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The small EF-hand Ca^{2+} binding protein S100A1 increases contractility and Ca^{2+} cycling in rat cardiac myocytes

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Abstract S100A1 is an interesting Ca^{2+} binding protein with respect to muscle physiology as it is preferentially expressed in cardiac muscle and colocalizes with the sarcolemmal and the sarcoplasmic reticulum membranes as well as with the sarcomere. It is therefore conceivable that S100A1 may play a specific role in the regulation of cardiac Ca^{2+} homeostasis and contractility. We therefore investigated the impact of adenoviral S100A1 overexpression on fractional shortening (FS%) and systolic Ca^{2+} transients in adult rat cardiomyocytes as well as of S100A1 protein on SERCA activity in skinned cell preparation. In our setting S100A1 gene transfer increased FS% by 55%, systolic Ca^{2+} amplitudes by 62%, while S100A1 protein increased SERCA activity by 28%. Importantly, the gain in systolic Ca^{2+} supply was not only seen on basal conditions but also with isoproterenol-stimulated Ca^{2+} cycling. Thus, S100A1 enhances cardiac contractility by increasing intracellular Ca^{2+} fluxes at least in part due to a modulation of SERCA. Since earlier observations demonstrated S100A1 protein levels to be increased in compensatory hypertrophy and significantly downregulated in end stage heart failure, these functional data suggest that S100A1 is a novel determinant of cardiac function whose expression levels are causally related to the prevailing contractile state of the heart.

Key words S100A1 – Ca^{2+} binding protein – Ca^{2+} homeostasis – contractility – cardiac

Introduction

A multitude of Ca^{2+} binding proteins are known to be involved in the regulation of cardiac Ca^{2+} homeostasis and contractile performance. While structure–function relationships of EF-hand Ca^{2+} binding proteins like troponin C, myosin light chains, and calmodulin have already been intensively studied (1), functional data are sparse as to the novel family of S100 proteins today representing the largest subgroup of EF-hand Ca^{2+} binding proteins. These proteins display the unusual property of solubility in 100% ammonium sulfate and were thus named S100 proteins (18). To date, 19 different S100 proteins constitute a multigenic protein family whose members are known to be expressed in a cell- and tissue-specific way and serve a wide variety of Ca^{2+}-dependent intracellular and extracellular functions (23). Due to

their unique biophysical characteristics S100 proteins are considered as Ca^{2+}-dependent regulators that according to their cell specific expression transduce the Ca^{2+} signal into cell specific actions (3).

S100A1 is a muscle specific member of the S100 protein family that shows the highest expression levels in cardiac tissue (15). Intriguingly, it colocalizes with the two principal membrane systems that govern Ca^{2+} homeostasis in the cardiac myocyte – the sarcolemmal and sarcoplasmic reticulum membranes – while immunohistochemical studies revealed that S100A1 decorates the contractile apparatus (2, 8, 15). These data imply that the highly conserved Ca^{2+} binding protein S100A1 may serve a fundamental role in the regulation of Ca^{2+} homeostasis and contractility. Kiewitz et al. (17) recently published that S100A1 is expressed as early as on day 8 during mouse embryogenesis with significant regional differences concerning its expression levels being higher in ventricular compared to atrial myocytes. Interestingly, this regionally differential expression of S100A1 is conserved in the adult pig heart, where S100A1 protein levels are also significantly higher in left ventricles as compared to right ventricular and atrial tissue (22). Furthermore, S100A1 appears to be differentially expressed in the course of cardiac failure with elevated protein levels in early compensated hypertrophy while S100A1 is significantly downregulated in the failing heart (5, 22). In order to elucidate intracellular functions of S100A1 and to study the physiological relevance of a differential expression of this Ca^{2+} binding protein we investigated the impact of S100A1 overexpression on contractile behavior and Ca^{2+} homeostasis in adult rat cardiomyocytes.

Methods

S100A1 adenovirus

Human S100A1 cDNA was amplified from a human heart cDNA library (Clontech HL 5506u), compared to human cDNA sequence (Gen Bank AC x58079) and introduced into a recombinant adenovirus using the pAdTrack-CMV/pAdEasy 1 system (11). Both the cDNA of S100A1 and of green fluorescent protein (GFP) – which served as reporter gene – were each under the control of a CMV promotor (S100A1). Adenovirus with GFP cDNA devoid of S100A1 served as GFP only virus (control).

