S100A1 Enhances the L-type Ca\textsuperscript{2+} Current in Embryonic Mouse and Neonatal Rat Ventricular Cardiomyocytes*

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S100A1 is an EF-hand type Ca\textsuperscript{2+}-binding protein with a muscle-specific expression pattern. The highest S100A1 protein levels are found in cardiomyocytes, and it is expressed already at day 8 in the heart during embryonic development. Since S100A1 is known to be involved in the regulation of Ca\textsuperscript{2+} homeostasis, we tested whether extracellular S100A1 plays a role in regulating the L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca}) in ventricular cardiomyocytes. Murine embryonic (day 16.5 postcoitum) ventricular cardiomyocytes were incubated with S100A1 (0.001–10 \textmu M) for different time periods (20 min to 48 h). I\textsubscript{Ca} density was found to be significantly increased as early as 20 min (from \(-10.8 \pm 1 \text{ pA/pF}, n = 18\), to \(-22.9 \pm 1.4 \text{ pA/pF}; +112.5 \pm 13\%\), \(n = 9\), \(p < 0.001\)) after the addition of S100A1 (1 \textmu M). S100A1 also enhanced I\textsubscript{Ca} current density in neonatal rat cardiomyocytes. Fluorescence and capacitance measurements evidenced a fast translocation of rhodamine-coupled S100A1 from the extracellular space into cardiomyocytes. S100A1 treatment did not affect cAMP levels. However, protein kinase inhibitor, a blocker of cAMP-dependent protein kinase A (PKA), abolished the S100A1-induced enhancement of I\textsubscript{Ca}. Accordingly, measurements of PKA activity yielded a significant increase in S100A1-treated cardiomyocytes. In vitro reconstitution assays further demonstrated that S100A1 enhances PKA activity. We conclude that the Ca\textsuperscript{2+}-binding protein S100A1 augments transsarcolemmal Ca\textsuperscript{2+} influx via an increase of PKA activity in ventricular cardiomyocytes and hence represents an important regulator of cardiac function.

In 1965, Moore (1) described a novel protein “soluble in 100% saturated ammonium sulfate solution” and therefore denoted it S100 protein. Today 21 different S100 proteins belonging to a multigene family of Ca\textsuperscript{2+}-binding proteins of the EF-hand type are known to be differentially expressed in a large number of cell types (2). S100A1 is found in the cytosol, displays highest expression levels in the heart (3), and is already expressed at day 8 postcoitum in embryonic mouse hearts (4). Although the physiological relevance of S100A1 proteins in healthy tissue is still debated, the evidence for a role of S100A1 as a regulator of cardiac function based on the observation that overexpression of S100A1 has led to a significant increase of contractility in adult cardiomyocytes (7). This was found to be related to an increased sarcoplasmatic reticulum Ca\textsuperscript{2+}-ATPase (8) and ryanodine receptor 2 activity (9) as well as to a decrease in myofilamental Ca\textsuperscript{2+} sensitivity (8) and might be explained by enhancement of PKA activity. The physiological relevance of S100A1 is further supported by data of Du and co-workers (10), who identified S100A1 as a regulator of cardiac reserve in a S100A1 mouse knock-out model. Accordingly, transgenic mice overexpressing S100A1 display enhanced myocardial contractility without developing hypertrophy (9).

New evidence suggests a role for S100A1 as a serum marker for myocardial ischemia (11). During heart attacks, S100A1 levels displayed a bell-shaped concentration time course with a fast rise followed by a rapid decline in serum. Being present in the serum compartment, S100A1 may have paracellular effects similar to those of other members of the S100 family (12). In fact, S100A1 translocation into the extracellular space was detected in the intact human heart after reperfusion following prolonged ischemia (13). Since S100 proteins are secreted and/or rapidly taken up by various cell types (12), we have examined the cellular effects of exogeneously applied S100A1 in murine embryonic and neonatal rat cardiomyocytes.

As S100A1 is assumed to be a crucial regulator of myocardial Ca\textsuperscript{2+} homeostasis, we were particularly interested whether S100A1 has any effect on I\textsubscript{Ca}, the key trigger mechanism for sarcoplasmatic Ca\textsuperscript{2+} release and the initiation of contraction. We have employed embryonic murine and neonatal rat cardiomyocytes, since adult cardiomyocytes rapidly dedifferentiate in culture (14). Both murine embryonic and neonatal rat cardiomyocytes are easy to isolate and can be cultivated for several days without changing functional characteristics (15). In particular, neonatal rat cardiomyocytes are known to recapitulate the regulatory components of adult cardiomyocytes (16, 17).

