Journal of Molecular and Cellular Cardiology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Alpha-2 adrenoceptors and imidazoline receptors in cardiomyocytes mediate counterbalancing effect of agmatine on NO synthesis and intracellular calcium handling☆

Alexander V. Maltsev [a], Yuri M. Kokoz [a,*], Edward V. Evdokimovskii [a], Oleg Y. Pimenov [a], Santiago Reyes [b], Alexey E. Alekseev [b,**]

[a] Institute of Theoretical and Experimental Biophysics, Russian Academy of Science, Institutskaya 3, Pushchino, Moscow Region 142290, Russia
[b] Division of Cardiovascular Diseases, Department of Molecular Pharmacology and Experimental Therapeutics, Stabile 5, Mayo Clinic, 200 1st Street SW, Rochester, MN 55905, USA

ARTICLE INFO

Article history:
Received 26 August 2013
Received in revised form 11 November 2013
Accepted 31 December 2013
Available online xxxx

Keywords:
Cell signaling
Heart
SR
eNOS
SERCA
Ryanodine receptor

ABSTRACT

Evidence suggests that intracellular Ca^{2+} levels and contractility of cardiomyocytes can be modulated by targeting receptors other than already identified adrenergic or non-adrenergic sarcolemmal receptors. This study uncovers the presence in myocardial cells of adrenergic α2 (α2-AR) and imidazoline I_1 (I_1R) receptors. In isolated left ventricular myocytes generating stationary spontaneous Ca^{2+} transients in the absence of triggered action potentials, the prototypic agonist of both receptors agmatine can activate corresponding signaling cascades with opposing outcomes on nitric oxide (NO) synthesis and intracellular Ca^{2+} handling. Specifically, activation of α2-AR signaling through PI_3 kinase and Akt/protein kinase B stimulates NO production and abolishes Ca^{2+} transients, while targeting of I_1R signaling via phosphatidylcholine-specific phospholipase C (PC-PLC) and protein kinase C (PKC) suppresses NO synthesis and elevates averaged intracellular Ca^{2+}. We identified that endothelial NO synthase (eNOS) is a major effector for both signaling cascades. According to the established eNOS transitions between active (Akt-dependent) and inactive (PKC-dependent) conformations, we suggest that balance between α2-AR and I_1R signaling pathways sets eNOS activity, which by defining operational states of myocellular sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) can adjust Ca^{2+} re-uptake and thereby cardiac inotropy. These results indicate that the conventional catalog of cardiomyocyte sarcolemmal receptors should be expanded by the α2-AR and I_1R populations, unveiling previously unrecognized targets for endogenous ligands as well as for existing and potential pharmacological agents in cardiovascular medicine.

1. Introduction

For many years, β-adrenoceptors were considered to be the exclusive type of sarcolemmal receptors transmitting catecholamine binding to potentiation of cardiac muscle contraction via cyclic AMP-dependent elevation of intracellular Ca^{2+} [1–3]. Later, the demonstration of cyclic AMP-independent increase in myocardial twitch contractile force made evident the presence of an additional population of α1-adrenoceptors in cardiomyocytes [2,4,5]. At present, a significant body of accumulated evidence suggests that certain neuromediators can modulate intracellular Ca^{2+} levels in cardiomyocytes, and presumably myocardial contractile force, by interacting with sarcolemmal adrenergic receptors different from β or α1 types of adrenoceptors.

Case in point is agmatine, the product of L-arginine decarboxylation and an intermediate in polyamine biosynthesis. Agmatine is synthesized in brain neurons, and release of this neurotransmitter from synaptosomes appears to be the source of its endogenous extracellular pool [6–8]. Levels of agmatine in plasma may vary, according to different studies, from 60 to 270 nM [9] or from 2.8 to 4.7 μM [10], depending on physiological or disease conditions. While the effects of such endogenous concentrations of agmatine on peripheral tissues remain only partially understood, profound cytoprotective effects have been observed following exogenous administration of agmatine [8]. Indeed, in addition to numerous effects in neuro-, nephro- and gastroprotection, with significant implications in the response to stress and trauma [8], agmatine has been also recognized to be cardioprotective [11]. Agmatine treatment given either pre- or post-ischemia enhanced hemodynamic recovery increasing cardiac performance after ischemia–reperfusion injury [12]. This protective effect of agmatine on the whole heart

* Correspondence to: Y.M. Kokoz, Institutskaya 3, Pushchino, Moscow Region 142290, Russia. Tel.: +7 4967 73 94 10; fax: +7 4967 33 05 53.
** Correspondence to: A.E. Alekseev, Stabile 5, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA. Tel.: +1 507 284 9501; fax: +1 507 266 9936.
E-mail addresses: goicr@rambler.ru (A.V. Maltsev), malat_88@mail.ru (Y.M. Kokoz), onletaet@gmail.com (E.V. Evdokimovskii), poleg237@rambler.ru (O.Y. Pimenov), reyesramirez.santiago@mayo.edu (S. Reyes), alekseev.alexey@mayo.edu (A.E. Alekseev).

