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Original article

S100A1 increases the gain of excitation–contraction coupling in isolated rabbit ventricular cardiomyocytes

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Abstract

The effect of S100A1 protein on cardiac excitation–contraction (E–C) coupling was studied using recombinant human S100A1 protein (0.01–10 μM) introduced into single rabbit ventricular cardiomyocytes via a patch pipette. Voltage clamp experiments (20 °C) indicated that 0.1 μM S100A1 increased Ca^{2+} transient amplitude by ~ 41% but higher or lower S100A1 concentrations had no significant effect. L-type Ca^{2+} current amplitude or Ca^{2+} efflux rates via the Na^+/Ca^{2+} exchanger (NCX) were unaffected. The rate of Ca^{2+} uptake associated with the SR Ca^{2+}-ATPase (SERCA2a) was increased by ~ 22% with 0.1 μM S100A1, but not at other S100A1 concentrations. Based on the intracellular Ca^{2+} and I_{NCX} signals in response to 10 mM caffeine, no significant change in SR Ca^{2+} content was observed with S100A1 (0.01–10 μM). Therefore, 0.1 μM S100A1 appeared to increase the fractional Ca^{2+} release from the SR. This result was confirmed by measurements of Ca^{2+} transient amplitude at a range of SR Ca^{2+} contents. The hyperbolic relationship between these two parameters was shifted to the left by 0.1 μM S100A1. [^{3}H]-ryanodine binding studies indicated that S100A1 increased ryanodine receptor (RyR) activity at 0.1 and 0.3 μM Ca^2. As with the effects on E–C coupling, 0.1 μM S100A1 produced the largest effect. Co-immunoprecipitation studies on a range of Ca^{2+}-handling proteins support the selective interaction of S100A1 on SERCA2a and RyR. In summary, S100A1 had a stimulatory action on RyR2 and SERCA2a in rabbit cardiomyocytes. Under the conditions of this study, the net effect of this dual action is to enhance the Ca^{2+} transient amplitude without significantly affecting the SR Ca^{2+} content.

Keywords: S100A1; Excitation–contraction (E–C) coupling; Calcium homeostasis; Cardiomyocytes

1. Introduction

S100A1 protein is a member of the EF-hand Ca^{2+}-binding S100 family and is expressed at high levels in the heart [8,9]. S100A1 has been found to be associated with the SR, mitochondria and contractile elements of cardiac tissue [8,9]. Interestingly, transgenic over-expression of S100A1 in the hearts of mice causes enhancement of cardiac function that remains after β-adrenergic receptor stimulation [15]. The over-expression of S100A1 via an adenovirus to neonatal cardiac myocytes in culture and to engineered heart tissue in vitro has demonstrated enhanced Ca^{2+} transients, improved SR Ca^{2+} uptake and contractility [14,24,25,27–29]. The S100A1 gene has also been knocked out in mice, and these animals have impaired cardiac contractile function [5]. Under pathophysiological conditions, the expression of S100A1 is down-regulated in end-stage heart failure but increased in pressure-overload hypertrophy [6,23]. Most recently, cardiac adenoviral S100A1 gene delivery has been proven to restore diminished S100A1 protein levels in failing rat myocardium in vivo and in vitro thereby normalizing defective contractile performance and impaired Ca^{2+} transients, respectively (Most et al., 2004). However, despite this work, no overall picture of the effects of S100A1 on cardiac Ca^{2+}-homeostasis has been described. S100A1 does enhance the activity of the RyR (type 1) in permeabilised skeletal muscle [16,26]. Similar effects on the cardiac RyR (type 2) have been observed in mouse cardiac SR vesicle preparations [15]. S100A1 has also been shown to stimulate SERCA2a-mediated SR Ca^{2+} uptake in permeabilised cardiac cells [14,15]. But the effects of

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S100A1 on the L-type Ca^{2+} current ($I_{Ca,L}$) or the Na^+/Ca^{2+} exchanger (NCX) have not been investigated. Hence, the aim of this study was to examine the effects of S100A1 on the Ca^{2+} fluxes associated with E–C-coupling in isolated rabbit ventricular myocytes to establish the subcellular mechanisms underlying the effects on the Ca^{2+} transient.

2. Methods

2.1. Expression and purification of human recombinant S100A1 protein

Expression and purification of recombinant human S100A1 protein (rh-S100A1) in E. coli was performed as previously described [16]. Purity of S100A1 protein preparations was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using XCell SureLock™ Mini-Cell (Invitrogen) and separated in a 4–20% gel with a Tris/taurine buffer system while relative mass was obtained by electrospray-ionisation mass spectroscopy (ESI-MS) as previously reported [16]. Silver staining of polyacrylamide gels was performed according to [19]. After dialysis against 10 mM HEPES, pH 7.4, aliquots of purified S100A1 protein were stored at – 80 °C.

2.2. Isolation of adult ventricular rabbit cardiomyocytes

Adult rabbit ventricular cardiac myocytes were isolated from New Zealand White rabbits by standard enzymatic dissociation as described previously [12,21]. Cardiomyocytes were re-suspended in a HEPES-based Krebs–Heinseleit solution containing (mM): NaCl (140), KCl (4), HEPES (5), $MgCl_2$ (1), $CaCl_2$ (1.8), glucose (11.1), pH 7.4 with NaOH.

2.3. Voltage clamp and intracellular [Ca^{2+}] measurements in rabbit cardiomyocytes

The isolated cardiomyocytes were superfused with a Krebs–Heinseleit solution (see above) with additional 5 μM TTX, 0.1 mM niflumic acid and 5 mM 4-AP at 19–20°C in a chamber mounted on the stage of an inverted microscope. Voltage clamp was achieved using whole cell patch technique with an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA, USA) in switch clamp (discontinuous) mode. Pipettes were filled with an intracellular solution of composition (mM): 20.0 KCl, 100.0 K Aspartate (DL), 20.0 TEA Cl, 10.0 HEPES, 4.5 $MgCl_2$, 4.0 Na_2ATP, 1.0 Na_2CrP, 2.5 EGTA (pH 7.25 with KOH), and were of resistance 3–6 MΩ. S100A1 recombinant protein (0.01–10 μM) was introduced into the cell via the patch pipette. The protein was added from a concentrated buffer stock solution to the pipette solution; control traces were recorded using a pipette solution with the buffer solution without recombinant protein. Intracellular [Ca^{2+}] was measured from Fura-2 fluorescence signals using a dual wavelength spectrophotometer method as previously described [7]. Cytosolic loading of Fura-2 was achieved by incubating cardiomyocytes with 5 μM Fura-2-AM at room temperature for 12 min.

