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Original article

Sarcoplasmic reticulum Ca^{2+} release in neonatal rat cardiac myocytes

Ulrich Gergs [a,*], Uwe Kirchhefer [b], Jan Buskase [b], Katharina Kiele-Dunsche [b], Igor B. Buchwalow [c], Larry R. Jones [d], Wilhelm Schmitz [b], Otto Traub [e], Joachim Neumann [a]

[a] Institut für Pharmakologie und Toxikologie, Medizinische Fakultät, Martin-Luther-Universität Halle-Wittenberg, 06112 Halle (Saale), Germany
[b] Institut für Pharmakologie und Toxikologie, Westfälische Wilhelms-Universität, Universitätsklinikum, 48149 Münster, Germany
[c] Institut für Hämatopathologie, Fangdieckstraße 75a, 22547 Hamburg, Germany
[d] Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[e] Institut für Genetik, Abteilung Molekulargenetik, Universität Bonn, 53117 Bonn, Germany

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ABSTRACT

In the neonatal mammalian heart, the role of ryanodine receptor (=Ca^{2+} release channel)-mediated sarcoplasmic reticulum (SR) Ca^{2+} release for excitation–contraction coupling is still a matter of debate. Using an adenoviral system, we overexpressed separately the junctional SR proteins triadin, junctin, and calsequestrin, which are probably involved in regulation of ryanodine receptor function. Infection of neonatal rat cardiac myocytes with triadin, junctin, or calsequestrin viruses, controlled by green fluorescent protein expression, resulted in an increased protein level of the corresponding transgenes. Measurement of Ca^{2+} transients of infected cardiac myocytes revealed unchanged peak amplitudes under basal conditions but with overexpression of calsequestrin and triadin caffeine-releasable SR Ca^{2+} content was increased. Our results demonstrate that an increased expression of triadin or calsequestrin is associated with an increased SR Ca^{2+} storage but unchanged Ca^{2+} signaling in neonatal rat cardiac myocytes. This is consistent with an ancillary role of the sarcoplasmic reticulum in excitation–contraction coupling in the developing mammalian heart.

1. Introduction

In the adult myocardium, Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR) via activation of ryanodine receptors (RyRs) is the main mechanism of cardiac excitation–contraction coupling [1,2]. The consecutive increase in cytosolic Ca^{2+} is responsible for muscle contraction [1,3]. For relaxation, Ca^{2+} is mainly removed from the cytosol by the action of SR Ca^{2+}-ATPase (SERCA) and to a lesser extent by the Na^{+}–Ca^{2+}-exchanger (NCX).

In the neonatal mammalian heart, Ca^{2+} influx and efflux via the sarcolemma are thought to be more important for contraction–relaxation cycling than Ca^{2+} release and uptake by the SR. This may have several reasons. For example, the SR of neonatal mammalian cardiomyocytes is poorly developed, as maturation of the T-tubule system is more a postnatal event. Fittingly, NCX is highly expressed in embryonic cardiac myocytes and may play an important role in excitation and contraction. As the SR develops, NCX is downregulated whereas the SR-proteins RyR, SERCA, calsequestrin (CSQ), and phospholamban are upregulated during postnatal development [4–8]. The permeation and gating kinetics of RyRs in newborn rats are identical to those of adult. Also, ligands like cytosolic Ca^{2+} and caffeine had similar effects on RyR function of newborn and adult rats [9].

While it is clear that the SR is less important in neonatal hearts than in adult heart, a significant role of the SR cannot be ruled out. For instance, the contractile response to ryanodine, which can inhibit Ca^{2+} release from the SR by acting on the RyR, was pronounced already in 1-day-old rat hearts [10]. In addition, Seki et al. reported that the SR already functions in the fetal rat heart and that the SR Ca^{2+} release is responsible for a considerable portion of the Ca^{2+} transients in fetal cardiomyocytes [11]. In the present study, we focused on the regulation of the SR Ca^{2+} release complex in neonatal cardiac myocytes. A disturbance of the stoichiometry of the RyR complex by overexpression of its regulatory proteins CSQ, cardiac triadin (TRD), or junctin (JCN) in adult cardiac myocytes in culture or in adult cardiac myocytes from transgenic mice caused impairment of SR Ca^{2+} release [12–18].

Here, we utilized adenoviral-mediated overexpression of TRD, JCN, and CSQ to change RyR function and therefore, to study the role of SR Ca^{2+} release in neonatal rat cardiac myocytes. Our hypothesis was that if the SR is important for Ca^{2+} handling in the neonatal heart,

* Corresponding author at: Institut für Pharmakologie und Toxikologie, Medizinische Fakultät, Martin-Luther-Universität Halle-Wittenberg, Magdeburger Str. 4, 06112 Halle (Saale), Germany. Tel.: +49 345 557 4093; fax: +49 345 557 1835.
E-mail address: ulrich.gergs@medizin.uni-halle.de (U. Gergs).

