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Biochemical and Biophysical Research Communications 300 (2003) 192–196
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Increased Ca^{2+} storage capacity in the sarcoplasmic reticulum by overexpression of HRC (histidine-rich Ca^{2+} binding protein)

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Received 18 November 2002

Abstract

The histidine-rich Ca^{2+} binding protein (HRC) is a high capacity Ca^{2+} binding protein in the sarcoplasmic reticulum (SR). Because HRC appears to interact directly with triadin, HRC may play a role in the regulation of Ca^{2+} release during excitation–contraction coupling. In this study, we examined the physiological effects of HRC overexpression in rat neonatal cardiomyocytes. Both caffeine-induced and depolarization-induced Ca^{2+} release from the SR were increased significantly in the HRC overexpressing cardiomyocytes. Consistently, the Ca^{2+} content, normally depleted from the SR in the presence of cyclopiazonic acid (CPA), remained elevated in these cells. In contrast, the density and the ryanodine-binding kinetics of the ryanodine receptor (RyR)/Ca^{2+} release channel were slightly reduced or not significantly altered in the HRC overexpressing cardiomyocytes. We suggest that HRC is involved in the regulation of releasable Ca^{2+} content into the SR.

Keywords: HRC; Ca^{2+} binding protein; Ryanodine receptor; Sarcoplasmic reticulum; Excitation–contraction coupling

The histidine-rich Ca^{2+} binding protein (HRC) is a high capacity, low affinity Ca^{2+} binding protein [1–3]. We previously showed that HRC is a multimeric complex residing in the lumen of the sarcoplasmic reticulum (SR) [4]. We also demonstrated that HRC binds to triadin and the binding occurs between the histidine-rich acidic repeat region of HRC and the KEKE motif in the lumenal region of triadin [5], a region also critical for binding to the well-characterized SR Ca^{2+} binding protein, calsequestrin [6–8]. We speculate that HRC, like calsequestrin, is involved in the regulation of Ca^{2+} release from the SR. However, the physiological role of HRC has not yet been studied extensively.

In this study, we utilized adenoviral-mediated gene transfer to induce HRC overexpression in rat neonatal cardiomyocytes and evaluated the physiological consequences. Our data show that HRC overexpression significantly enhances the SR Ca^{2+} storage capacity. This study provides the first evidence suggesting that HRC is indeed involved in Ca^{2+} homeostasis in the SR.

Materials and methods

Cardiomyocyte culture and transfection with recombinant adenoviruses. Isolation of cardiomyocytes from two- to three-day-old neonatal rats was performed as described earlier [9]. The isolated cells were resuspended in DMEM (Gibco-BRL) supplemented with 10% FBS (Gibco-BRL) and then plated on collagen-coated dishes (50 μg/ml) at a density of 200 cells/mm^2. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

The Adeno-X expression system kit (Clontech) was used to generate recombinant adenoviruses. A DNA fragment containing the full-length mouse HRC coding sequence (a gift from Dr. Ilona S. Skerjanc; University of Western Ontario, Canada) was subcloned into the pShuttle vector. The entire expression cassette from the resulting vector was then excised and inserted into the subcloning site of the Adeno-X viral DNA. This recombinant adenoviral plasmid was then transfected into HEK 293 cells to generate infectious viral particles. Cardiomyocytes were infected with recombinant adenoviruses for 2 h

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PII: S0006-291X(02)02829-2

at a multiplicity of infection (M.O.I) of 50 particles/cell and incubated for an additional 24–48 h to ensure transgene expression.

Immunoblotting. Cardiomyocytes were extracted with a lysis buffer (1% SDS, 10 mM Tris–Cl (pH 7.4)). About 20 μg of the extracted protein was separated by 8% SDS–PAGE and transferred to a nitrocellulose filter paper (Schleicher and Schuell). The paper was blocked in 5% nonfat milk for 1 h at room temperature and incubated with a polyclonal rabbit anti-mHRC antibody (1:10,000 dilution) overnight at 4 °C. Horseradish peroxidase-conjugated anti-rabbit IgG antibody was treated for 1 h at room temperature and the filter was developed using the ECL system (Introgene).

Immunostaining. Cardiomyocytes grown on collagen-coated cover slips were infected with the adenovirus for 24–36 h. The cells were fixed with 3.5% paraformaldehyde for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and then incubated in 5% BSA solution for 1 h at room temperature. The cells were sequentially incubated with polyclonal rabbit anti-mHRC antibody (1:500) and TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) (1:100) and then mounted with a Vectashield mounting medium (VECTA). Immunofluorescence was analyzed under a microscope equipped with a 100× objective lens and filters for epifluorescence (Olympus).