2.4. Electrophysiological protocols

2.4.1. E–C coupling protocol

Isolated rabbit cardiomyocytes were held at – 80 mV and the voltage stepped to – 40 mV (50 ms) to inactivate the remaining inward Na^+ current, before stepping to 0 mV (150 ms) (Fig. 1A). This protocol was repeated 40 times at a rate of 0.5 Hz to achieve steady state Ca^{2+} transients. SR Ca^{2+} content and NCX activity were then estimated by rapidly switching to 10 mM caffeine to cause SR Ca^{2+} release. In the continued presence of caffeine (20 s) the SR is unable to re-accumulate Ca^{2+}, and therefore, Ca^{2+}-removal is mainly via NCX. The time course of the decay of [Ca^{2+}] and the NCX-mediated inward current (I_{NCX}) represent rates of extrusion of Ca^{2+} from the cell predominately via NCX [4]. These signals were fitted to exponential decays over approximately 80% of their amplitude. The magnitude of non-NCX Ca^{2+}-removal mechanisms was estimated from the Ca^{2+} decay obtained by rapidly switching to 10 mM caffeine in the presence of 10 mM $NiCl_2$ [4].

2.4.2. Manipulation of SR Ca^{2+} load

(i) increased SR Ca^{2+} load: the holding potential was maintained at – 60 mV, thus reducing Ca^{2+} efflux via NCX activity, raising diastolic [Ca^{2+}], and therefore, SR content (ii) decreased SR Ca^{2+} load: 5 μmol l^{-1} thapsigargin was washed on to cardiomyocytes to reduce SR Ca^{2+} load. Three exposure times were used; 20, 40 and 100 s to achieve graded reduction in SR Ca^{2+} content.

2.4.3. NCX current density

Currents were measured in response to a 3 s ramp from – 120 to + 80 mV from a holding potential of – 80 mV following a previously published protocol [2]. The ramp protocol was performed at 0.1 Hz until steady state currents were achieved. Data from six ramps were averaged. The protocol was repeated in the presence of 5 mM $NiCl_2$ to measure the background current.

2.5. Western blots and co-immunoprecipitation studies

Co-immunoprecipitation studies were carried out to investigate S100A1 protein interaction with the L-type Ca^{2+}-channel (LTCC) α_1-subunit, RyR2, SERCA2a and NCX as described in detail elsewhere [15]. Briefly, β-escin permeabilised rabbit cardiomyocytes were incubated with vehicle or rh-S100A1 protein (1 μM) under conditions of low [Ca^{2+}] (1 mM EGTA) and high Ca^{2+} (1 mM) for 10 min. Preparations were then homogenised in buffer consisting of

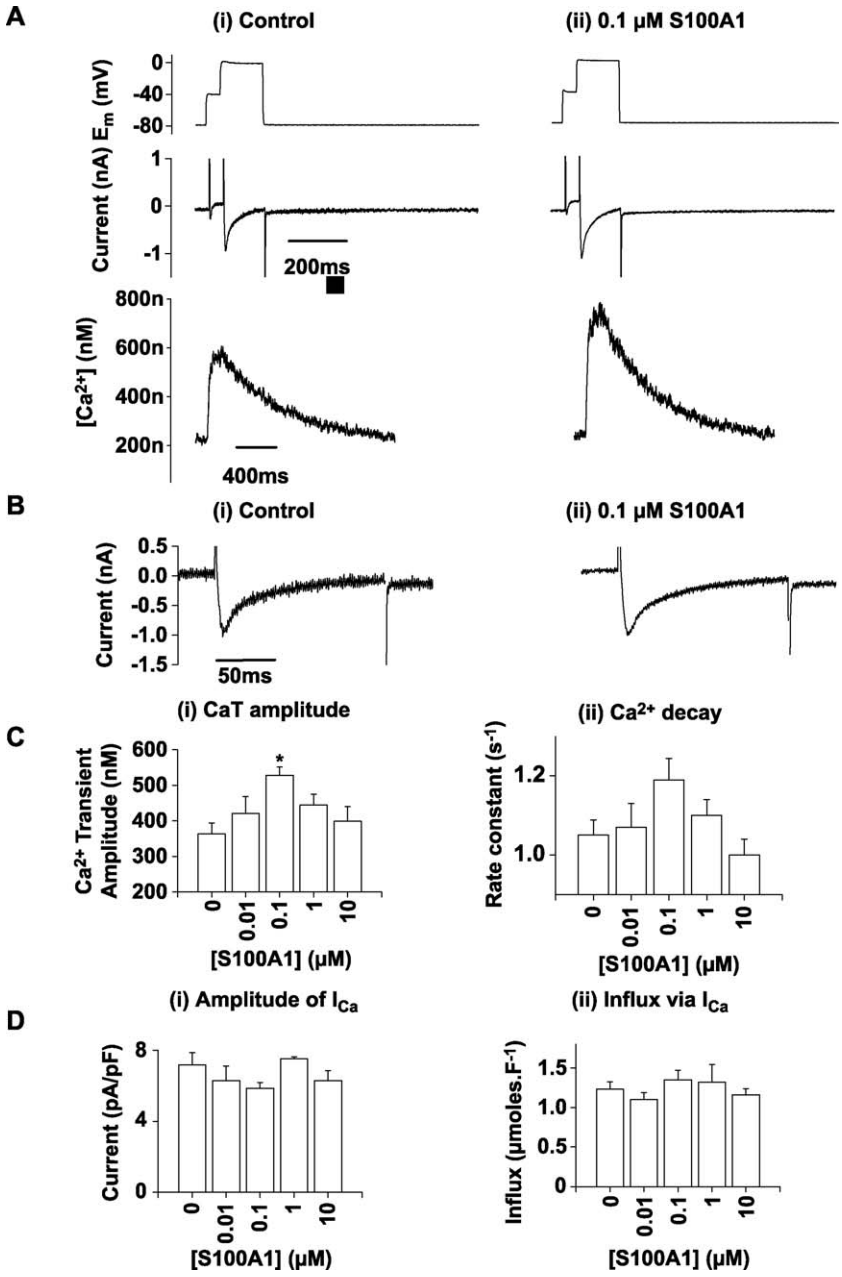

Fig. 1. Depolarisation induced Ca^{2+} transients recorded from control and S100A1 cardiomyocytes. Panel A shows records of membrane voltage (E_m), membrane current (I_m) and intracellular [Ca^{2+}] from single cardiomyocytes (average of four signals) under control conditions (i) and with the inclusion of 0.1 μM S100A1 in the patch pipette. Panel B, shows membrane current recorded on depolarisation from the pre-pulse potential of – 40–0 mV for 150 ms to illustrate the amplitude and time course of the L-type Ca^{2+} current. Panel C shows mean ± S.E.M. (n = 10) values of (i) Ca^{2+} transient amplitude and (ii) Rate constant of the decay of the Ca^{2+} transient. Panel D shows mean ± S.E.M. (n = 10) values of: (i) $I_{Ca,L}$ amplitude and (ii) $I_{Ca,L}$ integral.* Indicates significant difference from control ($P < 0.05$).