Preparation and transfection of adult ventricular cardiac myocytes

Ventricular myocytes from adult rat hearts were isolated using a collagenase method as previously published (21). In brief, animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and the aorta was rapidly cannulated after the hearts were excised and perfused with a rate of 8 ml/min in a Langendorff apparatus. Hearts were initially perfused with calcium-free AC medium (ACM) (pH 7.2) consisting of (in mM) 5.4 KCl, 3.5 $MgSO_4$, 0.05 pyruvate, 20 $NaHCO_3$, 11 glucose, 20 HEPES, 23.5 glutamate, 4.87 acetate, 10 EDTA, 0.5 phenol red, 15 butanedionemonoxime (BDM), 20 creatinine, 15 creatine phosphate (CrP), 15 taurine and 27 units/ml insulin under continuous equilibrium with 95% O_2/5% CO_2. After 5 min the perfusion was switched to ACM plus collagenase (0.5 U/ml, type A; Roche diagnostics GmbH, Germany) for 20 – 30 min. Finally, perfusion was changed to low Na^+, high sucrose Tyrode solution containing (in mM) 52.5 NaCl, 4.8 KCl, 1.19 KH_2PO_4, 1.2 $MgSO_4$, 11.1 glucose, 145 sucrose, 10 taurine, 10 HEPES, 0.2 $CaCl_2$ for 15 min. Thereafter left ventricles of digested hearts were cut into small pieces and subjected to gentle agitation to allow for dissociation of cells. Consequently, cells were resuspended in ACM without BDM in which 2 mM extracellular calcium ($[Ca^{2+}]_e$) was gradually reintroduced at 25 °C. Cardiac myocytes used for contractility and Ca^{2+} measurements were plated with a density of 30,000 cells/cm^2 on laminin-coated dishes followed by adenoviral transfection with either control (GFP only virus, multiplicity of infection (MOI) 20, 100, 200 plaque forming units (PFU)) or S100A1 virus (S100A1, MOI 20 PFU) and cultivated in HEPES-modified medium 199 (M199) (Sigma-Aldrich Corp., St.Louis, MO) for 24 hours.

Western blotting

After S100A1 transfection of rat cardiac myocytes the expression of human S100A1 was determined by Western blotting. Pooled fractions of both S100A1 and GFP transfected rat cardiac myocytes (3×10^5 cells) were homogenized at 4 °C in 3 w/v PBS with 5 mM EGTA and protease inhibitor mixture (1836170, complete Mini EDTA free, Roche Diagnostics GmbH, Germany) and centrifuged at 10,000 g for 15 min. Supernatant protein content was quantified with the BioRad DC Protein Assay (BioRad Laboratories, Richmond, CA). After fractionation of supernatant proteins (50 µg) by 12% SDS-PAGE (Invitrogen Corporation, Carlsbad, CA) as described by Ehlermann et al. (6) proteins were transferred to the PVDF membrane and probed either with a custom-made polyclonal anti-human-S100A1 antibody (SA 5632) or anti-GFP antibody (clontech, Heidelberg, Germany). Blots were developed with the Avidix™ chemoluminescence-detection system (Tropix, Applied Biosystems, Foster City, CA) and quantified by densitometry (ONE-D scan, 1.0 Scanalytics, CSP Inc.).

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Myocyte contractile parameters

Unloaded shortening of cardiomyocytes from four different preparations was determined under electrical field stimulation (1 Hz) at 37 °C with a video-edge detection system (Crescent Electronics, Sandy, Utah) 24 hours after transfection in M199 at room temperature. Data from five consecutive steady-state contractions were averaged for analysis of fractional shortening (FS%) in S100A1 transfected (S100A1) and GFP only virus transfected (control) cells. Measurements were only performed in cardiomyocytes with a parallel orientation to the electric field lines (12).

