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Ca^{2+} signaling in mouse cardiomyocytes with ablated S100A1 protein

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Abstract. S100A1 is a Ca^{2+}-binding protein expressed at high levels in the myocardium. It is thought to modulate the Ca^{2+} sensitivity of the sarcoplasmic reticulum (SR) Ca^{2+} release channels (ryanodine receptors or RyRs) and its expression has been shown to be down regulated in various heart diseases. In this study we used S100A1 knock-out (KO) mice to investigate the consequences of chronic S100A1 deficiency on Ca^{2+} cycling in ventricular cardiomyocytes. Confocal Ca^{2+} imaging showed that field-stimulated KO myocytes had near normal Ca^{2+} signaling under control conditions but a blunted response to β-adrenergic stimulation with 1 μmol/l isoproterenol (ISO). Voltage-clamp experiments revealed that S100A1-deficient cardiomyocytes have elevated I_{Ca} under basal conditions. This larger Ca^{2+} influx was accompanied by augmented Ca^{2+} transients and elevated SR Ca^{2+} content, without changes in macroscopic excitation-contraction coupling gain, which suggests impaired fractional Ca^{2+} release. Exposure of KO and WT cells to ISO led to similar maximal I_{Ca}. Thus, the stimulation of the I_{Ca} was less pronounced in KO cardiomyocytes, suggesting that changes in basal I_{Ca} could underlie the reduced β-adrenergic response. Taken together, our findings indicate that chronic absence of S100A1 results in enhanced L-type Ca^{2+} channel activity combined with a blunted SR Ca^{2+} release amplification. These findings may have implications in a variety of cardiac pathologies where abnormal RyR Ca^{2+} sensitivity or reduced S100A1 levels have been described.

Key words: Calcium — Excitation-contraction coupling — Sarcoplasmic reticulum — Ryanodine receptors — L-type Ca^{2+} channels — S100 proteins — EF-hand

Abbreviations: ISO, isoproterenol; KO, knock-out; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; SERCA, sarcoplasmic reticulum calcium pump; WT, wild type.

Introduction

S100A1 is a Ca^{2+}-binding protein of the EF-hand family. It is structurally related to other well-known Ca^{2+}-binding proteins such as calmodulin, troponin C and parvalbumin (Donato 2003; Marenholz et al. 2004; Wright et al. 2005; Heizmann et al. 2007; Most and Koch 2007; Davis et al. 2008; Schaub and Heizmann 2008). S100 proteins include at least 20 members involved in the regulation of diverse cellular functions, such as cytoskeletal organization, contraction, differentiation, and transcription. By forming dimers, S100 proteins can functionally link target proteins in a Ca^{2+}-dependent (and sometimes in a Ca^{2+}-independent) manner (Donato 2003; Wang et al. 2004; Wright et al. 2005; Santamaria-Kisiel et al. 2006).

Several *in vivo* findings support an important role of S100A1 in heart function. In the myocardium, S100A1 is present at high levels, and changes in its abundance were shown in states of heart failure and hypertrophy (Remppis et al. 1996; Ehlermann et al. 2000). It has been reported to interact with the cardiac sarcoplasmic reticulum (SR) Ca^{2+} pump (SERCA2a), with phospholamban and with titin, thereby modulating Ca^{2+} homeostasis and contractile performance of muscle cells (Kiewitz et al. 2003; Heizmann et al. 2007). Adenoviral S100A1 gene delivery normalized myocardial contractile function and Ca^{2+} handling in rats with postinfarction heart failure (Most et al. 2004). Mice with a targeted deletion of the S100A1 gene preserved normal cardiac function under baseline conditions, but showed impaired inotropic and lusitropic responses upon β-adrenergic stimulation (Du et al. 2002).

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On the cellular level, S100A1 is able to modulate Ca^{2+} homeostasis and contractility (Adhikari and Wang 2001; Most et al. 2001). In both, skeletal and heart muscle cells, it was shown that S100A1 directly binds to the SR Ca^{2+} release channels (ryanodine receptors; RyRs). Recently it was found that S100A1 can compete with calmodulin for its binding site in a Ca^{2+}-dependent manner on both RyR1 and RyR2 (Wright et al. 2008).

A range of quite diverse cardiac pathologies are thought to result from a destabilization of RyR gating, often noticeable as an increase in the cytosolic and/or SR luminal Ca^{2+} sensitivity of the RyRs. This is accompanied by a susceptibility to arrhythmias, often manifest as catecholaminergic polymorphic ventricular tachycardia (CPVT) (Marks et al. 2002; Jiang et al. 2005; George et al. 2007). Examples leading to destabilization of the RyRs are the hyperphosphorylation of the RyRs during heart failure (Marx et al. 2000; Marks 2002), but also redox modifications of the RyRs during oxidative stress (Sanchez et al. 2005) as well as several mutations identified recently in the cardiac RyR (Dulhunty et al. 2006; George et al. 2007). Possibly relevant for such conditions, S100A1 was found to limit accidental SR Ca^{2+} release by reducing the occurrence of spontaneous Ca^{2+} sparks in resting, permeabilized ventricular myocytes (Volkers et al. 2006). This feature makes the S100A1 protein a possible candidate for antiarrhythmic therapy based on RyR stabilization (Niggli 2007).

In order to gain insight into the consequences of changes of expression or even a chronic loss of S100A1, we studied Ca^{2+} cycling in ventricular cardiomyocytes isolated from S100A1-knock-out (KO) mice (Ackermann et al. 2006). Previously, we observed that the S100A1-KO mice developed prolonged ventricular repolarization and intraventricular conduction disturbances in response to sympathetic stimulation (Ackermann et al. 2008). Here we explored the role of the absence of S100A1 on the cellular level. We observed that the chronic absence of S100A1 results in an upregulation of the L-type Ca^{2+} current, possibly combined with a reduction of the RyR Ca^{2+} sensitivity. Moreover, the blunted β-adrenergic stimulation observed in these mice may be caused by the pre-existing upregulation of L-type Ca^{2+} currents under control conditions. Taken together our finding may have implications for several cardiac pathologies where reduced S100A1 levels and/or an abnormal RyR Ca^{2+} sensitivity have been described. Some preliminary data have been presented in abstract form (Gusev et al. 2006).