Intracellular Ca^{2+} measurement in a single cell. Cardiomyocytes were grown on coverslips, incubated for 45 min at 37 °C in a balanced salt solution (BSS) (140 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 10 mM Hepes, pH 7.2) containing 5 μM Fura-2 acetoxymethyl (AM) ester (Molecular Probes), and then washed with BSS solution to allow de-esterification of Fura-2-AM. We used a dual-wavelength spectrofluorometer with the excitation wavelengths at 340 and 380 nm and the emission at 510 nm, and measured fluorescence on an Olympus IX-70 microscope. Single-cell fluorescence spectra were continuously monitored at a sampling frequency of 50 Hz using a PTI spectrofluorometer (Photon Technology International) [10]. The change in intracellular Ca^{2+} in an individual cell was measured following exposure to 5 mM caffeine, 40 mM KCl, or 10 μM CPA.

Ryanodine-binding assay. Equilibrium ryanodine binding to whole homogenates was measured by incubating 200 μg of whole homogenate in 250 μl reaction mixture containing 20 mM MOPS (pH 7.4), 1 M KCl, various concentrations of [^{3}H]ryanodine (54.7 Ci/mmol), 1 mM EGTA, and 0.98 mM $CaCl_2$ for 2 h at 37 °C [11]. To measure nonspecific binding, we included a 100-fold excess of unlabeled ryanodine(Calbiochem). After incubation, 100 μl polyethylene glycol (PEG) solution (50 mM Tris–HCl, pH 7.4, 30% polyethylene glycol, and 1 mM EDTA) was added to each vial and incubation was continued for 5 min at room temperature. Proteins were precipitated for 5 min at 14,000 rpm in a microcentrifuge and the pellets were rinsed twice with 0.4 ml of the relevant ryanodine-binding buffer without radioactive ryanodine. The pellets were then solubilized in 100 μl Solune 350 (Packard) at 70 °C for 30 min and the solution was counted in 4 ml Picofluor (Packard) by liquid scintillation (Beckman Instruments).

Statistical analysis. Values are means ± SE. Significance was determined by Student's t test and p values of < 0.05 were considered statistically significant.

Results

Overexpression of mHRC in cardiomyocytes

Recombinant adenoviruses encoding β-galactosidase (AdLacZ) or mouse (m) HRC (AdHRC) were generated as described in Materials and methods and transduced into rat neonatal cardiomyocytes. Immunoblotting with a polyclonal antibody to mHRC revealed a single 120–130 kDa protein in cardiomyocytes transfected with AdHRC (Fig. 1A). Since the protein was absent in non-transfected cells or cells transfected with AdLacZ, the 120–130 kDa protein appeared to be mHRC (Fig. 1A). The apparent molecular weight of this protein is much greater than the expected molecular weight (79,860 Da) calculated from the mHRC amino acid sequence [12]. This phenomenon also occurred with rabbit HRC but has not yet been explained properly [1]. Immunostaining with the anti-mHRC antibody revealed a perinuclear expression pattern (Fig. 1B) that is typical for endoplasmic reticulum proteins. Thus, the majority of the adenoviral-encoded mHRC appears to be expressed in the physiologically relevant site.