(in mM): 120 KCl, 1 MgCl, 20 NaCl, 3 mercaptoethanol, 25 HEPES, pH 7.0, 1% Tween 20 and protease inhibitor mixture 1836170 (complete Mini EDTA free, Roche Diagnostics GmbH, Germany). Samples were run on SDS-PAGE and probed with antibodies for the series of target proteins (see below) and actin. For the co-immuno-precipitation studies, separate suspensions were incubated with bovine serum albumin-treated A/G-sepharose for 30 min and centrifuged to remove proteins bound nonspecifically to A/G-sepharose. The supernatants were then mixed with A/G-sepharose and precipitating antibodies either for LTCC α_1-subunit (Anti-$Ca_v1.2$, alomone labs, ACC-003; 1:100), ryanodine receptor (RyR) (Anti-RyR2, ABR MA3-925; 1:100), sarcoplasmic reticulum Ca^{2+}-ATPase (Anti-SERCA2a, ABR MA3-910, 1:100) and NCX (NCX, ABR MA3-926; 1:100) were added. The samples were incubated for 30 min and centrifuged (800 × g), and pellets were washed three times either with EGTA or Ca^{2+} containing buffer. Samples were resolved by 4–20% SDS-PAGE, transferred to a PVDF membrane and both stained for S100A1 (SA 5632) and precipitated proteins.

2.6. [^{3}H]-ryanodine binding

Single cell rabbit cardiomyocytes were enzymatically dissociated as previously described. The cells were washed in a solution containing (mM): 100 KCl, 25 HEPES , 1 EGTA, 10 creatine phosphate, 5.5 $MgCl_2$ and 5 ATP (pH 7.0 with KOH). Cells (minus S100A1, plus S100A1 at 0.1, 1.0 and 10 μM) were incubated in a binding buffer containing (mM): 500 KCl, 50 HEPES, 10 EGTA, 10 creatine phosphate, 5 ATP, 5 caffeine, protease inhibitors (Complete EDTA free, Roche Diagnostics), 5×10^{-6} [^{3}H] ryanodine (pH 7.0 with KOH) for 90 min at 37 °C. Each cell group was incubated in solution containing nanomolar to micromolar Ca^{2+}. At the end of the incubation period the samples were filtered on Whatman GF/B filters and washed with ice cold solution containing (mM): 240 KCl, 25 tris base (pH 8 with KOH). The amount of bound [^{3}H] ryanodine was determined by liquid scintillation counting. Unspecific binding was determined by incubation of cells with 50 μM cold ryanodine. Samples were prepared in duplicate and an average taken.

2.7. Statistical analysis

Data were expressed ± S.E.M. Comparisons were performed using unpaired Students' *t*-tests. Data from several concentrations of S100A1 were compared using one-way ANOVA followed by Tukey–Kramer multiple comparisons post test. Differences were considered significant where $P < 0.05$.

3. Results

3.1. S100A1 increases Ca^{2+} transient amplitude in voltage clamped rabbit ventricular cardiomyocytes

As illustrated in Fig. 1A and C(i), measurements of intracellular [Ca^{2+}] in voltage clamped cardiomyocytes showed that S100A1 protein added to the pipette solution significantly increased Ca^{2+} transient amplitude only at 0.1 μM (363 ± 40.7 nM vs. 518 ± 22.9 nM, control vs. S100A1, $P < 0.05$). At lower (0.01 μM) and higher (up to 10 μM) concentrations no significant change in transient was observed. No change in resting (end-diastolic) [Ca^{2+}] was observed throughout the range of S100A1 concentrations use (data not shown). The rate constant for the rate of decay of the Ca^{2+} transient (Fig. 1C (ii)) showed a trend to higher values at 0.1 μM S100A1, but none of the values reached statistical significance.

3.2. S100A1 does not significantly affect L-type Ca^{2+} current ($I_{Ca,L}$)

$I_{Ca,L}$ amplitude was not significantly altered with S100A1 added to the pipette (Fig. 1B(ii) and D(i)). $I_{Ca,L}$ was converted to a Ca^{2+} influx by integration of the current with respect to time. As with the amplitude, the integral of the current was not altered by S100A1 (Fig. 1D(ii)) suggesting no significant change in the time course of $I_{Ca,L}$.

3.3. S100A1 does not significantly augment cardiac SR Ca^{2+} content

The cause of the increased Ca^{2+} transient amplitude in S100A1-treated cardiomyocytes was further investigated by examining the characteristics of the caffeine-induced Ca^{2+} response. Ten milli molars of caffeine was rapidly applied within 2 s of the end of the train of 40Vage-clamp pulses (to ensure a steady state SR Ca^{2+} load). At a holding potential of – 80 mV, application of caffeine caused a rapid increase of intracellular [Ca^{2+}] as a result of Ca^{2+} release from the SR and a concomitant transient inward current carried by I_{NCX} (Fig. 2A). In the presence of S100A1, the peak of the caffeine-induced Ca^{2+} release and the peak transient inward current was not significantly different (Fig. 2B). The time-integral of the NCX-mediated inward current (i.e. the SR Ca^{2+} content) showed no significant difference across the range of S100A1 concentrations studied. These results suggest that S100A1 does not cause a significant change in SR Ca^{2+} content under these stimulus conditions.

3.4. S100A1 does not affect sarcolemmal Ca^{2+} extrusion

As shown in Fig. 2C, the average rate constants for the decay phase of [Ca^{2+}] and I_{NCX} were not altered, indicating the sarcolemmal Ca^{2+} extrusion (dominated by NCX activity) was not significantly altered by S100A1.

3.5. S100A1 does not affect both normal and reverse-mode NCX activity

These latter measurements apply to NCX in the forward-mode (Ca^{2+} extrusion). But at depolarised potentials, NCX reverses and acts as a means of Ca^{2+} entry (reverse-mode). I_{NCX} was measured from – 120 to + 80 mV using a voltage-ramp protocol as shown in Fig. 3A. Fig. 3B illustrates the NCX I–V curve for control myocytes and those dialysed 0.1 μM S100A1. As shown in Fig. 3, there is no significant difference in the magnitude of I_{NCX} both in the normal mode (negative to the reversal potential) and in the reverse-mode (positive to the reversal potential), similar results were observed for a range of S100A1 concentrations (0.01, 1 and 10 μM, results not shown).