Materials and Methods

Isolation of cardiomyocytes

Cardiac ventricular myocytes were isolated from adult S100A1-KO and the corresponding wild-type (WT) littermates using established enzymatic procedures (Mitcheson et al. 1998; Cerbai et al. 2000). Almost all experiments were carried out in males, besides experiments presented in Fig. 7, which were carried out in females. There was no sex-dependence difference observed in Ca^{2+} signaling or in responses to β-adrenergic stimulation. The generation, genetic characterization and breeding of S100A1-KO mice were described previously (Ackermann et al. 2006, 2008). All animal handling procedures were performed with permission of the State Veterinary Administration and according to the guidelines of the Swiss Animal Protection law. Briefly, mice were killed by cervical dislocation and excised hearts were mounted on a Langendorff perfusion system. After perfusion with nominally Ca^{2+}-free solution for 3–5 min, collagenase II (14 U/ml, Worthington type 2), protease (0.2 U/ml, Sigma, type XIV) and 50 μmol/l Ca^{2+} were added to the solution. After further 6–9 min of perfusion hearts were removed from the Langendorff apparatus and the ventricles cut in small pieces, followed by gentle trituration to obtain a cell suspension. Subsequently, the Ca^{2+} concentration was slowly raised to the normal level used in our experiments (1.8 mmol/l).

Confocal imaging and cytosolic Ca^{2+} ($[Ca^{2+}]_i$) measurements

Isolated cardiac myocytes were loaded by exposure to 4 μmol/l fluo-3 acetoxymethyl (AM) ester for 25 min or with 50 μmol/l fluo-3 through the patch-clamp pipette. For experiments using field-stimulation, cells were electrically paced using a pair of field electrodes (at 1 Hz to match the voltage-clamp experiments, 60–80 V/cm, 2 ms duration). Fluo-3 was excited with the 488 nm line of an argon-ion laser. Fluorescence was detected at > 500 nm with a confocal laser-scanning microscope operating in line-scan mode (500 lines/s, μRadiance, Bio-Rad, Nikon DIC H 60× water-immersion objective). The fluorescence was normalized and expressed as F/F_0, where F_0 is the baseline fluorescence at the beginning of each recording.

Electrophysiology

Cells were voltage-clamped in the whole-cell configuration of the patch-clamp technique and held at –80 mV. Current/voltage (I-V) relationships were measured with the following protocol: four prepulses (from –40 to 10 mV, 300 ms, 1 Hz) and a delay of 2 s were followed by a series of incremental test potentials (ranging from –30 to +80 mV, 300 ms, at 10 mV intervals). In order to measure the time-dependent evolution of I_{Ca} currents, cells were held at –80 mV, depolarized (at 0.2 Hz) to –40 mV for 300 ms (to inactivate I_{Na}), and further depolarized to 0 mV for 300 ms to elicit I_{Ca}. The time course of I_{Ca} was corrected for run-down by a monoexponential fit applied to the first 5 min in control conditions and verified by the observed I_{Ca} after wash-out of isoproterenol (ISO). The amount of Ca^{2+} released from the SR following application of 20 mmol/l caffeine was estimated from the peak amplitude of the Ca^{2+}-transient or by integrating the Na^+-Ca^{2+} exchange

current (I_{NCX}). All recordings were made at room temperature (20–22°C).

Solutions

The extracellular perfusion solution contained (in mmo/l): NaCl 140, KCl 5, $CaCl_2$ 1.8, $BaCl_2$ 0.5, CsCl 1, HEPES 10, Glucose 10, pH 7.4 (adjusted with NaOH). The patch pipettes were filled with intracellular solution composed of (in mmol/l): 120 CsAsp, 20 TEA-Cl, 5 K_2ATP, 20 HEPES and 0.05 K_5fluo-3, pH 7.2 (adjusted with CsOH). Ba^{2+}, TEA and Cs^+ were added to allow proper quantification of I_{Ca} without overlapping K^+-currents. Where indicated 0.1 µmol/l of S100A1 protein was included in the pipette solution. In these cases, the measurements were performed at least 4 min after break-in.

Data analysis

Data are presented as mean ± S.E.M. obtained from *n* different cells. Statistical differences between data sets were evaluated by Student's *t*-test. Significance was defined at $p < 0.05$.

Results

Dialysis of S100A1 into WT cardiomyocytes results in increased SR Ca^{2+} content

As an initial assessment for the acute effect of S100A1 protein in our conditions, we included S100A1 in the patch pipette filling solution (Fig. 1). The purpose of this experiment was to verify whether S100A1 restrains CICR (Ca^{2+}-induced Ca^{2+}-release),

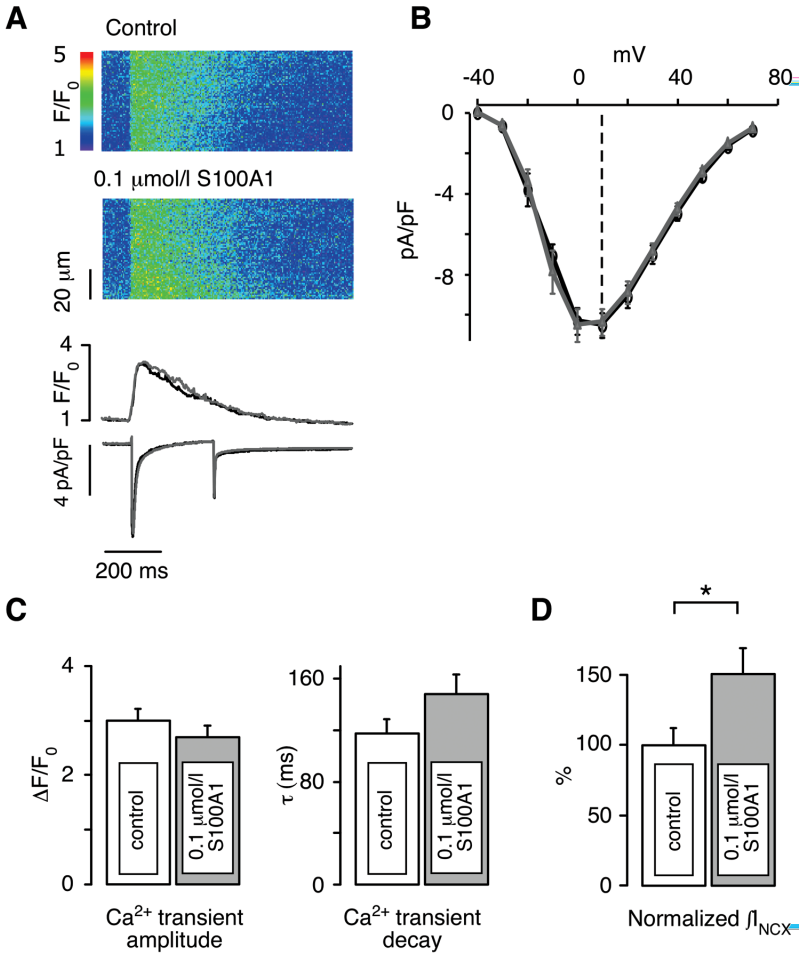


Figure 1. Addition of S100A1 protein increases SR Ca^{2+} load but does not influence the I_{Ca} and Ca^{2+} transients in WT cells. **A.** Line-scan images and averaged profile plots of Ca^{2+} transients and simultaneously recorded I_{Ca} at 0 mV. **B.** I-V relationship measured later than 4 min after beginning of the experiment. **C.** Averaged Ca^{2+} transient amplitude and decay time constant (control: $n = 8$; 0.1 µmol/l S100A1: $n = 7$). **D.** SR Ca^{2+} content is estimated by 20 mmol/l caffeine application and integration of I_{NCX} (integrated charge: 100 ± 12% *vs.* 150 ± 18%, control: $n = 4$, 0.1 µmol/l S100A1: $n = 7$). Empty bars, control; gray and filled bars, 0.1 µmol/l S100A1 included in the pipette solution. * $p < 0.05$.