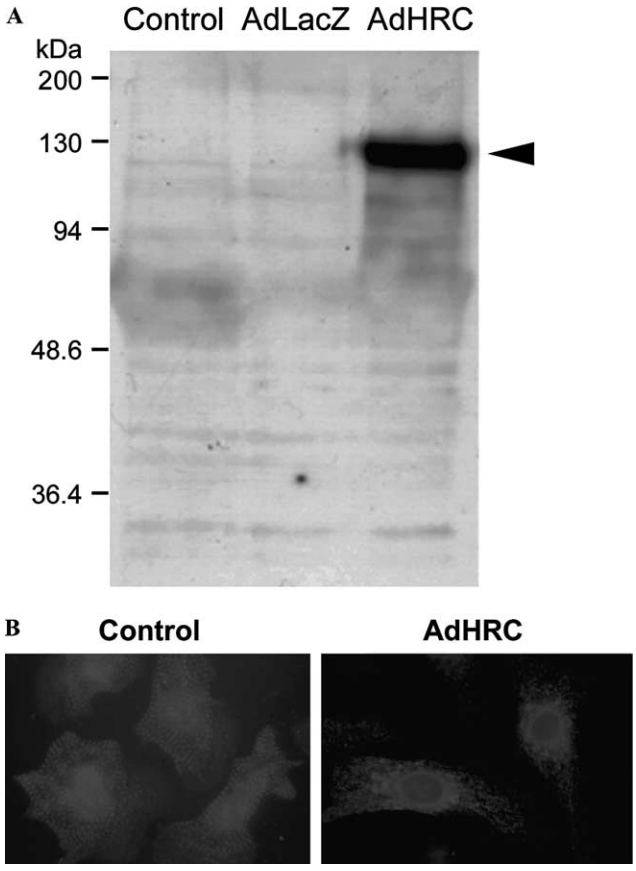

Fig. 1. Overexpression of mHRC in rat neonatal cardiomyocytes. (A) Cell lysates (20 μg) of non-transfected cardiomyocytes (control) and cardiomyocytes transfected with AdLacZ or AdHRC were separated on 8% SDS–PAGE, blotted, and probed with anti-mHRC antibody. The 120–130 kDa mHRC protein is indicated by an arrow. (B) The mHRC was visualized by immunofluorescence in non-transfected cardiomyocytes (control) and cardiomyocytes transfected with AdHRC. Note the perinuclear expression pattern of mHRC.

Caffeine-induced Ca^{2+} release is enhanced by HRC overexpression

We first measured caffeine-induced Ca^{2+} release from the SR in individual cardiomyocytes to determine if HRC overexpression alters the SR Ca^{2+} storage capacity.

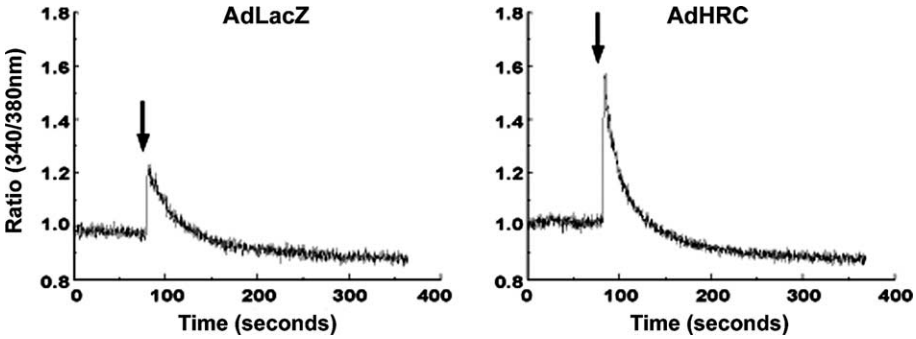

Fig. 2. Caffeine-induced Ca^{2+} release in cardiomyocytes. Cardiomyocytes were transfected with either AdLacZ or AdHRC and cultured for two more days. Culture conditions were identical for Fig. 2–5. Caffeine (5 mM) was added to the media in the absence of extracellular Ca^{2+} to trigger Ca^{2+} release through the RyR/Ca^{2+} release channel. Each trace is representative of 10–11 independent experiments. Arrows indicate the timing of the addition of caffeine.

Caffeine (5 mM) was added to the culture media in the absence of extracellular Ca^{2+} to open the RyR/Ca^{2+} release channel and to allow the release of Ca^{2+} from the SR into the cytoplasm (Fig. 2). The addition of caffeine caused a transient increase in Ca^{2+} release from the SR. However, the peak amplitude of the caffeine-induced Ca^{2+} release in cardiomyocytes transfected with AdHRC was significantly higher than the peak in control cardiomyocytes (340/380 nm signal: 0.60 ± 0.03 in HRC overexpressing cells ($n = 9$) and 0.25 ± 0.03 in control cells ($n = 10$)). Therefore, the SR Ca^{2+} storage capacity appears to have been increased significantly in the HRC overexpressing cardiomyocytes.

Depolarization-induced Ca^{2+} release is enhanced by HRC overexpression

The depolarization of cardiomyocyte plasma membranes causes an extracellular Ca^{2+} influx through the L-type Ca^{2+} channels (DHPRs), resulting in increased cytosolic Ca^{2+} content, which in turn triggers the release of intracellular Ca^{2+} from the SR through the RyR/Ca^{2+} release channel [13–16]. To determine if HRC overexpression alters this depolarization-induced Ca^{2+} release from the SR, we induced the depolarization of the plasma membrane by adding 40 mM KCl to the media in the presence of 0.5 mM extracellular Ca^{2+}, and measured the transient Ca^{2+} current (Fig. 3). As in the caffeine-induced Ca^{2+} release, the peak amplitude of the depolarization-induced Ca^{2+} release in the HRC overexpressing cardiomyocytes was significantly higher than the amplitude in control cardiomyocytes (340/380 nm signal: 0.60 ± 0.02 in HRC overexpressing cells ($n = 10$) and 0.37 ± 0.01 in control cells ($n = 11$)). Thus, the SR Ca^{2+} storage capacity appears to have been augmented in the HRC overexpressing cardiomyocytes.