3.6. S100A1 effects SERCA-mediated Ca^{2+} extrusion

The decay of the Ca^{2+} transient elicited by the voltage clamp pulse (Fig. 1) is a result of Ca^{2+} uptake via SERCA and Ca^{2+} extrusion across the sarcolemma. Although SERCA is the dominant mechanism, sarcolemmal extrusion is significant, particularly at room temperature [4,22]. The rate of Ca^{2+} uptake by SERCA can be estimated from the Ca^{2+} transient by subtracting the rate constant associated with sarcolemmal

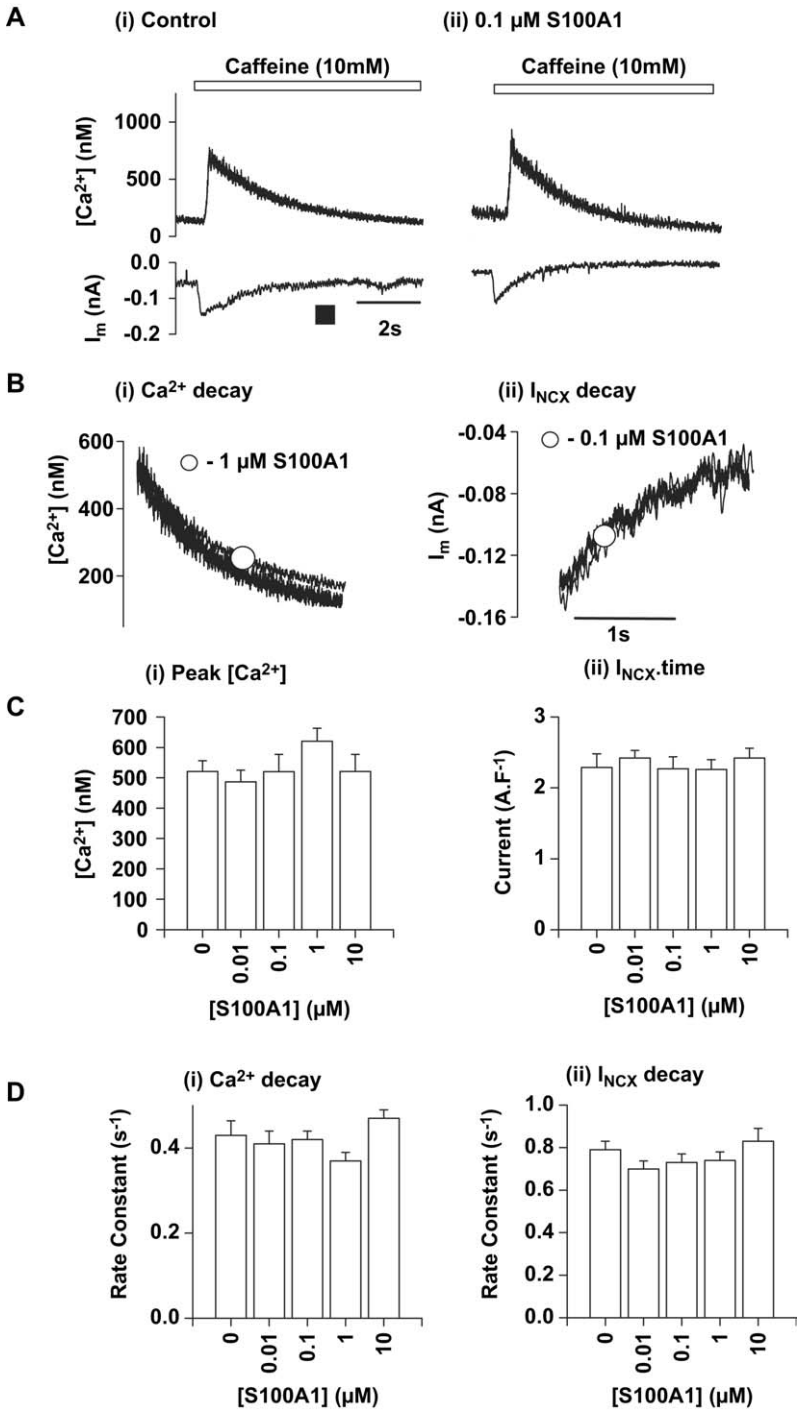

Fig. 2. Characteristics of caffeine-induced SR Ca^{2+} release and the corresponding membrane currents on rapid application of 10 mmol l^{-1} caffeine. Panel A: records of intracellular [Ca^{2+}] and membrane current recorded on application of 10 mM caffeine in control and S100A1-treated cardiomyocytes. Panel B (i), superimposed declining phase of intracellular [Ca^{2+}] signal from control and S100A1-treated cardiomyocytes (taken from panel A). Panel B (ii) superimposed records of the decline phase of membrane currents in response to rapid application of 10 mM caffeine in control and S100A1 (marked) cells (taken from panel A). Panel C, mean ± S.E.M. values: (i) Peak [Ca^{2+}] for control (n = 11) and S100A1: 0.01 μM (n = 12); 0.1 μM (n = 13); 1 μM (n = 8); 10 μM (n = 8) (ii) Peak I_{NCX} for control (n = 12) and S100A1: 0.01 μM (n = 13); 0.1 μM (n = 16); 1 μM (n = 9) 10 μM (n = 5); (iii) I_{NCX}. time integral for control (n = 10) and S100A1: 0.01 μM (n = 13); 0.1 μM (n = 10); 1 μM (n = 6); 10 μM (n = 6). Panel D: mean ± S.E.M. values: (i) rate constant for the decay of the caffeine-induced Ca^{2+} transient for control (n = 10) and S100A1: 0.01 μM (n = 16); 0.1 μM (n = 12); 1 μM (n = 7); 10 μM (n = 6). (ii) Rate constant for the decay of I_{NCX} for control (n = 7) and S100A1: 0.01 μM (n = 12); 0.1 μM (n = 10); 1 μM (n = 7); 10 μM (n = 4).

extrusion (measured in caffeine) from the rate constant observed during the transient [3,4]. This can be done on a cell-to-cell basis; the results of these calculations are shown in Table 1. A significantly higher SERCA-mediated Ca^{2+} uptake rate was observed at 0.1 μM S100A1, at 0.01 μM and 1 μM S100A1, the rate of Ca^{2+} uptake was not different from