Calibration and measurement of Ca^{2+} transients

Intracellular Ca^{2+} transients of rat cardiac myocytes were calibrated in situ and measured as previously described (10). Briefly, isolated cardiomyocytes were washed in M199 (Sigma-Aldrich Corp., St. Louis, MO) and incubated with 2 μM Fura2-AM (Sigma-Aldrich Corp., St. Louis, MO) for 20 min at room temperature. Cells were washed two times in M199 and calibration and fluorescence measurements were carried out at room temperature using an inverse Olympus microscope (IX70) with a UV filter connected to a monochromator (Polychrome II, T.I.L.L. Photonics GmbH, Germany). Cells were electrically stimulated with 1 Hz, excited at 340/380 nm and fluorescence emission was detected at 510 nm with 18-ms intervals. The signal was processed with T.I.L.L.VISION software (v. 3.3) and analyzed with LabView (National Instruments, Munich, Germany). Data from five consecutive steady-state transients were averaged for analysis of Ca^{2+} transient amplitude ([nM]). Calibration was performed in 50 Fura2-AM loaded rat ventricular myocytes that yielded a minimal ratio (R_{min}) of 0.20 ± 0.07 and a maximal ratio (R_{max}) of 2.39 ± 0.35, while β and K_d were estimated to be 4.97 ± 0.41 and 224 ± 18 nM, respectively. Free intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ was calculated by the equation of Grynkiewicz et al. (7): $[Ca^{2+}]_i = K_d \times \beta\ (R - R_{min})/(R_{max} - R)$. Ca^{2+} transients were investigated on basal conditions and throughout a stepwise increase of isoproterenol concentrations (10^{-9} – 10^{-6} M). Measurements were restricted to cardiomyocytes with a parallel orientation to the electric field lines (12).

S100A1 protein

Native S100A1 protein was purified from porcine left ventricular myocardium as previously described (4) with a yield of 2 mg S100A1/100 g wet weight (> 95% purity). S100A1 protein was tetramethyl-rhodamine (TAMRA) labelled by Eurogentec (Belgium). S100A1 protein was dialyzed against 10 mM HEPES, pH 7.4 and stored in aliquots at –80 °C.

Sarcoplasmic Ca^{2+} uptake studies

Sarcoplasmic Ca^{2+} uptake was performed as described elsewhere (24) with some modifications. Briefly, isolated adult rat ventricular cardiomyocytes (1.5×10^6) were exposed to 0.1 mg/ml β-escin at room temperature for 1 min and resuspended in a cytosolic mock solution (in mM: 120 KCl, 5 MgATP, 15 CrP, 1 $MgCl_2$, 25 HEPES, 20 K_2Oxalate, 0.05 K_2EGTA, pH 7.0). Cells were placed in a cuvette (1.5 ml) and equilibrated with 0.01 mM Fura2 (Sigma-Aldrich Corp., St. Louis, MO), 20 μM oligomycin and 5 μM ruthenium red in mock solution by stirring. Ca^{2+} uptake measurements were started after the addition of 10 μl of 10 mM $CaCl_2$ which resulted in ~0.8 to 1.5 μM free $[Ca^{2+}]_i$ within the cuvette. The decay of the Fura2 fluorescence within the cuvette was recorded at 30 Hz with a spinning wheel spectrophotometer (Cairn Research) at room temperature. The signal was low-pass filtered (–3 dB at 30 Hz) and digitized for later analysis. Ca^{2+} uptake rate (dCa^{2+}/dt; $pmolCa^{2+}/s$) for 1.5×10^6 cells was calculated from the time constant tau (τ) of extrasarcoplasmic $[Ca^{2+}]$ decline. τ was obtained from best-fit one-exponential decay from experiments where free $[Ca^{2+}]_i$ in the cuvette exceeded 1 μM. This was the case in 8 out of 14 experiments. The relationship between given calcium concentrations and the resulting fluorescence ratios was established with a series of calibration experiments and analyzed according to Grynkiewicz et al. (7). For S100A1 interventions permeabilized cells were preincubated with 1 μM S100A1 for 30 min while S100A1 storage buffer served as control. Free access of S100A1 protein to intracellular space was confirmed by the application of 1 μM rhodamine-labelled S100A1 in cytoplasmic mock solution and 2 μM carbonyl-cyanide p-trifluoromethoxyphenylhydrazone (FCCP) to block nonspecific staining of mitochondria by rhodamine. Controls were carried out with rhodamine dye only. After three subsequent washing steps with mock solution fluorescence was excited at 542 nm and detected at 568 nm with a confocal microscope (FV500, Olympus; 40X).

Statistical analyses

Data are presented as mean ± SEM. Unpaired student's *t*-test and a two way repeated ANOVA analysis were performed to test for differences between groups. A value of $P < 0.05$ was accepted as statistically significant.