reduces the SR Ca^{2+} leak and may thereby lead to elevated SR Ca^{2+}. Furthermore, we wanted to examine whether the protein stimulates the SERCA2a (which could be another mechanism resulting in an elevation of the SR Ca^{2+} content), and we wanted to make sure that the L-type Ca^{2+} current was not significantly influenced by S100A1 in our conditions. For these experiments we collected confocal line-scan images of fluo-3 fluorescence from cardiomyocytes while simultaneously recording L-type Ca^{2+} currents with the whole-cell patch-clamp technique, after allowing at least 4 min for the pipette solution to equilibrate with the cell interior. Inclusion of 0.1 µmol/l S100A1, a concentration which has previously been shown to exhibit a maximal effect on the Ca^{2+} transient (Kettlewell et al. 2005) did neither change I_{Ca} (Fig. 1A,B), nor did it increase the Ca^{2+} transient amplitude (Fig. 1A,C). However, the protein led to an elevated SR Ca^{2+} content, as estimated by integrating I_{NCX} in response to application of 20 mmol/l caffeine (Fig. 1D: 100 ± 12% *vs.* 150 ± 18%), confirming results obtained with adenoviral transfection of S100A1 (Most et al. 2005) (but see Kettlewell et al. 2005). Since I_{Ca} was not changed, the elevated SR Ca^{2+} content could be explained either by a transiently reduced Ca^{2+} release (leading to some SR Ca^{2+} accumulation) or by an accelerated SR Ca^{2+} uptake. However, the slowing of the Ca^{2+} transient decay argues against a stimulation of the SERCA2a and rather suggests that a suppression of CICR by acutely applied S100A1 leads to elevated SR Ca^{2+} content (Fig. 1C). Also note that despite a higher SR Ca^{2+} load during S100A1 dialysis, the efficiency of I_{Ca} to trigger CICR remained the same, which implies a reduction in fractional Ca^{2+} release.

Ca^{2+} signals in field stimulated WT and KO cardiomyocytes

The results above suggest that addition of S100A1 protein acutely reduces fractional SR Ca^{2+} release, possibly *via* its reported inhibitory action on the RyRs observed at resting Ca^{2+} concentrations (Volkers et al. 2006). But what will be the consequences of a chronic absence of the S100A1 protein and the possibly resulting hyperactivity of the RyRs? To answer this question we carried out experiments in cardiomyocytes obtained from mice with ablated S100A1 protein (S100A1-KO mice; Ackermann et al. 2006). These mice are viable, fertile and have no overt phenotypic changes. The hemodynamic measurements revealed essentially normal cardiac function (systolic and diastolic pressures, contraction and relaxation) in the absence of S100A1 under baseline conditions. Also there was no upregulation of other S100 or other Ca^{2+} signaling proteins (Ackermann et al. 2008).

When ventricular cardiomyocytes isolated from WT and KO mice (6–7 months old) were field-stimulated, the amplitudes of the Ca^{2+} transients were slightly larger in KO cells (Fig. 2).

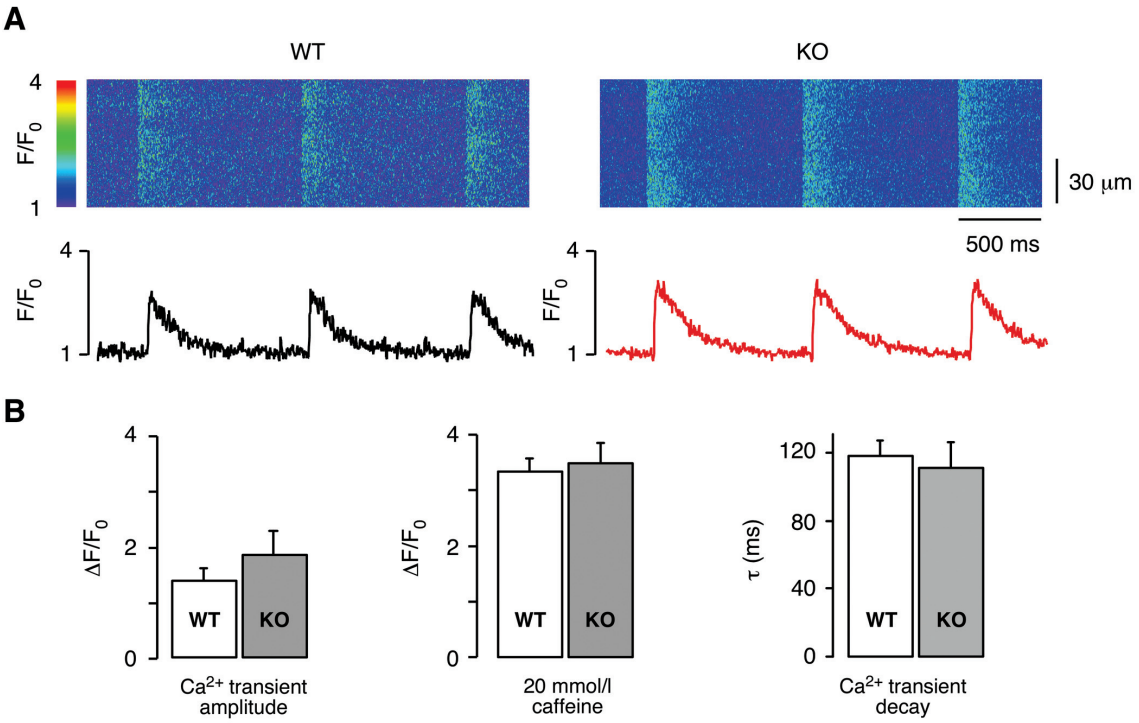


Figure 2. Field stimulated WT and KO cardiomyocytes behave similarly. **A.** Line-scan images and corresponding profile plots of Ca^{2+} transients in WT and KO cardiomyocytes. **B.** Averaged Ca^{2+} transient amplitudes, caffeine responses and Ca^{2+} transient decay time constants. The Ca^{2+} transients were slightly larger in KO cells, but here were no significant differences (WT: n = 12; KO: n = 8).