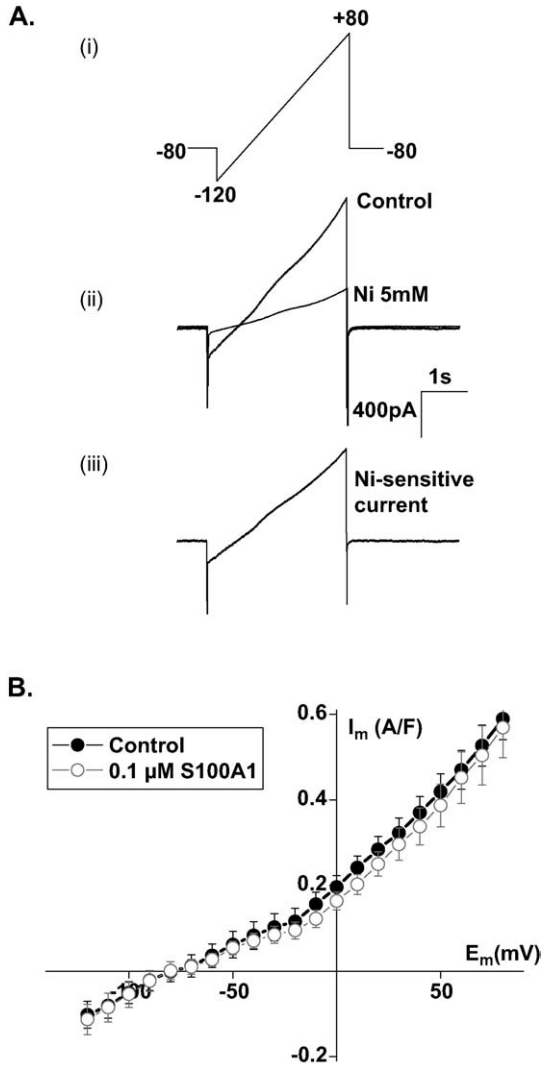

Fig. 3. I_{NCX} current–voltage relationship. Panel A: The voltage-ramp signal and accompanying membrane currents before and after addition of 5 mM Ni^{2+}. The difference current is shown below the individual currents. This Ni^{2+} sensitive current is taken as I_{NCX}. Panel B: Representative I_{NCX} traces. Panel C: Averaged *I–V* relationship: Control: $n = 12$, 0.1 μM S100A1 $n = 11$.

Table 1
Mean values of rate constants for the decay of transients

	Rate constant (s^{-1})		
	(a) CaffCaT	(b) CaT	SERCA (b–a)
Control	0.42 ± 0.035	1.06 ± 0.073	0.63 ± 0.014
0.01 μM S100A1	0.41 ± 0.031	1.07 ± 0.061	0.68 ± 0.035
0.1 μM S100A1	0.42 ± 0.020	1.19 ± 0.056	0.77 ± 0.038*
1 μM S100A1	0.37 ± 0.020	1.10 ± 0.039	0.72 ± 0.050

(a) Caffeine-induced Ca^{2+} release (CaffCaT); (b) Depolarisation induced Ca^{2+} transient (CaT). The SERCA values were calculated by subtracting CaffCaT rate constant from CaT rate constant measured from individual cardiomyocytes. * Indicates, significant difference from control ($P < 0.05$).

control. These results suggest that part of the action of S100A1 (at 0.1 μM) is stimulation of SERCA-mediated Ca^{2+} uptake.

3.7. S100A1 increases cardiac E–C coupling gain

To determine the relationship between SR Ca^{2+} content and Ca^{2+} transient amplitude, measurements were made at a range of SR loads. As shown in Fig. 4, the plot of I_{NCX} integral (SR Ca^{2+} content) and Ca^{2+} transient amplitude for the control group generated an approximately hyperbolic relationship. Analysis of $I_{Ca,L}$ indicated that there were no significant changes in the amplitude or time course of this current in any of the data-sets. Therefore, the hyperbolic relationship described by the control data represent the relationship between SR Ca^{2+} content and the ability of $I_{Ca,L}$ to trigger Ca^{2+} release from the SR; i.e. E–C coupling 'gain' [1,17]. The data from the measurements made with 0.1 μM S100A1 in the pipette solution describe a relationship that lies to the left of the control group. S100A1 appears to increase Ca^{2+} transient amplitude by increasing the fractional release of Ca^{2+} from the SR.

For comparison, agents known to modulate Ca^{2+} induced Ca^{2+} release (CICR) from the SR were studied. Caffeine (0.5 mM) [18] and tetracaine (100 μM) [20] did not change the steady state Ca^{2+} transient amplitude, but significantly altered the SR Ca^{2+} content. This result contrasts with the effects of S100A1, here increased fractional SR Ca^{2+} release is manifest as an increase in the Ca^{2+} transient with no detectable changes in SR Ca^{2+} content. The reason for this discrepancy is discussed later.

3.8. S100A1 increases [^{3}H]-ryanodine binding of RyR2

To investigate the effect of S100A1 on RyR2 activity [^{3}H] ryanodine (^{3}H-Ry) was measured at range free Ca^{2+} concentrations. As shown in Fig. 5A, increasing [Ca^{2+}] caused a progressive increase in ^{3}H-Ry binding up to a saturating value observed at a free Ca^{2+} of ~ 20 μM. When the assay was repeated in the presence of S100A1, ^{3}H-Ry binding was increased. A shift of the sigmoidal relationship to the left can be observed in Fig. 5A, but a clearer impression of the effects of S100A1 is gained by examining the results at each [Ca^{2+}]. As shown in Fig. 5B, 0.1 μM S100A1 significantly increased ^{3}H-Ry binding at 0.1 μM and 0.3 μM Ca^{2+}. At higher concentrations, the effect was less pronounced. At 10 μM, ^{3}H-Ry binding at 0.1 μM Ca^{2+} was significantly less than control. A similar dome-shaped relationship was observed at 0.3 μM Ca^{2+}. At 20 μM Ca^{2+}, the effect of S100A1 was complex, with 10 μM significantly increasing maximal ^{3}H-Ry binding. This data suggest that 0.1 μM S100A1 increases Ca^{2+}-dependent RyR2 activity. The effects of high concentrations of S100A1 (10 μM) is complex with both inhibition and stimulation evident different [Ca^{2+}].

3.9. S100A1 interacts with SR but not sarcolemmal Ca^{2+}-effector proteins

To further substantiate the differential effect of S100A1 protein on Ca^{2+}-regulatory proteins in the heart, the interaction of the S100A1 with major SR and sarcolemmal Ca^{2+}-channels and transporters was investigated by co-immunoprecipitation. As shown in Fig. 6A, neither exposure to EGTA nor to Ca^{2+} alters the Western blot signal of any of