Results

Adenoviral transfection of rat cardiac myocytes

To test the impact of either adenoviral burden or GFP itself on Ca^{2+} transients we transfected cardiac myocytes with a MOI of 20, 100, and 200 PFU. Figure 1A shows that with a MOI of 20 PFU Ca^{2+} amplitudes were indistinguishable from wild-type cells, while a further increase in virus load was accompanied by a decrease in Ca^{2+} cycling in GFP transfected cardiac myocytes. Therefore a MOI of 20 was used for further investigations which lead to a nearly 100% transfection rate in both the S100A1 (Fig. 1B) and GFP control (Fig. 1D) transfected cardiac myocytes as assessed by GFP fluorescence.

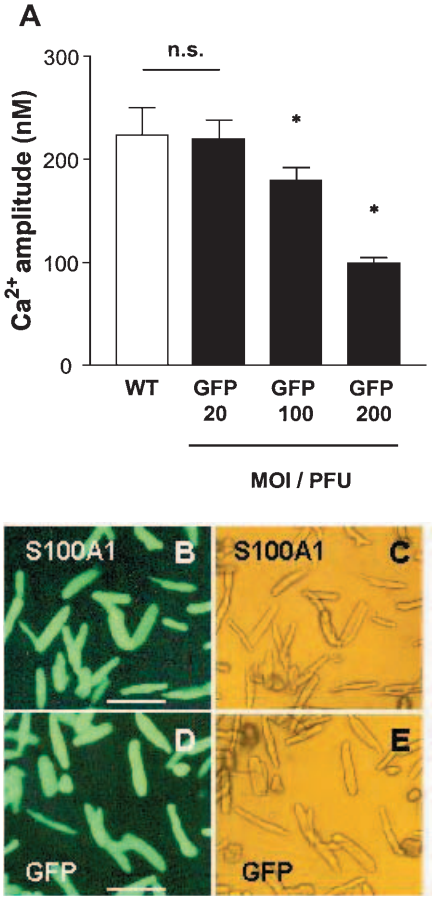

Fig. 1 Effect of increasing dosage of adenovirus on intracellular Ca^{2+} cycling and efficiency of adenoviral-mediated gene transfer to primary cardiomyocytes. **A** Impairment of intracellular Ca^{2+} handling in ventricular cardiomyocytes due to progressive doses of GFP only virus (GFP) > 20 MOI/PFU compared to non-transfected wild-type cardiomyoctyes (WT). Calibrated Ca^{2+} transient amplitudes were assessed 24 hours following administration of adenovirus. **B–E** Representative transmission and GFP emission images taken from adult rat ventricular cardiomyocytes 24 h following adenoviral transfection with a MOI of 20 PFU, which was the lowest titer required for 100% infection rate. **B** 510-nm emission and **C** transmission in S100A1-transfected cardiomyocytes (S100A1), **D** 510-nm emission and **E** transmission in GFP only virus-transfected cardiomyocytes (GFP) (Bar = 100 μm). Data are mean ± SEM. *, $P < 0.05$ vs. WT (n = 80; 20 cardiomyocytes each from four different hearts were studied).

S100A1 increases unloaded shortening of rat cardiac myocytes

Twentyfour hours after adenoviral S100A1 gene-transfer, a robust expression of human S100A1 was documented