Estimates of SR Ca^{2+} loading during field-stimulation by rapid application of caffeine revealed no significant difference in the SR Ca^{2+} content. The time-course of the Ca^{2+} transient decay also did not change (110 ± 15 ms in KO *vs.* 117 ± 9 ms in WT), which suggests unaltered SERCA2a activity. Taken together the minimal differences in Ca^{2+} signaling between WT and KO cells indicate that there may be compensatory mechanisms which developed to allow the cells to cope with the lack of S100A1 and its consequences.

Ca^{2+}transients and L-type Ca^{2+}currents are larger in S100A1-KO cells

While experiments with field-stimulation maintain the cells more closely to physiological conditions, they do not allow to control several experimental variables, such as the action potential duration (including its beat-to beat variability) and the intracellular environment. In order to obtain experimental control over these parameters we used the whole-cell patch-clamp technique in combination with confocal Ca^{2+} imaging.

Fig. 3A shows simultaneously recorded representative Ca^{2+} transients and I_{Ca} in WT and KO cells, elicited by applying a depolarizing step from –40 to 0 mV for 200 ms. As summarized in Fig. 3B, the membrane capacitance of KO and WT cells was similar, whereas the amplitudes of Ca^{2+} transients and I_{Ca} were significantly larger in KO cardiomyocytes ($\Delta F/F_0$: 2.9 ± 0.2 *vs.* 3.5 ± 0.2; I_{Ca}: –5.0 ± 1.0 pA/pF *vs.* –8.2 ± 1.0 pA/pF, for WT and KO, respectively).

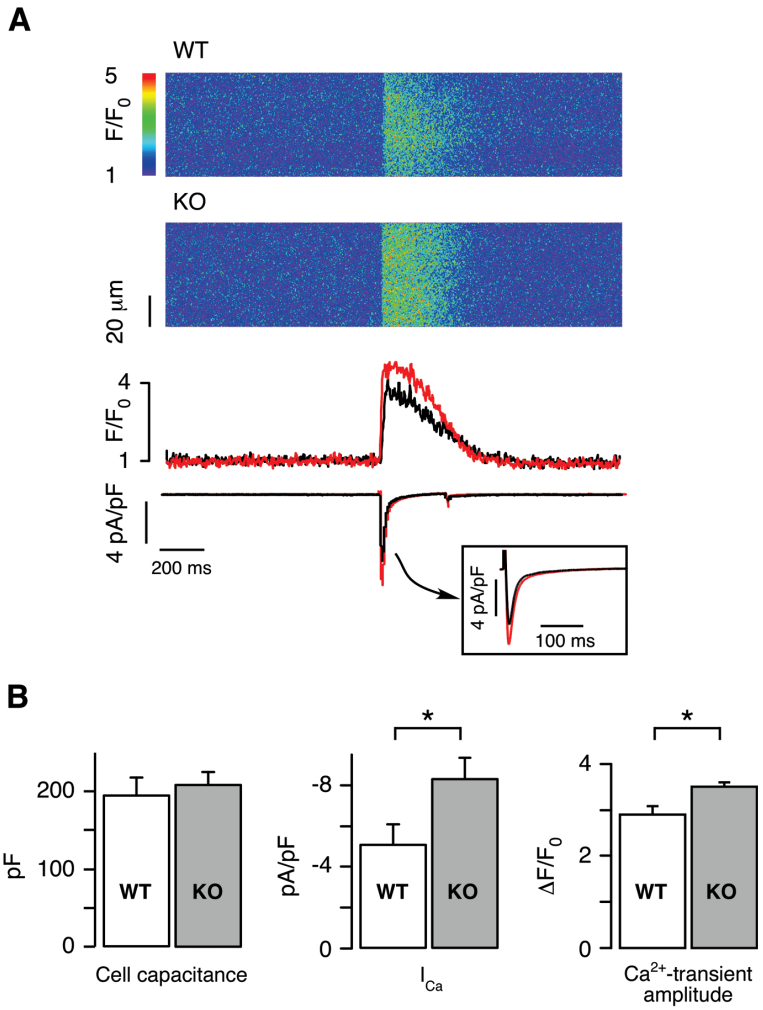
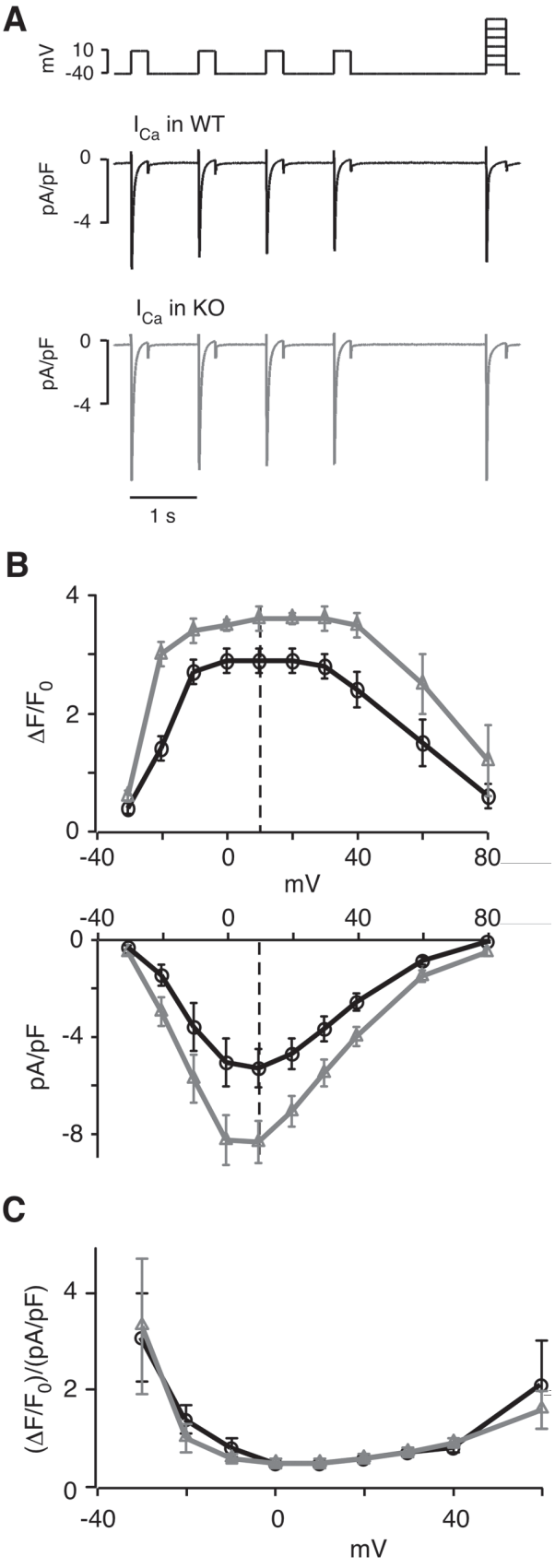