MATERIALS AND METHODS

Cell Preparation—Murine embryonic cardiomyocytes were obtained from superovulated mice of the HUM:OF1 strain as described (18, 19). Spontaneously beating neonatal Sprague-Dawley rat cardiomyocytes were isolated and purified as reported earlier (20). The purity of the preparation was increased by using the selective adhesion technique (21). All experiments were carried out according to the guidelines provided by the University of Cologne animal welfare committee.
**S100A1-mediated Modulation of I\(_{\text{Ca}}\)**

*Single Cell Ca\(^{2+}\) Measurements—Ca\(^{2+}\) measurements were performed as previously described (7). In brief, freshly isolated neonatal rat cardiomyocytes were loaded with the Ca\(^{2+}\) indicator Fura 2-AM (5 \mu M) for 30 min. Cytosolic Ca\(^{2+}\) was determined employing a monochromator on the excitation and a CCD camera-based system (TILLPhotonics®, Planegg, Germany) on the emission side attached to an Olympus inverted microscope. Cells were paced at 1 Hz using a commercial stimulator (Phywe Systeme GmbH, Göttingen, Germany).

**Electrophysiology—**\(I_{\text{Ca}}\) was measured with the whole-cell patch clamp technique as reported in detail earlier (22, 23). Briefly, for voltage-clamp recordings, the solutions contained the following: internal solution, 120 mM CsCl, 3 mM MgCl\(_2\), 5 mM MgATP, 10 mM EGTA, 5 mM HEPES, pH 7.4 (CsOH); external solution, 120 mM NaCl, 5 mM KCl, 3.6 mM CaCl\(_2\), 20 mM tetraethylammonium-Cl, 1 mM MgCl\(_2\), 10 mM HEPES, pH 7.4 (TEAOH). Only cells with an increase of \(I_{\text{Ca}}\) density >5% after drug application were considered responders. As reported previously (22), \(I_{\text{Ca}}\) did not show significant run down (see also Fig. 6). As a control, heat-inactivated S100A1 (95 °C, 1 h) was used, and the effect on \(I_{\text{Ca}}\) was evaluated. Since no significant changes of \(I_{\text{Ca}}\) density in embryonic mouse cardiomyocytes were observed 20 min after incubation, we employed, if not otherwise stated, unstimulated cells as control. For the analysis of inactivation, biexponential fits using a simplex optimization algorithm were applied. All measurements were performed 48 h after dissociation.

**S100A1 Protein—**Recombinant human S100A1 protein was purified as described (24). Briefly, after EDTA extraction of recombinant bacteria, S100A1 was purified by octyl-Sepharose hydrophobic interaction and HiTrapQ anion exchange chromatography. The protein concentration was determined using a commercial Bradford protein assay (BioRad). Stock concentrations ranged between ~150 and 500 \mu M. Vehicle alone did not affect \(I_{\text{Ca}}\) (data not shown). Recombinant human S100A1 was labeled custom-based by Eurogentec® (Belgium) with rhodamine dye (rhod-S100A1). Unbound rhodamine dye used for control experiments was inactivated by 50 mM Tris to exclude nonspecific binding. As controls, cardiomyocytes were either not stimulated or exposed to heat-inactivated S100A1 protein for capacitance, confocal imaging, and PKA activity measurements in cell homogenates.

**Detection of S100A1 Translocation—**Cells exposed to rhod-S100A1 were excited (1 ± 0.05 Hz) at 550 nm, employing a monochromator. The emitted fluorescence was recorded with a CCD camera. Inactivated rhodamine served as control. After the extracellular addition of rhod-S100A1 (1 \mu M), intra- and extracellular fluorescence intensities were measured on-line. When fluorescence intensities reached plateau levels, excessive dye was washed out with Tyrode’s solution. Intracellular fluorescence intensities were estimated by subtracting background fluorescence and autofluorescence. Furthermore, intracellular accumulation of S100A1 was assessed using confocal laser-scanning microscopy (LSM 410; ×63 Plan Neofluar objective; Zeiss) employing the following configuration: wavelength 543 nm, beam splitter FT 488/543 and LP 570.

**Capacitance Measurements—**Endocytotic uptake of S100A1 was monitored by combining the whole-cell patch clamp technique with on-line capacitance measurements (HEKA-Pulse 8.4 software). Capacitance was determined every 5 s. To avoid interference by sudden changes of capacity, the cellular resistance (\(R_c\)) was monitored on-line (25).