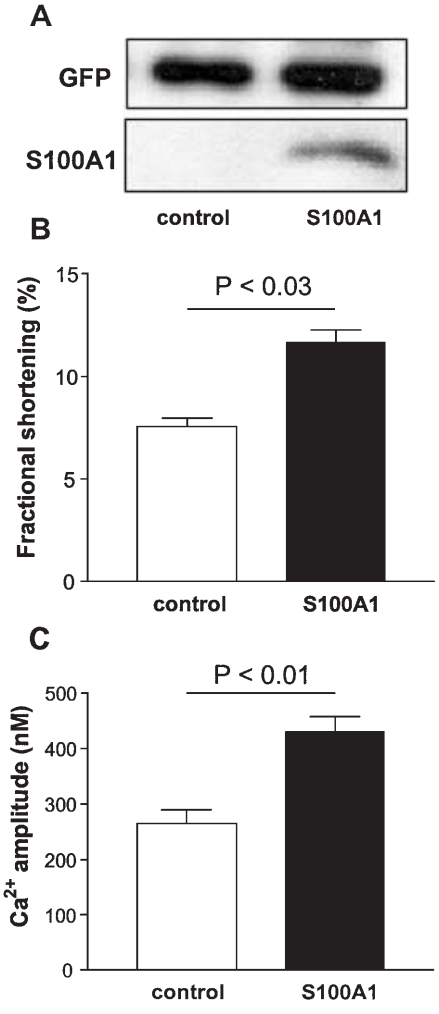

Fig. 2 A–C Effect of S100A1 adenoviral-mediated gene transfer on GFP and S100A1 protein expression, cellular contractility, and intracellular Ca^{2+} cycling in GFP only virus (control) and S100A1 transfected (S100A1) rat cardiomyocytes. **A** Representative SDS/PAGE analysis of homogenates from control and S100A1-transfected cardiomyocytes. Application of 20 PFU of S100A1 adenovirus per cell leads to a robust overexpression of human S100A1 protein compared to control. Equal transscriptional activity of both GFP (control, MOI 20/PFU) and S100A1 (S100A1, MOI 20/PFU) adenovirus was controlled by GFP protein expression. **B** Significant increase of fractional shortening and **C** intracellular Ca^{2+} transient amplitude following S100A1 overexpression in rat cardiomyocytes. Data are mean ± SEM (n = 80; 20 cardiomyocytes each from four different hearts were studied).

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(Fig. 2A). Similar GFP expression both in S100A1 transfected and control cells confirmed equal transcriptional activity of both applied adenoviruses. Due to the low abundance of S100A1 in rat heart (16) and the restricted number of cardiac myocytes that were investigated we failed to detect intrinsic rat S100A1 protein (Fig. 2A) but found a robust expression of S100A1 protein in S100A1 transfected cells. Contractile parameters were evaluated in field stimulated cardiac myocytes (1 Hz) using a video-edge detection system. Fractional shortening (FS%) increased by roughly 55% from 7.65 ± 0.41% in controls to 11.69 ± 0.62% in S100A1 overexpressing cells (S100A1) ($P < 0.03$) (Fig. 2B).

S100A1 improves Ca^{2+} cycling in rat cardiac myocytes

Since the contractile state of the cardiac myocyte critically depends on intracellular Ca^{2+} homeostasis, we next sought to understand whether S100A1 exerts its effects by modulating intracellular Ca^{2+} transients in cardiac myocytes. For this purpose Ca^{2+} transients were measured in Fura2AM-loaded rat cardiac myocytes after S100A1 gene delivery (S100A1) and control transfection with GFP only virus (control). S100A1 overexpression clearly upregulated systolic Ca^{2+} amplitude by 62% resulting in a systolic Ca^{2+} peak of 430 ± 29 nM compared to 265 ± 25 nM in controls ($P < 0.01$) (Fig. 2C). Figure 3 demonstrates that both control and S100A1 overexpressing cells showed a regular increase of systolic Ca^{2+} amplitudes in response to isoproterenol stimulation. Interestingly the S100A1-associated gain in systolic Ca^{2+} supply at basal conditions was maintained throughout the series of isoproterenol stimulation indicating that S100A1 augments β-adrenergic response.

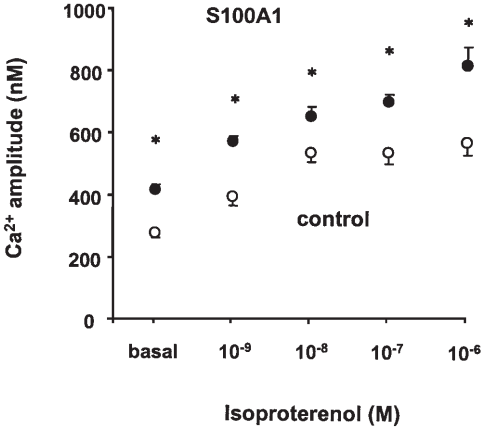

Fig. 3 Influence of S100A1 overexpression on isoproterenol-stimulated intracellular Ca^{2+} fluxes. S100A1 overexpression (S100A1) significantly augmented both basal and β-adrenergic-stimulated Ca^{2+} transient amplitudes in rat ventricular cardiomyocytes. Data are mean ± SEM. *, $P < 0.05$ vs. control (GFP only virus) (n = 80; 20 cardiomyocytes each from four different hearts were studied).