Figure 3. Ca^{2+} transients and I_{Ca} are increased in patch-clamped KO cardiomyocytes under control conditions. **A.** Line-scan images and averaged plots of Ca^{2+} transients and corresponding I_{Ca} at 0 mV (insert: expanded I_{Ca} at test potential) in WT (black) *vs.* KO (red). **B.** Averaged membrane capacitance, Ca^{2+} transient amplitude and I_{Ca} in WT (empty bars) *vs.* KO (filled bars). (WT: $n = 8$; KO: $n = 7$). * $p < 0.05$.



For measurements of I-V relationships, cells were held at –40 mV and after four prepulses to +10 mV the test potential was applied (Fig. 4A, upper trace). Depolarization elicited Ca^{2+} currents, which inactivated within 300 ms (Fig. 4A, lower traces). The I-V relationship of summarized data confirmed significantly larger I_{Ca} amplitudes in KO cells at all voltages tested (–5.3 ± 0.8 pA/pF *vs.* –8.2 ± 0.9 pA/pF at V_m = 10 mV, Fig. 4B, upper panel). There was no significant shift in the voltage-dependence of the current. The corresponding Ca^{2+} transients were also larger in KO cells, indicating a larger Ca^{2+} influx and, presumably, Ca^{2+} release during the same voltage protocol (Fig. 4B, lower panel; $\Delta F/F_0$: 2.9 ± 0.2 *vs.* 3.6 ± 0.2 at 10 mV). Comparison of the decay kinetics of Ca^{2+} transients with similar amplitudes revealed no significant difference (120 ± 13 ms in WT *vs.* 98 ± 7 ms in KO, respectively). This again argues against an involvement of SERCA2a modulation during the compensatory changes.

To assess the excitation-contraction (EC)-coupling efficiency we also determined the macroscopic EC-coupling gain, which is defined as the amplitude of Ca^{2+} transients normalized for the I_{Ca} amplitude (Fig. 4C). EC-coupling gain was not different between WT and KO cells, suggesting that the same amount of Ca^{2+} influx will trigger the same SR Ca^{2+} release. However, as estimated from the amplitude of caffeine-induced Ca^{2+} transients, the Ca^{2+} content of the SR was significantly increased in KO cells (Fig. 5A,B, left; $\Delta F/F_0$: 3.2 ± 0.2 *vs.* 4.0 ± 0.2). Quantification of the SR Ca^{2+} load by integrating I_{NCX} revealed a similar increase in SR Ca^{2+} content in KO cells (Fig. 5A,B, right; 100 ± 6% *vs.* 121 ± 6%). Taken together, both types of analyses indicated a more than 1.2-fold increase in SR Ca^{2+} content in KO *versus* WT cells. At the same time, the Ca^{2+} dependence and the time-course of I_{NCX} did not change (864 ± 63 ms in WT (n = 10) *vs.* 862 ± 45 in KO (n = 11), not shown), suggesting no changes in NCX function.

Thus, despite elevated SR Ca^{2+} content the KO cardiomyocytes have an unchanged macroscopic EC-coupling gain. This is not expected and implies a reduced fractional Ca^{2+} release at a given current.

Upregulation of I_{Ca} upon β-adrenergic stimulation is diminished

Previously, it has been noted that S100A1-KO mice have impaired inotropic responses and relaxation upon β-

◀ **Figure 4.** Macroscopic EC-coupling gain in KO cardiomyocytes is unchanged. **A.** Voltage protocol and I_{Ca} in WT (black) and KO (grey) cells. **B.** I-V relationship and corresponding Ca^{2+} signals were measured in voltage-clamped cardiomyocytes (WT: n = 8, black circles; KO: n = 7, grey triangles). **C.** EC-coupling gain calculated as $(\Delta F/F_0)/I_{Ca}$ for the experiments shown in panel B.

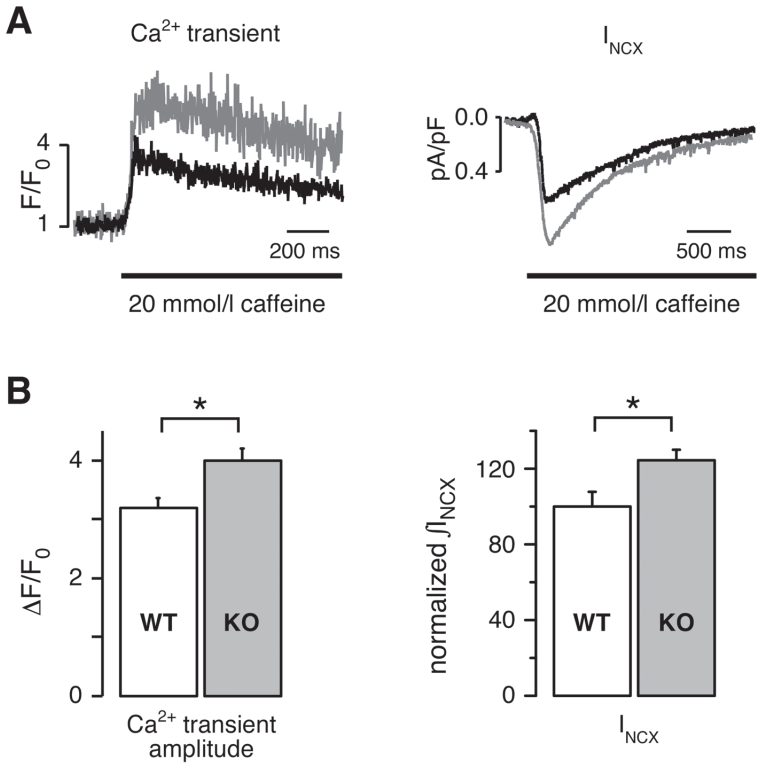


Figure 5. SR Ca^{2+} content is increased in KO cardiomyocytes. **A.** SR Ca^{2+} content measured in voltage-clamped cardiomyocytes in response to 20 mmol/l caffeine application in WT (black) and KO (gray) cardiomyocytes. Left: Profile plots of caffeine-induced Ca^{2+} transients. Right: I_{NCX} and its normalized integral. **B.** Averaged Ca^{2+} transient amplitude (left) and normalized $\int I_{NCX}$ for caffeine response (WT: $n = 10$; KO: $n = 11$).

adrenergic stimulation (Du et al. 2002). Although in our study we observed unchanged inotropic and lusitropic responses of the heart to β-adrenergic stimulation, this stimulation resulted in cardiac repolarization and conduction impairments *in vivo* (Ackermann et al. 2008). In the light of the larger Ca^{2+} currents observed in KO cells in the present study (see above), we were interested to examine whether this was related to the mechanism responsible for the dysfunction of β-adrenergic pathways in S100A1-KO ventricular cardiomyocytes.