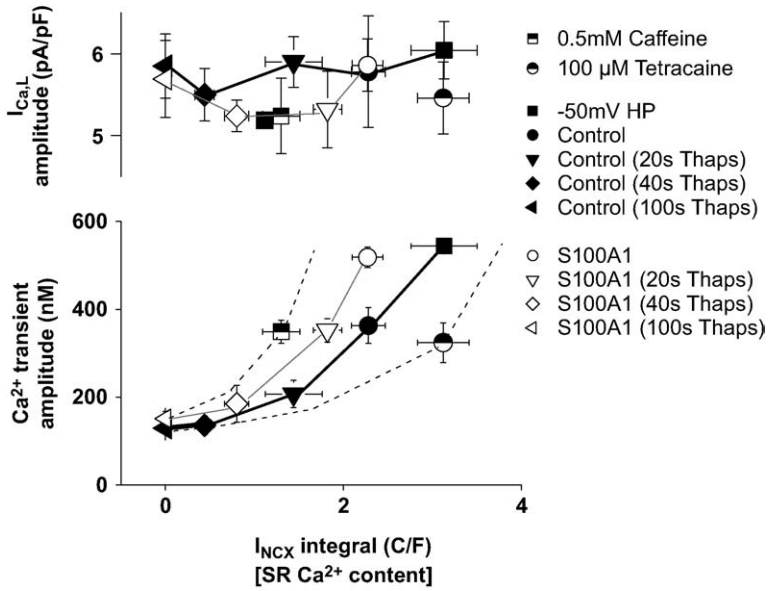

Fig. 4. SR gain curves for control and S100A1 cell groups. Relationship between I_{NCX} integral (an index of SR Ca^{2+} content) and Ca^{2+} transient amplitude and $I_{Ca,L}$ amplitude for cardiomyocytes from the control group (Control!, $n = 10$; 5 μmol l^{-1} thapsigargin 20 s exposure, $n = 8$; 40 s exposure $n = 8$; 100 s exposure $n = 8$; holding potential of –50 mV, $n = 10$ and S100A1 (0.1 μM) $n = 10$; 5 μmol l^{-1} thapsigargin 20 s exposure, $n = 8$; 40 s exposure $n = 7$; 100 s exposure $n = 8$.

the putative target proteins. Only the native S100A1 signal and the signal after incubation with 1 μM rh-S100A1 protein show a Ca^{2+}-dependent enhancement of the signal.

Co-immunoprecipitation preparations (Fig. 6B) revealed Ca^{2+}-dependent interactions of S100A1 protein with the RyR2 and SERCA2a. Neither LTCC nor NCX showed significant co-immunoprecipitation signals with S100A1 protein.

4. Discussion

Transgenic- and adenoviral-mediated over-expression of the Ca^{2+}-binding protein S100A1 in murine hearts and isolated ventricular rat and rabbit cardiomyocytes, respectively, has been shown to enhance myocardial contractile performance in vivo and in vitro [14,15,24,25]. Although these studies provided clear evidence that the S100A1 mediated positive inotropic effect relied on enhanced intracellular Ca^{2+} cycling, the underlying subcellular mechanisms were unresolved. Thus, this study subsequently analysed the effects of exogenous S100A1 protein on the major trans-sarcolemmal and trans-SR Ca^{2+} fluxes involved in cardiac E–C coupling. The current study shows that 0.1 μM S100A1 increased the amplitude of the Ca^{2+} transient mediated through enhanced E–C coupling gain in isolated rabbit cardiomyocytes under voltage clamp conditions. These effects are in addition to those of endogenous S100A1 protein and are designed to provide an insight into the subcellular physiological actions of the protein.

4.1. S100A1 does not affect Ca^{2+} fluxes via $I_{Ca,L}$ and NCX

Ca^{2+} influx via the $I_{Ca,L}$ and Ca^{2+} efflux via NCX were unaffected by S100A1 (0.01–10 μM). Direct examination of current–voltage relationship of NCX failed to show affects of S100A1 in either normal or reverse-modes (Fig. 3). The functional data were supported by biochemical data failing to show any interaction between S100A1 and either the α1-subunit of the LTCC or NCX (Fig. 6). Thus the effects of S100A1 on Ca^{2+} cycling in rabbit cardiomyocytes appear to be mediated entirely by effects on SR. It was surprising that the larger amplitude of the Ca^{2+} transient were not accompanied by enhanced rate of inactivation of the $I_{Ca,L}$. It would appear that under the conditions of the study, the effect cannot be distinguished from the inter-cell variation of $I_{Ca,L}$ inactivation.

4.2. S100A1 increases Ca^{2+} transient amplitude and SERCA activity

Under the conditions of this study, only 0.1 μM S100A1 protein significantly enhanced the Ca^{2+} transient amplitude in ventricular cardiomyocytes, whereas the effect at 0.01 and 1 μM S100A1 was smaller and not statistically significant. In line with these results, maximal effects of S100A1 protein on Ca^{2+} transients in skeletal muscle fibers where also found at 0.1 μM [16]. However, in contrast to the narrow range of effective S100A1 concentrations in our experimental setting, S100A1 significantly increased Ca^{2+} transients in permeabilised skeletal muscle fibers throughout a range of 0.01–10 μM in a bell-shaped manner [16]. Given the fact that chemical permeabilisation of skeletal muscle fibers resulted in depletion of endogenous S100A1 protein [16], it seems conceivable that in our experimental setting endogenous S100A1 protein might interfere with exogenous S100A1 protein at different cytosolic target sites in cardiomyocytes thereby altering the effective threshold of exogenous S100A1 protein. However, we cannot exclude that at