S100A1 protein increases SR Ca^{2+} uptake in skinned rat cardiac myocytes

Having shown that S100A1 improves intracellular Ca^{2+} handling it was intriguing to investigate whether S100A1 protein would improve SERCA activity, since sarcoplas-

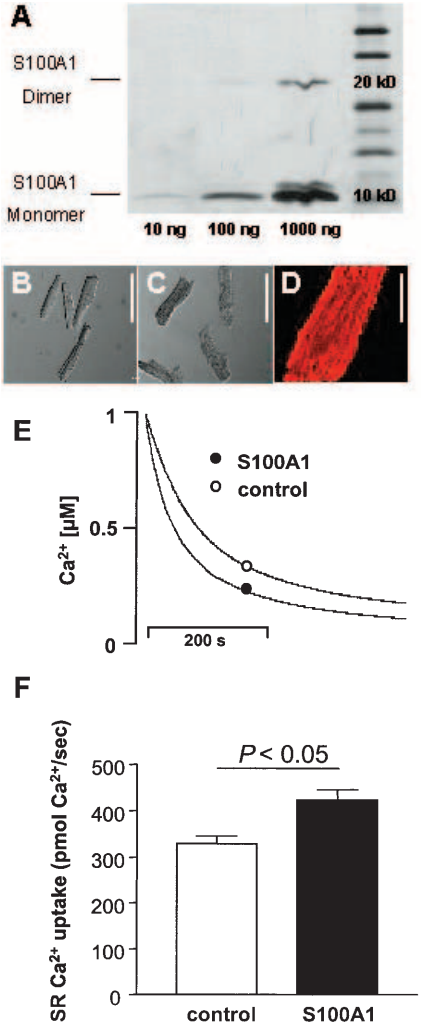

Fig. 4 Purification of native S100A1 protein and effect of S100A1 protein on sarcoplasmic reticulum (SR) Ca^{2+} uptake in oxalate-equilibrated permeabilized cardiomyocytes. **A** Representative silver staining of purified porcine S100A1 protein (10, 100 and 1000 ng) on SDS/PAGE. **B** and **C** Representative morphology of adult rat ventricular cardiomyocytes after isolation (**B**) and after chemical skinning with β-escin (**C**) (Bar = 100 µm). **D** Application of rhodamine-labeled S100A1 protein (1 µM) to skinned ventricular cardiomyocytes confirmed the free access of S100A1 protein to intracellular structures while control experiments with rhodamine dye revealed no staining (data not shown). Picture was taken after 5 min of incubation with dye-labeled S100A1 protein thus showing the rapid diffusion of the protein across the permeabilized sarcolemmal membrane (Bar = 25 µm). **E** Representative superimposed original tracings of $[Ca^{2+}]_i$ decline taken from cuvettes containing 1.5 × 10^6 permeabilized cardiac myocytes in a total volume of 1.5 ml after preincubation with 1 µM S100A1 protein (•) compared to control buffer (○). **F** Calculated SR Ca^{2+} uptake from the time constant (τ) of $[Ca^{2+}]_i$ decline for 1 µM S100A1 protein and control buffer at 1 µM free [Ca2+]i. Data are mean ± SEM (n = 8).

mic reticulum Ca^{2+} uptake represents a rate limiting step in cardiac contraction-relaxation cycle. For this purpose native S100A1 protein was purified (Fig. 4A) and we took advantage of the oxalate-equilibrated permeabilized cardiomyocyte model which allows one to specifically investigate SR function (24). In this model β-escin skinned wild-type cardiomyocytes are suspended in a cytosolic mock solution supplemented with Fura2. While SR oxalate loading facilitates SR Ca^{2+} uptake, ryanodine receptors and mitochondria are blocked by ruthenium red and oligomycin, respectively. SERCA activity is then measured indirectly by the decay of Fura2 signal ratio over time after starting the Ca^{2+} induced Ca^{2+} uptake by adding Ca^{2+} to the solution. As permeabilization did not afflict rod-shaped morphology of cardiac myocytes (Fig. 4C) as compared to freshly isolated cells (Fig. 4B), it resulted in a rapid intracellular accumulation of dye labelled S100A1 (1 μM) only after 5 min (Fig. 4D). After a period of 30 min preincubation with 1 μM S100A1 protein, we measured an accelerated decay of Fura2 signal ratio (Fig. 4E) reflecting an increased SR Ca^{2+} uptake rate by S100A1 (Fig. 4F). Calculated Ca^{2+} uptake rate at 1 μM free $[Ca^{2+}]_i$ was significantly increased by 28% from 329 ± 17 pmol/s in control to 423 ± 23 pmol/s in S100A1-treated cells ($P < 0.05$) (Fig. 4F).