Experiments with field-stimulated myocytes revealed a less pronounced response of Ca^{2+} signaling in KO myocytes during β-adrenergic stimulation with ISO (1 μmol/l) (Fig. 6A,B). Interestingly, KO and WT cells reached the same maximal Ca^{2+} transient amplitude during β-adrenergic stimulation. However, based on these results alone it is difficult to make conclusions regarding the origin of the reduced regulation by β-adrenergic stimulation.

The whole-cell voltage-clamp technique allows a direct quantification of the I_{Ca} stimulation mediated by phosphorylation during application of ISO. As before, cells were held at –80 mV and in order to measure I_{Ca}, voltage jumps to 0 mV were applied at 0.2 Hz (after a prepulse to –40 mV to inactivate I_{Na}). I_{Ca} was expressed as difference between peak and steady-state current after inactivation (Fig. 7A). In control conditions I_{Ca} was larger in KO than in WT cells (Fig. 7B, left: -3.9 ± 0.4 *vs.* -6.3 ± 0.2 pA/pF). However, after application of 1 μmol/l ISO for 5 min, the amplitude of I_{Ca} reached similar maximal current levels in both types of cells (Fig. 7B, middle: -9.0 ± 1.2 *vs.* -10.0 ± 1.5 pA/pF). The corresponding Ca^{2+} transient amplitudes behaved in a similar way as I_{Ca} with a more pronounced increase of the signals in WT than in KO cells (Fig. 7C). There was slight reduction of EC-coupling gain in WT cells during application of ISO, whereas it remained unchanged in KO cells (Fig. 7D). This could arise from the reduced increase in (redundant) Ca^{2+} current in KO cells (see below for Discussion). Taken together these observations suggest, that β-adrenergic signaling is not impaired in S100A1-KO cells but that in basal conditions the Ca^{2+} channels of KO cardiomyocytes are in a pre-activated state and therefore less available for further stimulation.

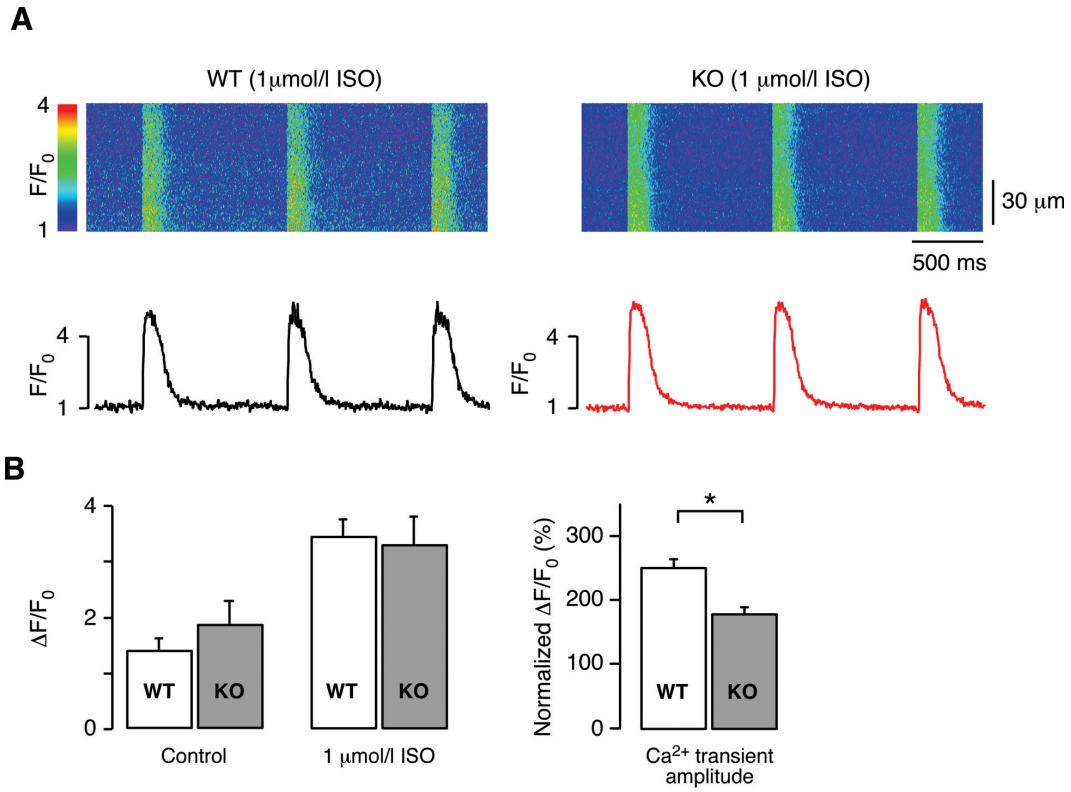


Figure 6. KO cells have blunted β-adrenergic receptor response. **A.** Line-scan images and corresponding averaged profile plots of Ca^{2+} signals in WT and KO cardiomyocytes during field-stimulation. **B.** Averaged Ca^{2+} transient amplitudes before and after 5 min of 1 μmol/l ISO application, relative changes in Ca^{2+} transient amplitude (WT: $n = 12$;. KO: $n = 8$, control values are replotted from Fig. 1). * $p < 0.05$.

Discussion

In KO mouse models the resulting phenotype is quite often the result of both, the loss of function of an ablated protein, and the compensatory gain of function of several other proteins and/or mechanisms. Our study primarily investigated the consequences of a chronic absence of the S100A1 protein on the Ca^{2+} homeostasis in mouse cardiomyocytes. However, the initial change of function due to the KO may be extrapolated to some extent from known acute effects of the protein in question. Therefore, we initially examined how Ca^{2+} signaling was altered by the addition of exogenous S100A1 protein to the patch pipette filling solution.