Extent of SR Ca^{2+} depletion is increased by HRC overexpression

Next, we determined if the extent of SR Ca^{2+} depletion was affected by HRC overexpression. The pretreatment of cardiomyocytes with the SERCA inhibitor, cyclopiazonic acid (CPA), in the absence of extracellular Ca^{2+}, depleted almost entirely the SR Ca^{2+} content (Fig. 4). However, the peak amplitude in HRC overexpressing cardiomyocytes was significantly higher than the amplitude in control cardiomyocytes (340/380 nm signal: 0.20 ± 0.01 in HRC overexpressing cells ($n = 6$) and 0.09 ± 0.01 in control cells ($n = 5$)). These results are

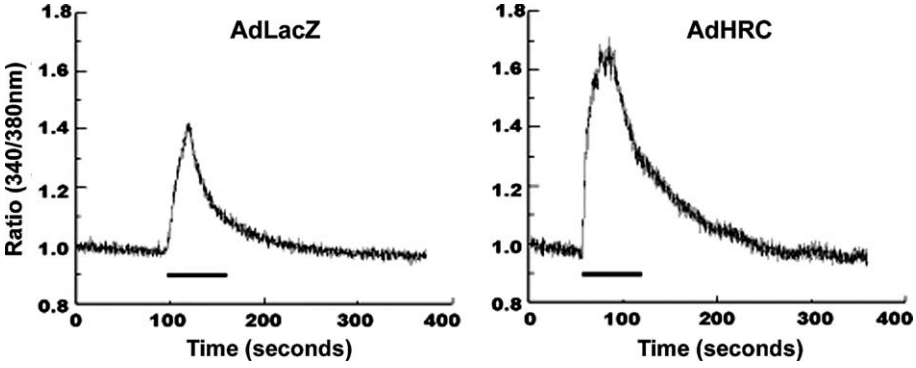

Fig. 3. Depolarization-induced Ca^{2+} release in cardiomyocytes. KCl (40 mM) was added to the media to induce the plasma membrane depolarization in the presence of 0.5 mM Ca^{2+}. Each trace is representative of 9–10 independent experiments. Bars indicate the timing of the addition of KCl.

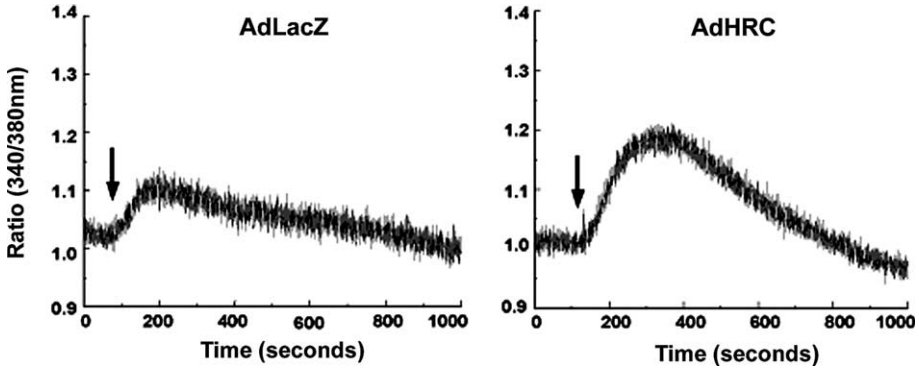

Fig. 4. Depletion of SR Ca^{2+} in cardiomyocytes. Depletion of Ca^{2+} across the SR membrane was measured using 10 μM cyclopiazonic acid (CPA), a SERCA inhibitor. Each trace is representative of 5–7 independent experiments. Arrows indicate the timing of the addition of CPA.

consistent with the caffeine- and depolarization-induced increases in SR Ca^{2+} release.