**Cellular cAMP—**Intracellular cAMP concentrations were measured using a quantitative cAMP assay system with a nonacetylated EIA procedure as described elsewhere (26). Controls and S100A1-treated cell homogenates (1 \mu M, 20 min) were analyzed for cAMP concentrations.

To confirm the validity of the assay, cAMP levels were also determined in untreated and S100A1-treated cells after maximal stimulation by isoprenaline (ISO; 1 \mu M) (27) and isobutylmethylxanthine (IBMX; 200 \mu M) (28).

**PKA Activity—**For the determination of PKA activity in purified cell homogenates of S100A1-treated and control embryonic cardiomyocytes, a nonradioactive assay kit (Promega PepTag-Assay®) was used (29). Cell homogenates were prepared using a standard homogenization buffer containing a protease/phosphatase inhibitor mixture. Following incubation of homogenates and a synthetic PKA substrate (Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly)) in reaction buffer and electrophoretic separation of phosphorylated substrate, the intensities of the specific gel bands were quantified using densitometry. For controls unstimulated cell homogenates and cells stimulated with heat-inactivated S100A1 were used.

To gain mechanistic insight into the molecular function of S100A1, highly purified regulatory (R) and/or catalytic (C) subunits of PKA from different suppliers (Promega and Sigma) were incubated with or without cAMP or purified recombinant S100A1 in the presence of 2 \mu Ci of \(\gamma\)-\[^{32}\]P\]ATP (tube as described previously (30). The reaction mixtures comprised 40 mM Tris-HCl (pH 7.4), 10 mM MgCl\(_2\), 1 mM dithiothreitol and 3 \mu g of histone H1 as the substrate. The reaction was allowed to proceed for 30 min at 30 °C before proteins were separated by SDS-PAGE. Western blotted membranes were exposed to Fuji imaging plates, and autoradiographic signals were quantitated with a FLA-5000 Fuji-Imager® (Raytest, Straubenhardt, Germany).

**Statistical Analysis—**To evaluate statistical significance, we have performed analysis of variance with subsequent Tukey or Dunney tests. Where applicable, paired or unpaired Student’s \(t\) tests were used. Values below 0.05 were considered significant. Results are given as means ± S.E.

**RESULTS**

**S100A1 Enhances Peak \(I_{\text{Ca}}\) Density and Cytosolic Ca\(^{2+}\) Transients—**To investigate the effect of S100A1 on \(I_{\text{Ca}}\), purified S100A1 protein was added to isolated murine embryonic and neonatal rat cardiomyocytes 20 min to 48 h before \(I_{\text{Ca}}\) was measured using the whole-cell patch clamp technique. The addition of S100A1 resulted in pronounced enhancement of peak \(I_{\text{Ca}}\) density in embryonic mouse cardiomyocytes as compared with controls (Fig. 1, a and b). A significant stimulatory effect of S100A1 was already detected at concentrations of 0.1 \mu M and higher (Fig. 1c), whereas the most robust S100A1-induced enhancement of \(I_{\text{Ca}}\) was observed using 10 \mu M S100A1 (−15.1 ± 1.4 pA/pF, +34.9 ± 12.5%, 48 h, \(n = 20\)) compared with controls (-11.2 ± 0.5 pA/pF, \(n = 45\)). The overall stimulatory effect, however, did not significantly differ between concentrations ranging from 0.1 to 10 \mu M. The amplitude of peak \(I_{\text{Ca}}\) was significantly increased as early as 20 min after the addition of S100A1 (1 \mu M) to −22.9 ± 1.4 pA/pF (+112.5 ± 13%, \(n = 9\), \(p < 0.001\); Fig. 1d) as compared with controls (−10.8 ± 1 pA/pF, \(n = 18\)). At later time points, the stimulatory effect of S100A1 on \(I_{\text{Ca}}\) current density decreased but remained significantly enhanced (−18 ± 0.9 pA/pF, +66.9 ± 8%, 48 h, \(n = 12\), \(p < 0.001\)). Heat-inactivated S100A1 protein (1 \mu M) was without effect on \(I_{\text{Ca}}\) density (−8.1 ± 1.4 pA/pF, 20 min, \(n = 9\), \(p > 0.05\)) compared with controls (−9.1 ± 1.6 pA/pF, \(n = 6\)). Parallel to our findings in murine embryonic cardiomyocytes, S100A1 (1 \mu M, 20 min) also induced a pronounced enhancement of \(I_{\text{Ca}}\) density (−19.7 ± 2.3 pA/pF, +47 ± 16.9%,
n/H11005 8, p/H11021 0.02) in neonatal rat cardiomyocytes (Fig. 2, a–d); decreased but stable levels of the S100A1-mediated stimulatory effect on $I_{Ca}$ were found after 48 h ($18.4 \pm 1.5 \text{ pA/pF}, 37.8 \pm 11.1\%$, $n = 9, p < 0.04$) (Fig. 2c) as compared with controls ($-13.4 \pm 1.1 \text{ pA/pF}, n = 15$).