Acute interaction of S100A with Ca^{2+} signaling proteins

It has been published previously that at diastolic $[Ca^{2+}]_i$ the spontaneous activity of RyRs, which underlies most of the SR Ca^{2+} leak, is reduced by adding human S100A1, at least in rabbit cardiomyocytes (Volkers et al. 2006). Interestingly, it has also been shown that the S100A1 protein can potentiate triggered Ca^{2+} release *via* RyRs and modify the SERCA2a activity (Du et al. 2002; Most et al. 2003; Kettlewell et al. 2005). Recent publications identified a possible interaction with the calmodulin-binding domain of the RyRs, which may underlie this modulation by S100A1 (Prosser et al. 2008; Wright et al. 2008). The total concentration of S100A1 in murine heart has been estimated to be approximately 10 μmol/l (Du et al. 2002). However, most of the S100A1 is bound to other proteins and the actual free concentration in the cytosol is unknown but likely to be much lower. Since the acute application of 0.1 μmol/l S100A1 has been described to exhibit the strongest effect (Kettlewell et al. 2005), we also used this concentration in this series of experiments.

As shown in Fig. 1, our main finding in these experiments was that the intracellular application of S100A1 led to an increase in the SR Ca^{2+} content. However, we did not observe some of the other reported changes, such as stimulation of the triggered Ca^{2+} release or of the SERCA2a function. In addition, the amplitude and time-course of I_{Ca} remained unaltered. Therefore, among the previously reported S100A1 effects on Ca^{2+} signaling a reduction of the SR Ca^{2+} leak at rest is the most likely explanation for these findings, and is

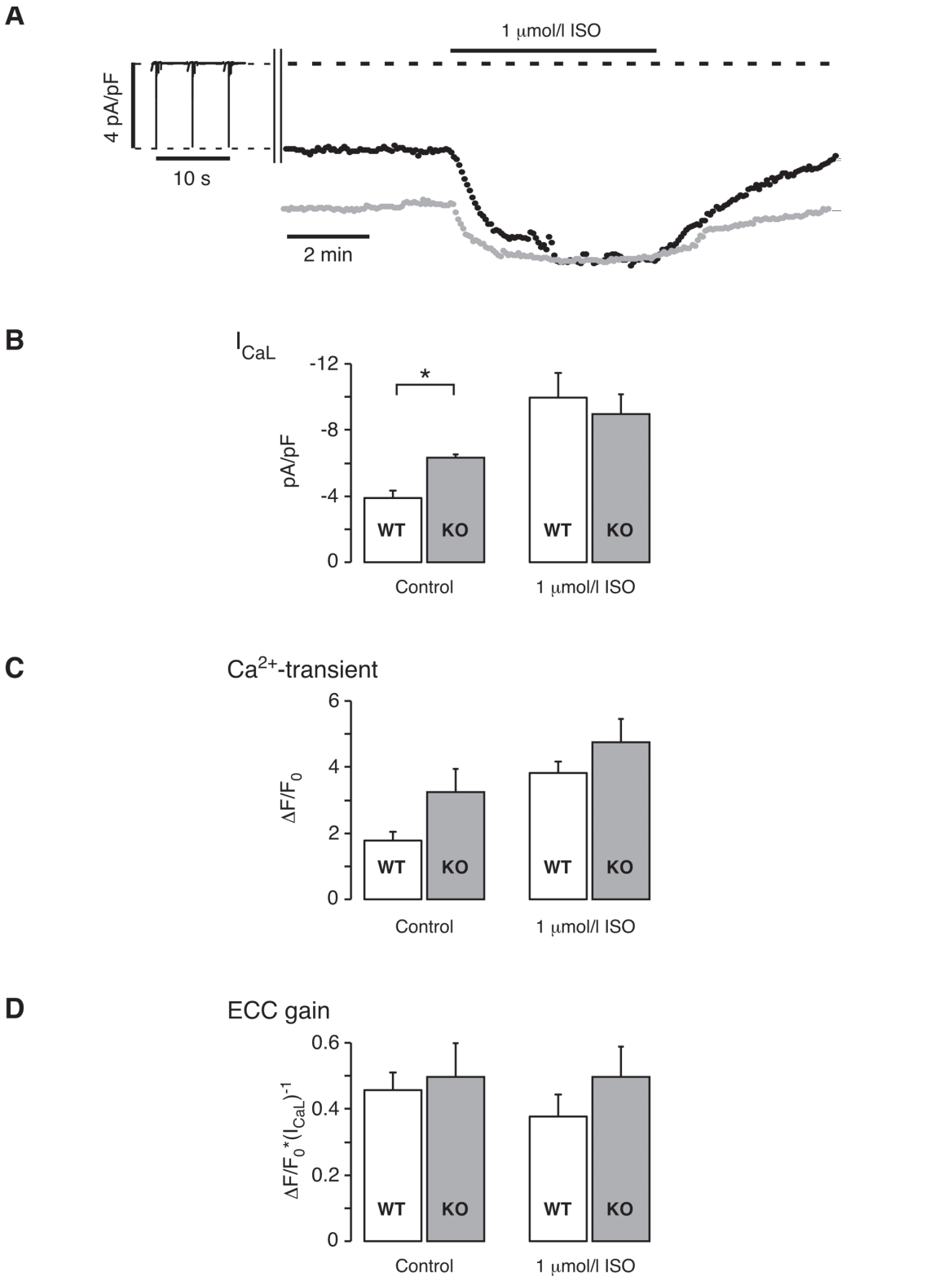


Figure 7. A. Time-dependent evolution of the I_{Ca} peak amplitude in basal conditions and during β-adrenergic stimulation by 1 μmol/l ISO in a WT (black) and a KO (grey) cardiomyocyte. I_{Ca} amplitudes were measured at 0 mV (every 5 s) and corrected for run-down. **B.** Averaged I_{Ca} peak amplitude immediately before (left) and 5 min after ISO (right). **C.** and **D.** Corresponding Ca^{2+} transient amplitudes and EC-coupling gains before (left) and after ISO (right). (WT: $n = 4$; KO: $n = 3$).

consistent with the reported inhibition of RyR2 by S100A1 at diastolic $[Ca^{2+}]_i$. Because of the autoregulatory features of CICR for varying Ca^{2+} triggers and SR Ca^{2+} releases, it is expected that, in the steady state, the total amount of Ca^{2+} released from the SR self-adjusts and does not change much (Trafford et al. 2002). A significant SERCA2a stimulation by S100A1 seems unlikely under our conditions, as the Ca^{2+} transients did not decay faster in the presence of the protein.

Based on these observations we extrapolated that the absence of S100A1 in developing KO cardiomyocytes might initially lead to an elevated SR Ca^{2+} leak. A persistent SR Ca^{2+} leak could result in a reduced SR Ca^{2+} content, and subsequently in diminished Ca^{2+} signal amplitude leading to abnormal heart function. However, these S100A1-KO mice do not present any overt hemodynamic changes at rest (Ackermann et al. 2008), suggesting that some adaptive mechanisms may have developed to compensate for the chronic absence of S100A1. In order to preserve normal cardiac function, any adaptive remodeling would presumably attempt to maintain a normal amplitude and time-course of the Ca^{2+} transient (although other scenarios are also conceivable, such as compensatory changes of myofilament Ca^{2+} sensitivity).

