induce a low-level and transient ER stress, with a distinct conveyance into cell macroautophagy and viability: whereas nifedipine triggers a macroautophagic process in CMs and ultimately promotes apoptosis, mibefradil exerts the opposite effect, by decreasing the autophagic flux and not affecting cell death. Thus, we identify L-type and T-type channels as new targets for macroautophagy regulation of CMs, and provide new clues to the beneficial actions reported in clinical trials for T-type channel blockers, particularly against pathophysiological conditions involving a maladaptive autophagy.

2. Materials and methods

2.1. Primary cell culture and treatments

The investigation with experimental animals conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by our Experimental Animal Ethic Committee. Neonatal rat ventricular cardiomyocytes (CMs) were obtained from the heart of 0–3 days old Sprague-Dawley rats. In brief, heart ventricles were removed, minced into 1 mm^3 pieces, and digested with type-2 collagenase (Worthington Biochemical, Freehold, NJ, USA). The dissociated cells were resuspended in supplemented DMEM (Dulbecco's minimum essential medium, see below), and fibroblasts were removed by pre-plating for 45 min twice. After pre-plating, the supernatant (cardiomyocytes) was recovered and cells were plated in supplemented DMEM (see below) on P35 plates at a density of 1×10^6 cells/well.

2.2. Culture media

CMs were cultured in M199:DMEM low glucose (1 g/L) 1:3, 10% horse serum, 5% fetal calf serum (FCS), 1 mM Hepes and 1% penicillin/streptomycin. This medium was supplemented with 5-bromo-2′-deoxyuridine (100 μM) for the initial 24 h, to suppress fibroblast growth. For starvation experiments, cells were maintained in DMEM glucose-free, serum-free medium (Sengupta et al., 2009).

All media and supplements were from Invitrogen (Carlsbad, CA, USA). Culture media was changed every 24 h, to minimize basal cell stress. VGCC blockers and calcium modulators were dissolved in DMSO or water as appropiate and kept at −80 °C, until the day of the experiment.

2.3. Drugs

Bafilomycin A_1 was obtained from Tocris Biosciences (Bristol, UK). Thapsigargin was from Alomone Labs Ltd. (Jerusalem, Israel). VGCC antagonists nifedipine and mibefradil were purchased from Sigma–Aldrich.

2.4. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

To quantify cell death we employed the *Tunel In Situ Cell Death Detection Kit* (TMR red) from Roche Applied Science (Indianapolis, IN, USA), according to the manufacturer's instructions for adherent cells. Cell nuclei were counter-stained with Hoechst 33258 10 μg/mL (Molecular Probes, Invitrogen), cover-slipped with Mowiol (Calbiochem, La Jolla, USA), and analyzed by fluorescence microscopy (Olympus IX70, Tokyo, Japan) equipped with epifluorescence optics and a camera (Olympus OM-4 Ti). Fields were selected at random and a minimum 300 cells/coverslip were counted at 200× magnification. Percentage of Tunel-positive cells was calculated as Tunel-positive cell number/total nuclear (Hoechst staining).

2.5. Fluorescence microscopy on live cells

Nuclei were labeled with 10 μg/mL of Hoechst 33258 (Molecular Probes) for 5 min at 37 °C. After 3 washes in phosphate-buffered saline (PBS), either monodansylcadaverine (MDC, 50 μM, 30 minutes, from Sigma–Aldrich) or Lysotracker red (LR, 100 nM, 2 minutes, from Molecular Probes, Invitrogen) were applied, and that was followed by 3 more washes in PBS. Micrographs were obtained using an inverted Olympus IX70 microscope as in the previous section.

2.6. RT-PCR

Total RNA was isolated from cultured cells using a Nucleospin RNA/protein isolation kit (Macherey-Nagel, Cultek, Madrid, Spain). Messenger RNA was reverse transcribed (RT) to cDNA (25 °C for 10 min, 42 °C for 1 h and 95 °C for 5 min) using Superscript II reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA). For each sample, reactions from which reverse transcriptase was omitted were run to control for contaminating DNA. Primers were designed to be complementary to the published rat sequences (see http://srs.ebi.ac.uk and http://www.ncbi.nlm.nih.gov) for each of the pore-forming subunits of the VGCCs. Table 1 contains primer sequences and product sizes.

2.7. XBP-1 splicing assay

Under ER stress conditions, XBP-1 mRNA is processed by unconventional splicing and translated into a functional transcription factor (Iwawaki and Akai, 2006). The procedure for XBP-1 splicing assay was adapted from previous literature (Nakamura et al., 2006). XBP-1 processing is characterized by excision of a 26-bp sequence from the coding region of XBP-1 mRNA. To distinguish the unspliced from the spliced band, the PCR product was digested with Pst-1 for 2.5 h at 37 °C, because the cleaved product contains a Pst-1 restriction site. The resulting digests were run on a 3% agarose gel, to resolve a digested product of 290 bp. In contrast the products amplified from spliced XBP-1 mRNA are resistant to digestion and remained 473 bp long.

2.8. Quantitative RT-PCR (Q-PCR)

1 μg of total RNA was used to generate cDNA with Superscript II reverse transcriptase as above. 1 μl of each synthesized cDNA was analyzed by quantitative RT-PCR (ABI Prism 7000 HT sequence detection system, Applied Biosystems). All assays were based on TaqMan hydrolysis probes labeled with FAM (green fluorescent fluorophore 6-carboxyfluorescein). Samples were assayed in triplicate for each gene, and the mean expression was used during