Discussion

It is widely accepted that the failing heart displays characteristic changes as to Ca^{2+} homeostasis with a decreased systolic Ca^{2+} supply and a diastolic Ca^{2+} overload that finally results in a compromised contractile performance of the heart (9). The current study now demonstrates that an increased protein level of the small Ca^{2+}-binding protein S100A1 is sufficient to clearly increase both systolic Ca^{2+} supply and fractional shortening of adult rat cardiac myocytes. A manipulation of S100A1 gene expression might therefore in the future prove beneficial as a novel positive inotropic intervention.

Because the transfection marker GFP is able to negatively modulate the contractile state of cardiac myocytes (14), we first sought to define an adenoviral dosage that would not interfere with intracellular Ca^{2+} cycling. In our experimental setting a MOI of 20 PFU yielded a nearly 100% transfection rate without affecting Ca^{2+} cycling in comparison to wild-type cardiac myocytes, thus, excluding collateral side effects by either the virus or GFP. Consequently all experiments were performed with this viral dosage. As rat myocardium shows a considerably lower S100A1 abundance we were not able to detect intrinsic rat S100A1 protein levels in extracts from a restricted number of cultured rat cardiomyocytes. We could thus not specify the relative increase of total S100A1 protein levels in S100A1 transfected cells but found a robust expression of human S100A1 in rat cardiac myocytes, while GFP expression was equal in both controls and S100A1 transfected cells. Moreover, as S100A1 overexpression did not change intrinsic S100A1 levels in rabbit cardiac myocytes (19), we do not expect it to be the case in rat cardiac myocytes either.

The manipulation of S100A1 expression was accompanied by a significant increase in fractional shortening based on a significant gain in systolic peak Ca^{2+} concentrations. Since we recently found that the S100A1 associated gain in contractility was neither accompanied by a change in cellular cAMP levels nor was it suppressed by carbachol interventions in rabbit cardiac myocytes (19), we hypothesized that S100A1 might augment β-adrenergic signaling at the level of its target proteins. It is thus of interest that S100A1 overexpression was indeed not only sufficient to raise basal Ca^{2+} transient amplitude but also augmented isoproterenol-stimulated Ca^{2+} cycling.

Since SERCA is a central regulator of the contraction-relaxation cycle, we next tested the impact of native S100A1 protein on the activity of SERCA taking advantage of the model of skinned cardiac myocytes. Having demonstrated that the intracellular milieu is readily accessible for S100A1 after β-escin skinning, we found that the administration of S100A1 leads to a significant increase of sarcoplasmic reticulum Ca^{2+} uptake. S100A1 thus activates a critical determinant of cardiac contractility. Due to earlier studies where we tested S100A1 peptides devoid of the Ca^{2+}-binding moieties (20), we can exclude a mere Ca^{2+}-buffering effect of S100A1 in this experimental setting. Thus the activation of SERCA by S100A1 would increase systolic Ca^{2+} peaks in response to an increased SR Ca^{2+} loading and simultaneously facilitate relaxation. As however S100A1 has been shown to specifically interact with the ryanodine receptor (25) resulting in enhanced SR Ca^{2+} release (20), it is conceivable that this Ca^{2+}-binding protein exerts its effects not only by modulating SERCA activity but also by interfering with a key element of cardiac excitation-contraction coupling. Further studies will thus have to clarify this point.

Although rat and rabbit display considerable species-related differences with respect to cytosolic Ca^{2+} homeostasis (13), cardiac myocytes of both species revealed a similar gain in Ca^{2+} turnover and contractile performance in response to increased human S100A1 levels (19). This implies that the highly conserved Ca^{2+} binding protein S100A1 serves basic functions in muscle physiology. As an earlier study demonstrated that S100A1 is significantly downregulated in human end stage heart failure (22) our findings support the notion that protein levels of S100A1 might also be positively correlated with the prevailing cardiac performance. Further studies are now warranted to investigate whether an upregulation of S100A1 in the failing heart would suffice to normalize

cardiac Ca^{2+} cycling and thus improve contractile performance as a novel positive inotropic intervention.

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