Chronic and adaptive changes resulting from the ablation of S100A1

Several findings of the present study can be reconciled with the evolution of adaptive mechanisms aiming to counterbalance a putatively enhanced SR Ca^{2+} leak at rest. For example, while Ca^{2+} transients were only slightly larger and SR Ca^{2+} content was not changed in field-stimulated intact cells (Fig. 2), voltage-clamp experiments revealed that S100A1-deficient cardiomyocytes had elevated I_{Ca} under control conditions (Figs. 3, 4). This larger Ca^{2+} influx was accompanied by augmented Ca^{2+} transients and subsequently resulted in elevated SR Ca^{2+} content (Figs. 3–5). Thus, the voltage-clamp technique may unmask a subtle difference in Ca^{2+} signaling by enabling better control of experimental variables (e.g. intracellular environment, membrane potential). While Ca^{2+} transients in patch-clamp conditions are proportional to the I_{Ca} and the duration of the depolarization, the shape and duration of the action potential is somewhat variable and uncontrolled during field-stimulation. Since we and others found that S100A1 supplementation has no immediate effect on the I_{Ca}, the enhanced current most likely reflects an adaptive response of the cardiomyocytes. Also, the somewhat paradoxical similarities of Ca^{2+} signaling changes during acute application of S100A1 and those seen in its chronic absence suggest, that the resulting phenotype of the KO animals is more defined by adaptive mechanisms than by the lack of S100A1 itself.

Excitation-contraction coupling gain

The reliability of EC-coupling can be estimated from the EC-coupling gain, which is calculated by dividing the Ca^{2+} signal amplitude by the peak Ca^{2+} current. Surprisingly, the macroscopic EC-coupling gain was not different in KO myocytes, despite the elevated SR Ca^{2+} content. However, the EC-coupling gain and the fractional SR Ca^{2+} release is known to normally depend on SR content (Shannon et al. 2000), among other factors, such as RyR Ca^{2+} release sensitivity and release synchronization (Polakova et al. 2008). But the larger Ca^{2+} current observed in KO myocytes could also contribute to lower EC-coupling gain. Recent studies have shown that large Ca^{2+} currents can lead to redundant Ca^{2+} entry (i.e. not triggering Ca^{2+} release), thereby reducing EC-coupling gain without changes in RyR properties (Altamirano and Bers 2007). In any case, the finding that the ECC gain remained unchanged despite an elevated SR Ca^{2+} content suggests that at any given current the total SR Ca^{2+} release was similar in WT and KO myocytes. In other words, the fractional SR Ca^{2+} release was reduced in the KO cardiomyocytes (Figs. 4, 5). In the present study, this could be explained, for example, by a reduced Ca^{2+} sensitivity of the RyRs. This, in turn, could result from the lack of the reported EC-coupling gain stimulation by S100A1 (Kettlewell et al. 2005). Alternatively, it could also be a consequence of an adaptive change subsequent to S100A1 ablation, possibly to compensate for an abnormal diastolic SR Ca^{2+} leak at rest (Volkers et al. 2006), or from an elevated redundancy of Ca^{2+} entry *via* L-type Ca^{2+} current. Taken together, a slight shift of the Ca^{2+} signaling towards being more I_{Ca}-based occurred in KO myocytes, which means a larger I_{Ca} but a reduced fractional SR Ca^{2+} release. Under voltage clamp conditions, where the cell is forced into a square-shaped artificial action potential, this shift in the Ca^{2+} signaling towards more Ca^{2+} current becomes more obvious and will subsequently result in a more substantial Ca^{2+} extrusion *via* the NCX, because the Ca^{2+} transients are also larger. Thus, under voltage-clamp conditions the system converges to a new steady state where Ca^{2+} removal *via* NCX again equals influx, even without changes in NCX expression levels (Ackermann et al. 2008).

Possible implications of reduced S100A1 levels

As it was mentioned above, there are several diseases and conditions that have abnormal Ca^{2+} sensitivity of the RyRs as a common end-point (Marks et al. 2002; Benkusky et al. 2004; George et al. 2007). For example direct mutations in the RyRs or associated proteins, such as calsequestrin (di Barletta et al. 2006) or subsequent to PKA-dependent phosphorylation or hyperphosphorylation of the RyRs (Marx et al. 2000; Lindegger and Niggli 2005). Hyperphosphorylation

is thought to occur after chronic β-adrenergic stimulation, such as during congestive heart failure. Furthermore, redox modifications of the RyRs tend to increase their Ca^{2+} sensitivity in various conditions of cellular stress, when excessive amounts of reactive oxygen species (ROS) are produced (Pessah et al. 2002; Sanchez et al. 2005; Jung et al. 2008). If more than one of these sensitizing conditions occurs simultaneously, either alone or together with elevated SR Ca^{2+} load, the CICR mechanism may become unstable. Thus, in the S100A1-KO myocytes, the chronically elevated L-type Ca^{2+} current, in combination with a tendency to overload the SR with Ca^{2+}, may represent an adaptive mechanism that has some proarrhythmogenic potential. In some of those conditions, overexpression of S100A1 might actually have a beneficial effect (Most and Koch 2007).

Modulation by β-adrenergic stimulation

The larger Ca^{2+} current in KO cells under control conditions is a remarkable finding (Figs. 3, 4). The mechanism causing this upregulation is not yet identified. But our observation of virtually identical currents in WT and KO cells during maximal β-adrenergic stimulation suggest a change in the availability of Ca^{2+} channels in KO cells for further potentiation. This could result, for example, from a modification of the L-type Ca^{2+} channels under control conditions. A possibility for enhanced I_{Ca} is posttranslational modifications of the channels (Catterall 2000), such as phosphorylation by PKA, or other protein kinases (McDonald et al. 1994; Puri et al. 1997; Kamp and Hell 2000; Hulme et al. 2004; Sun et al. 2006). The time-dependent development of the I_{Ca} amplitude during application of 1 μmol/l ISO suggests that the L-type Ca^{2+} channels were already pre-activated in KO myocytes by a mechanism similar to PKA-mediated phosphorylation of the channels (Fig. 6C,D), but other possibilities underlying this stimulation cannot be excluded at present.

In summary, our findings give insight into the intrinsic plasticity of Ca^{2+} handling and adaptation mechanisms that maintain the Ca^{2+} balance in response to a chronic S100A1 deficit. While keeping the system working at rest these adaptive changes may contribute to the disturbances of heart function and the development of arrhythmias following β-adrenergic stimulation.

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