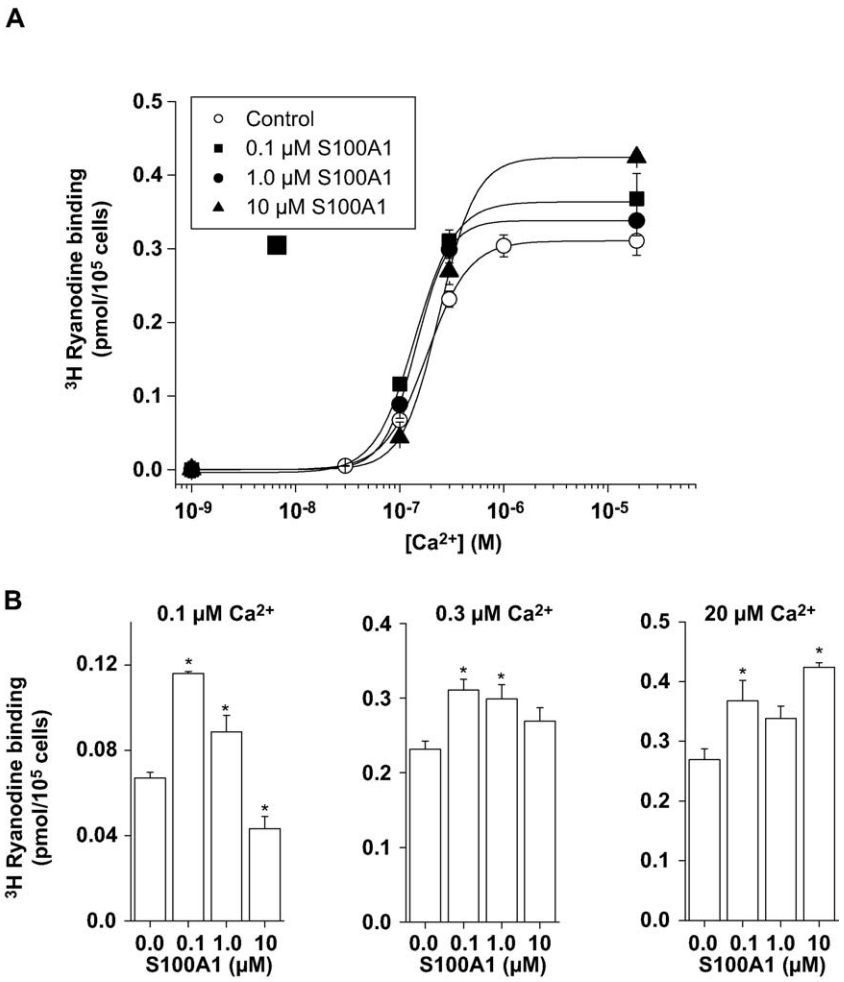

Fig. 5. Effects of S100A1 on [^{3}H]-ryanodine binding. Panel A: mean values of [^{3}H]-Ryanodine (pmol per 10^5 cells). Measurements made at four different [Ca^{2+}] in the presence of 5 mM ATP and 5 mM caffeine. Solid lines are best-fit logistic curves to the data points. Measurements made in the absence and presence of S100A1 (0.1, 1 and 10 μM). Panel B, the same data plotted at each [Ca^{2+}]. * Indicates significant difference from control ($P < 0.05$).

least supra-normal levels of S100A1 protein used in this study may not mimic the physiological action of this protein.

Nevertheless, effects mediated through 0.1 μM S100A1 are in line with numerous recent studies showing that increasing cardiac intracellular S100A1 protein levels resulted in significantly enhanced cytosolic Ca^{2+} turnover. The rate constant for Ca^{2+} uptake associated with SERCA activity was significantly higher at this level of S100A1 (Table 1). This suggests that enhanced SERCA activity was responsible for the enhanced Ca^{2+} transient. This effect was not reflected in a significant increase of the overall rate constant of the Ca^{2+} transient although there was a trend to higher values at 0.1 μM S100A1. Inter-cell variation within the experimental group obscured the enhanced rate constant between experimental groups. Previous work has shown that S100A1 directly stimulates SERCA2a activity and SERCA2a-mediated Ca^{2+} uptake into the oxalate equilibrated cardiac SR [14]. The mechanism of this stimulation is unknown but the effect remains after β-adrenergic stimulation [14]. A direct interaction between S100A1 and the SR Ca^{2+}-pump is suggested by recent findings showing both co-immunoprecipitation and co-immunolocalisation of S100A1 protein with human, murine and rat SERCA2a protein, respectively [10]. A similar co-immunoprecipitation of S100A1 with rabbit SERCA2a was observed in the current study (Fig. 6). Based on the 'gain' curve shown in Fig. 4, an increase of Ca^{2+} transient amplitude to ~ 130% (0.1 μM S100A1) would be associated with an increase in the SR Ca^{2+} content to ~ 150%. Therefore, the absence of a significant enhancement of SR Ca^{2+} content with S100A1 (0.1 μM) was initially puzzling. This would suggest that other aspects of E–C coupling are altered in parallel to generate the altered 'gain' status observed in S100A1.

4.3. S100A1 increases RyR2 activity and increases fractional SR Ca^{2+} release

[^{3}H]-ryanodine binding to RyR2 was enhanced by S100A1 protein (0.1 μM) suggesting that S100A1 enhances the open probability of the cardiac SR Ca^{2+} release channel. The evidence of a direct interaction between S100A1 and RyR2 was further supported by the Ca^{2+} sensitive co-immunoprecipitation studies shown in Fig. 6, which has previously shown for rat and murine RyR2 [27–29]. A similar effect of S100A1 has been reported for skeletal muscle ryano-

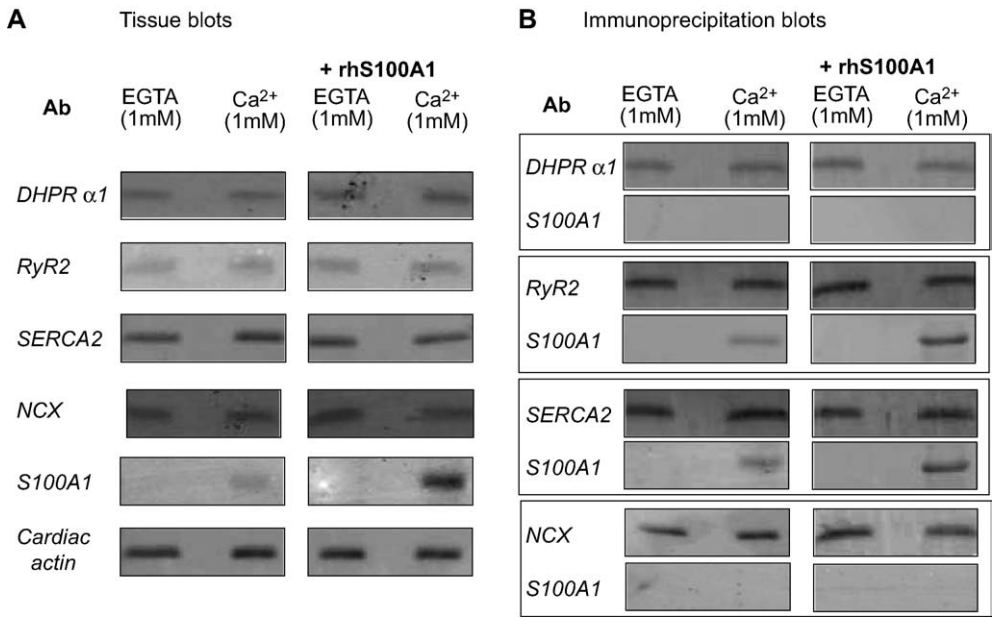

Fig. 6. Ca^{2+}-dependent interaction of S100A1 protein with specific cardiac proteins. Panel A, representative Western blots for, L-type Ca^{2+}-channel (DHPRα1), RyR2, SERCA2a, NCX, S100A1 and actin prepared from β-escin permeabilised rabbit ventricular cardiomyocytes after treatment with EGTA (low Ca^{2+}) and high Ca^{2+} (1 mM). Samples were incubated under control conditions and in the presence of 1 μM rhS100A1(A(ii)). Panel B shows representative blots after immunoprecipitation procedure. Each gel is probed with protein specific antibody and with S100A1 antibody. Both native and S100A1 protein interacts in a Ca^{2+}-dependent manner with RyR2 and SERCA2a. Note that the amount of co-precipitated S100A1 protein is increased in the rhS100A1-treated group indicating that added S100A1 protein can increase the amount of Ca^{2+}-dependent bound protein to RyR2 and SERCA2a. In contrast, neither DHPRα1 nor NCX co-immunoprecipitates with native or S100A1 protein, respectively, under. Control experiments with A/G-sepharose alone neither yielded precipitation of Ca^{2+}-regulatory proteins nor S100A1 (data not shown). This data are representative of three independent cell preparations.