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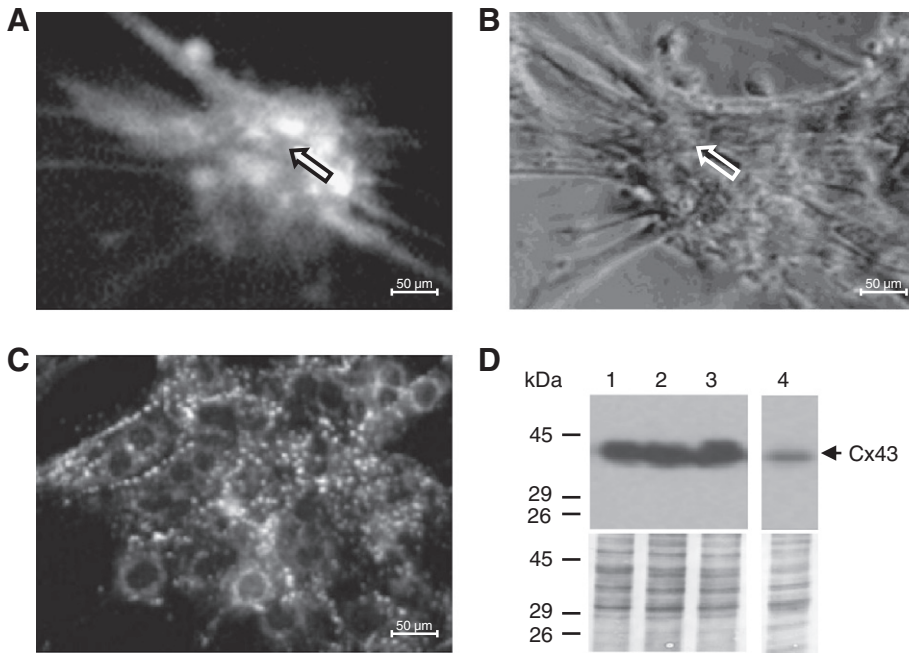

Fig. 1. Gap-junctional intercellular communication and Cx43 expression in neonatal rat cardiac myocytes. Gap-junctional communication was measured by the microinjection-dye transfer method with Lucifer Yellow (LY) at day 5 of culture (A, B). LY was detected by fluorescence microscopy (A). The corresponding phase contrast picture is shown (B). The arrows indicate the injected cell. Immunostaining of Cx43 demonstrated the localization of Cx43 at myocyte cell–cell contacts (C). Western blot analysis (D) revealed high expression of Cx43 in cultured cardiac myocytes at day 3 (lane 1), 4 (lane 2), and 5 (lane 3) of culture. For comparison, expression of Cx43 in cardiac fibroblasts at day 5 of culture is shown (lane 4). Ponceau S staining demonstrates uniform protein loading.

overexpression of the main known regulators of the Ca^{2+} release channel should alter Ca^{2+} transients. However, if for instance the NCX were solely involved in Ca^{2+} handling no effect would be expected. Our results support a role of the SR for Ca^{2+} storage but not cycling in immature cardiomyocytes.

2. Materials and methods

2.1. Generation of cDNAs

Total RNA was extracted from dog hearts using Trizol-Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. For cardiac CSQ and JCN the entire coding region was amplified using standard RT-PCR methodology. The cDNA of the cardiac isoform of TRD was isolated as described previously [19].

2.2. Recombinant adenoviruses

Recombinant adenoviruses carrying the cDNA of CSQ, TRD, or JCN (Ad-CSQ, Ad-TRD, Ad-JCN) were generated and amplified as described before [13,20]. As shuttle vector pAdTrack-CMV containing the reporter gene GFP was used. Both, GFP and the genes of interest were expressed under control of a separate CMV promoter. A green fluorescent protein (GFP)-only virus (Ad-Control) was used as control adenovirus.

2.3. Neonatal rat cardiac myocytes

Myocytes from hearts of 1–3-day-old Wistar rats were isolated using the neonatal cardiomyocyte isolation system purchased from Worthington Biochemical Corporation (CellSystems, St. Katharinen, Germany) following the manufacturer's instructions. The dissociated cells were transferred to collagen-coated plates with a final density of 125,000 cells per cm^2 and cultured as described before [13].

2.4. Preparation of cell lysates

Adenovirus infected cultured cells were washed with PBS at 4 °C and collected in lysis buffer containing 10 mM histidine (pH 7.4), 250 mM sucrose and 1% (w/v) SDS. Remaining adenoviruses were inactivated by boiling the lysate for 5 min. Finally, solubilization was completed by sonication for 30 s (VirTis, Gardiner, USA). The lysate was cleared by centrifugation for 10 min at 14,000 ×*g*.

2.5. Western blot analysis

For Western blot analysis, cell lysates were prepared and SDS sample buffer according to Laemmli was added [21]. Aliquots of 100 µg protein were loaded per lane and Western blots were performed as described previously [13]. The following primary antibodies were used: polyclonal rabbit anti-calsequestrin, polyclonal rabbit anti-triadin, polyclonal rabbit anti-junctin (all kindly provided by Larry Jones, Indianapolis, USA), and polyclonal rabbit anti-Cx43 [22].

2.6. Immunocytochemical procedures and microscopy

For immunocytochemical analysis, neonatal cardiac myocytes were plated onto collagen-coated glass coverslips and infected as described above. The immunocytochemical procedure was described previously [23]. Briefly, cells were immunoreacted overnight at 4 °C with rabbit primary antibodies recognizing CSQ, TRD or JCN and mouse primary antibodies recognizing SERCA (kindly provided by Larry Jones, Indianapolis, USA) or the Golgi apparatus (anti 58 kDa Golgi protein: Sigma-Aldrich, Munich, Germany). Bound primary antibodies were detected with Cy3-conjugated (Dianova, Hamburg, Germany) or Alexa Fluor 488-conjugated (Invitrogen, Darmstadt, Germany) anti-rabbit or anti mouse IgG. Finally, samples were counterstained with DAPI (4′6′-diamidine-2′-phenylindole dihydrochloride, 5 µg/ml PBS; Sigma-Aldrich, Munich, Germany). Separate images for DAPI staining (blue), Cy3-immunolabeling (red), Alexa 488-immunolabeling (green) and for GFP fluorescence (green) of the