0022-2828/$ – see front matter © 2014 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.yjmcc.2013.12.030

apparently results from activating molecular targets in both central and peripheral control systems, including different subtypes of imidazoline receptors (IR) and α2-adrenergic receptors (α2-AR) in neuronal and vascular tissues, and is accompanied by norepinephrine release and nitric oxide (NO) production [11,13,14]. On the other hand, it has been demonstrated that agmatine can inhibit voltage-dependent Ca^{2+}-influx and reduce intracellular Ca^{2+} in ventricular cardiomyocytes [15–17], suggesting cardiac striated muscle cells as an additional site for the agmatine-dependent protective mechanisms. Little is known, however, about the presence of imidazoline and α2-receptors in cardiomyocytes, as well as about the signaling mechanisms evoked by agmatine-receptor interactions in cardiac muscle tissue.

This study, performed in isolated cardiomyocytes, revealed the presence of α2-AR and I_1 imidazoline receptors (I_1R) that, when targeted by agmatine, can activate corresponding signaling cascades with opposing outcomes on intracellular NO synthesis and Ca^{2+} levels. Therefore, these results indicate that the conventional myocellular set of receptors should be expanded by the adrenergic α2 and non-adrenergic I_1 receptor populations.

2. Methods

2.1. Cell isolation

All procedures were performed according to the institutional requirements for the care and use of laboratory animals. Ventricular myocytes were isolated by enzymatic dissociation as previously described [18]. Briefly, cardiectomy was performed in pentobarbital-anesthetized (1 mL/100 mg body weight i.p.) Sprague Dawley and Wistar rats. Hearts were retrogradely perfused for 3–5 min with DMEM + 10 mM HEPES medium (pH 7.25). After stabilization of cardiac contractions, perfusion was continued with basic medium containing (in mM): NaCl, 80; KCl, 10; KH_2PO_4, 1.2; $MgSO_4$, 5; glucose, 20; taurine, 50; HEPES, 10; L-arginine, 1, pH 7.25, supplemented with 2.5 mM EGTA, which was replaced by 20 mg/100 mL of protease Type XIV (Sigma), 100 mg bovine serum albumin (fraction V, Sigma), and 140 μM $CaCl_2$ following cardiac arrest. After 10 min, ventricles were separated from atria and cut into small fragments in basic medium enriched with 200 μM $CaCl_2$. Single cells were then isolated by stirring (at 37 °C) in basic medium supplemented with protease Type XIV (Sigma) and collagenase IV (2.5 mg/10 mL; Worthington Biochemical Corp.). The aliquots were removed at 15–20 min intervals until the tissue was entirely digested. Isolated cardiomyocytes were precipitated by centrifugation (600–800 rpm, 1 min), washed twice and stored in basic medium containing 200 μM $CaCl_2$.

2.2. RT-PCR

RT-PCR was performed with samples from isolated left ventricular myocytes using Tertsik amplifier (DNATechnology, Russia) in buffer containing (in mM): KCl, 50; dNTPs, 0.25; Mg^{2+}, 2.5; Tris–HCl, 10; pH 8.3, supplemented with 250 nM of each primer, and 2.5 U of Taq-polymerase.

2.2.1. RNA isolation

The precipitate of cardiomyocytes was supplemented with 10 volumes of lysis buffer containing guanidine thiocyanate 4 M, sodium citrate 25 mM, laurylsarcosyl 0.5%, and β-mercaptoethanol 0.1 M. The lysate was supplemented with 1/10 of the volume of AcONa 2 M (pH 5.0), 1 part of water-saturated (acid) phenol, and 1/5 of the volume of chloroform-isoamyl alcohol mixture (24:1), and incubated for 15 min at 4 °C. After centrifugation at 2000 *g*, the supernatant was diluted by 2.5 volumes of 96% ethyl alcohol, and kept overnight at −20 °C. After centrifugation at 12,000 *g* for 5 min, mRNA precipitates were washed with 70% ethanol, air-dried and dissolved in 20 μL of diethylpyrocarbonate (DEPC)-treated water.