dine receptor (RyR1) [16,26]. Several small modulatory proteins including S100A1 are known to interact with and modulate RyR2 activity [11,13]. The mechanism of action and binding site of S100A1 on RyR2 is not known and requires further study. The 'gain curve' plot (SR Ca^{2+} content vs. Ca^{2+} transient amplitude) shown in Fig. 4 suggests that S100A1 (0.1 μM) significantly increases the fractional release of Ca^{2+} from the SR. This effect is consistent with the enhanced Ca^{2+}sensitive ^{3}H-Ry binding observed in Fig. 5. However, as illustrated in Fig. 4, agents, known to enhance RyR2 activity, and therefore, CICR (caffeine) do not affect the Ca^{2+} transient amplitude in the steady state, instead SR Ca^{2+} content is reduced [18]. Additional effects of S100A1 on Ca^{2+} uptake into the SR is thought to be the basis for the discrepancy with agents that purely modulate CICR.

4.4. The sum of effects on SERCA2a and RyR2 can explain the effects on E–C coupling

As illustrated in Fig. 7, the combination of SERCA-mediated and RyR2-mediated effects provides an explanation for effects of S100A1. Increased SERCA activity alone would normally result in an increase in both Ca^{2+} transient amplitude and SR Ca^{2+} content together (Fig. 7A). Increased RyR2 sensitivity would normally preserve Ca^{2+} transient amplitude and decrease SR Ca^{2+} content (Fig. 7B). As illustrated, the combination of these two effects can result in an increase in Ca^{2+} transient amplitude with little change in SR Ca^{2+} content (Fig. 7C).

There is currently no explanation for the biphasic dose–response effects of S100A1. Examination of the effects of S100A1 on RyR activity indicates a complex dose-dependence (Fig. 5). This data were obtained under conditions designed to maximise RyR open probability, and therefore, it is difficult to extrapolate to the intracellular conditions of a beating cardiomyocyte. The effects on SERCA-mediated Ca^{2+} uptake based appeared maximal at 0.1 μM with a minimal fall-off at higher S100A1 concentrations (Table 1). Therefore, on the basis of this study it would appear that the action of S100A1 on RyR2 is the dominant cause of the complex dose–response relationship. Further work using S100A1 levels above and below normal values is required understand the physiological role of this protein in more detail.

In summary, this is the first study to examine the subcellular mechanisms of S100A1 on intracellular Ca^{2+} fluxes in cardiac muscle. The data indicate that the effect of S100A1 on the Ca^{2+} transient is a balance of separate effects on SR Ca^{2+} release and uptake pathways apparently independent of trans-sarcolemmal Ca^{2+} fluxes. An interesting consequence of the two former actions is increased Ca^{2+} transient amplitude while minimising the increase in SR Ca^{2+} content. This interesting combination of effects resulting in enhanced E–C coupling gain could be useful as model for designing novel S100A1 based inotropic therapeutic strategies for the heart.

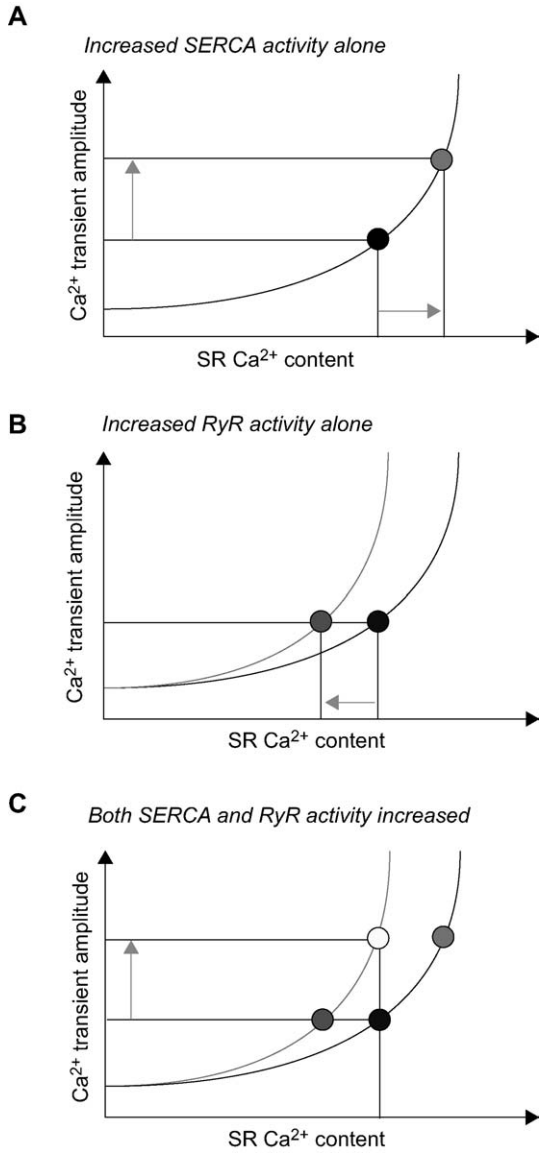

Fig. 7. Model of S100A1 effects on cardiac Ca^{2+}-handling. Schematic of the relationship between SR Ca^{2+} content (intergral of I_{NCX}) vs. Ca^{2+} transient amplitude. Panel A illustrates that manoeuvres that increase the Ca^{2+} content of the SR (e.g. stimulation of SERCA2A) will increase Ca^{2+} transient amplitude from control (filled circle) to a new value (gray circle). The two data points are on a common underlying hyperbolic relationship representing the E–C coupling 'gain' relationship. Panel B illustrates the effects of manoeuvres that increase the Ca^{2+} sensitivity of the CICR mechanism. The shift of the gain curve to the left results in a maintained Ca^{2+} transient amplitude (control = filled circle to grey circle = increased gain). This illustrates identical Ca^{2+} transient amplitude but decreased SR Ca^{2+} content. Panel C illustrates a result of combined SERCA2a stimulation and increased sensitivity of CICR. With effects of appropriate magnitude it is possible to increase Ca^{2+} transient amplitude from control (open circle) to a new value (black circle) with no significant effects on SR Ca^{2+} content. Gray points indicate the individual effects, on RyR2 and SERCA2A activity.

However, the effects of S100A1 protein are almost certainly not limited to the SR since S100A1 also co-localises both with mitochondria and contractile filaments in the heart [8,9]. Additional studies are underway to address the impact of S100A1 on the functional interplay between the SR and these compartments.

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