Density of RyR/Ca^{2+} release channel and its open state are not significantly altered by HRC overexpression

The increase in the SR Ca^{2+} release may be due to an increase in the density and/or the open state of RyR/Ca^{2+} release channel. We measured ryanodine binding to whole cell lysates at various [^{3}H]ryanodine concentrations. As the [^{3}H]ryanodine concentration increased from 0 to 16 nM, ryanodine binding to cell lysates was rapidly saturated (Fig. 5). The maximal binding (B_{max}) and dissociation constant (K_d) of ryanodine binding were calculated by iterative computer fitting using the equation $Y = B_{max}X[X/(K_d + X)]$ [17]. The RyR density, as determined by B_{max}, was slightly lower in HRC overexpressing cardiomyocytes than in control cardiomyocytes (0.1742 ± 0.0011 in HRC overexpressing cells ($n = 4$) and 0.2072 ± 0.0052 in control cells ($n = 4$)). The K_d of ryanodine binding was not significantly altered (0.6992 ± 0.0185 in HRC overexpressing cells ($n = 4$) and 0.7062 ± 0.0542 in control cells ($n = 4$)). We conclude that HRC overexpression does not critically alter the density and/or ryanodine-binding kinetics of the RyR/Ca^{2+} release channel.

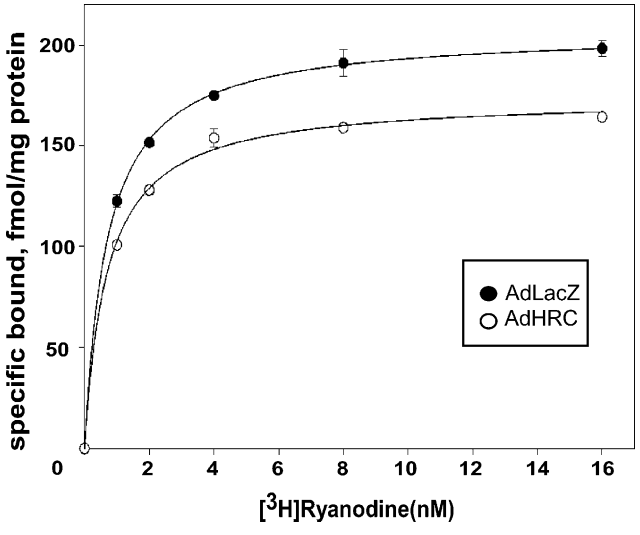

Fig. 5. Equilibrium ryanodine binding of whole lysates of cardiomyocytes. Equilibrium ryanodine-binding assays were performed with 200 μg of whole lysates of myocytes transfected with AdLacZ (●) or AdHRC (○). The curves are fitted with the results of four independent experiments.

Discussion

We have presented evidence suggesting that HRC is a SR lumenal protein. We initially demonstrated that HRC is protected from tryptic digestion and biotinylation in intact SR but not in solubilized SR [4]. We then showed that HRC interacts with triadin, a component of the excitation–contraction coupling machinery and that binding occurs between the histidine-rich acidic repeat region of HRC and the lumenal region of triadin [5]. Here, we present data showing that HRC overexpression significantly enhances the Ca^{2+} storage capacity in the SR. Since HRC is a high capacity, low affinity Ca^{2+} binding protein like calsequestrin, HRC may serve as a Ca^{2+} store in overexpressed cardiomyocytes. These findings suggest that HRC is indeed a SR lumenal protein.

Transgenic mice overexpressing calsequestrin in the heart appeared to have an increased SR Ca^{2+} storage capacity [18,19]. This finding is consistent with our data demonstrating that HRC overexpression enhances the SR Ca^{2+} storage capacity. However, in sharp contrast, the Ca^{2+}-induced Ca^{2+} release was impaired in the cardiomyocytes of the calsequestrin overexpressing transgenic mice. The discrepancy may be due to the different experimental approaches; we conducted a rather short-term experiment while gross changes in gene expression may have modified the excitation–contraction coupling machinery in the transgenic mice.

The patho-physiological function of HRC as well as calsequestrin is largely unknown except that the human HRC gene was localized to a region corresponding to 19q13.3. Since the locus for myotonic muscular dystrophy resides in the region 19q13.2–13.3, HRC has been considered as a candidate gene for the disease [20]. Overexpression of calsequestrin in the heart resulted in mild to severe cardiac hypertrophy which might be caused by enhanced Ca^{2+} handling [18,19]. Similarly, we observed a mild cardiac hypertrophy in the transgenic mice overexpressing HRC in the heart (W.J. Park et al., unpublished data), suggesting that HRC might play a similar role as calsequestrin during excitation–contraction coupling. Since HRC is much less abundant than calsequestrin in the SR, it may act as a backup Ca^{2+} store. However, we cannot rule out the possibility that HRC plays an other unknown role during excitation–contraction coupling. Further, other experimental approaches employing knock-out mice or RNAi could potentially elucidate these physiological functions of HRC.

Acknowledgments

This work was supported by the BK21 program, grants from the Korean Ministry of Science and Technology (Critical Technology 21 on "Life Phenomena and Function Research", 00-J-LF-01B-54), and the Korea Science Foundation (1999-1-20700-001-5).

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