2.2.2. Synthesis of the first-strand cDNA

The first-strand cDNA was synthesized using a MMLV Reverse Transcriptase kit (Eurogen, Russia). The solution containing 14 μL of mRNA and 4 μL of oligo $d(T)_{18}$ primer was incubated for 2 min at 70 °C, cooled at 4 °C and supplemented with 8 μL of 5× buffer, 4 μL of dNTP mixture (10 mM of each), 4 μL of DTT (20 mM), 4 μL of sterile water, and 2 μL of MMLV revertase (200 U). cDNA synthesis was performed with 40 μL of the reaction mixture for 60 min at 37 °C and stopped by heating at 70 °C for 10 min.

2.2.3. Primers

The primers used for α2A (*adra2a*) were: forward TGGACCAAGACA GAAGGAAATGA, reverse CAAGTGGTGCCTCAGCGAAT (NCBI GenBank: NM_012739.3, predicted amplicon length: 80 bp); for α2B (*adra2B*): forward CTAGCCTTGATCTTCCTCTTGCTT, reverse CAGGTCCTCCTGGG AAATGTC (NM_138505.2, 70 bp); for α2C (*adra2c*): forward TCATGG GCGTGTTCGTACTG, reverse CCTCACGGCAGATGCCATA (NM_138506.1, 72 bp); and for Nischarin (*nisch*): forward CCAGCCTGCCAAAAGCAG, reverse CAGATTCGGCTGCGGC (XM_001057307.3, 242 bp).

2.3. Western blotting

Isolated rat ventricular cardiomyocytes were lysed in hypotonic buffer containing NaCl 20 mM, Tris–HCl 20 mM (pH 7.4) and 1% Triton X-100, supplemented with protein inhibitors and centrifuged at 2500 *g* for 5 min. Proteins were separated by 10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane (0.45 μm; Santa-Cruz, sc-3724) for immunoblotting. Rabbit polyclonal primary antibodies against α2A (Santa Cruz, sc-28983, 1:100 dilution), α2B (Abcam, ab151727, dilution 1:2000) and α2C (Abcam, ab46536, 1:200 dilution) adrenoceptors as well as goat polyclonal primary antibody against the I_1 imidazoline receptor component Nischarin (Santa Cruz, sc-47236, 1:100 dilution) were used to probe immunoreactive proteins, followed by counterstaining with a horseradish peroxidase (HRP)-conjugated anti-rabbit (Santa Cruz, sc-2004, 1:300 dilution) and anti-goat (Santa Cruz, sc-2020, 1:300 dilution) secondary antibody, respectively. HRP signals were detected using 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate (Amresco, E733) and film-captured.

2.4. Immunocytochemistry

Freshly isolated cardiac cells were plated onto coverglass and fixed with 3% paraformaldehyde followed by permeabilization and blocking of non-specific immunoreactive sites in PBS containing 0.2% Triton X-100 and 1% bovine serum albumin. The presence of α2A-AR or I_1R was probed by incubating permeabilized cells with primary antibodies (rabbit sc-28983 or goat sc-47236, respectively; both from Santa Cruz) overnight at 4 °C followed by incubation with FITC-conjugated secondary IgG antibody (anti-rabbit: sc-2090, 1:300 dilution and anti-goat: sc-2024, 1:300 dilution, both from Santa Cruz) for 2 h at room temperature. Cell nuclei were stained with DAPI (2 μg/mL for 10 min) prior to mounting and imaging using a Leica TCS SP5 laser scanning confocal microscope (Leica, Germany).

2.5. Measurements of intracellular Ca^{2+} and nitric oxide

Intracellular levels of Ca^{2+} and NO were determined in isolated cardiomyocytes using a Leica TCS SP5 laser scanning confocal microscope (Leica, Germany) or an inverted Eclipse TS100F microscope equipped with a fluorescent module T1-FM (Nikon, Japan), respectively. Cells were superfused with Hanks balanced salt solution (HBSS) containing (in mM): NaCl, 138; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $MgCl_2$, 0.5;

Please cite this article as: Maltsev AV, et al, Alpha-2 adrenoceptors and imidazoline receptors in cardiomyocytes mediate counterbalancing effect of agmatine on NO synthesis and intracellular calcium handling, J Mol Cell Cardiol (2014), http://dx.doi.org/10.1016/j.yjmcc.2013.12.030