Similar to the effects of β-adrenergic stimulation on $I_{Ca}$ in terminally differentiated cardiomyocytes (31), S100A1 (1 μM, 20 min) caused a slight left shift of the threshold potential and current voltage ($I/V$) relationship of $I_{Ca}$ (Fig. 2, b and d) in cardiomyocytes derived from neonatal rats. In addition, we also observed an increase of the fast inactivation component of $I_{Ca}$ (Fig. 2e), probably due to an augmented influx of Ca$^{2+}$ and ensuing increase of Ca$^{2+}$-induced Ca$^{2+}$ release (31).

To determine the physiological relevance of the S100A1-mediated stimulation of $I_{Ca}$, intracellular Ca$^{2+}$ was measured in electrically stimulated neonatal rat cardiomyocytes. S100A1 (1 μM, 48 h) led to significantly augmented amplitudes of Ca$^{2+}$ transients with $195.7 \pm 7.2 \text{ nmol}$ ($n = 51$ cells, 204 Ca$^{2+}$ transients) versus $146.4 \pm 6.1 \text{ nmol}$ ($n = 29$ cells, 116 Ca$^{2+}$ transients) in controls. This is due to a significant increase of peak Ca$^{2+}$ concentrations (controls: $244 \pm 6 \text{ nmol}$ versus $278 \pm 7 \text{ nmol}$ for S100A1-treated cells, $p < 0.02$) and lowered resting Ca$^{2+}$ levels ($98 \pm 5 \text{ nmol}$ versus $82 \pm 4 \text{ nmol}$, $p < 0.02$). Thus, extracellular application of S100A1 causes a prominent enhancement of both peak $I_{Ca}$ density and cytosolic Ca$^{2+}$ transients in cardiomyocytes.

### S100A1 Rapidly Passes the Cell Membrane

To explore the possibility that S100A1 is able to diffuse into the cell, fluorescence imaging experiments were performed using rhod-S100A1 and inactivated rho-

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**FIGURE 1.** S100A1 enhances peak $I_{Ca}$ in embryonic (day 16.5 postcoitum) murine cardiomyocytes. a and b, representative current traces and $I/V$ curves of $I_{Ca}$ recorded from a control (a) and a S100A1-treated (b) (1 μM, 48 h) cardiomyocyte. $I_{Ca}$ was evoked by applying voltage pulses from $-80$ to $+50$ mV ($HP = -80$ mV) at a frequency of 0.2 Hz after an initial prepulse from $-80$ to $-40$ mV to inactivate $I_{Na}$. c, significant enhancement of $I_{Ca}$ density was detected at S100A1 concentrations of ≥0.1 μM (48 h). d, S100A1 (1 μM) enhanced $I_{Ca}$ density from 20 min up to 48 h after application.

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$n = 8, p < 0.02$ in neonatal rat cardiomyocytes (Fig. 2, a–d); decreased but stable levels of the S100A1-mediated stimulatory effect on $I_{Ca}$ were found after 48 h ($-18.4 \pm 1.5 \text{ pA/pF}, 37.8 \pm 11.1\%$, $n = 9, p < 0.04$) (Fig. 2c) as compared with controls ($-13.4 \pm 1.1 \text{ pA/pF}, n = 15$).
S100A1-mediated Modulation of $I_{Ca}$

FIGURE 2. S100A1 increases $I_{Ca}$ density in neonatal rat cardiomyocytes. 

a and b, original traces and I/V curves of $I_{Ca}$ in a control (a) and S100A1-stimulated (b) cardiomyocyte (1 μM, 2 h). c, the S100A1 effect was observed between 20 min and 48 h of exposure. d, S100A1 (1 μM, 20 min) induced a slight left shift of the threshold potential and current voltage (I/V) relationship of $I_{Ca}$ (normalized). e, enhancement of the fast inactivation of $I_{Ca}$ by S100A1 (1 μM, 20 min). The insets show fitting of representative normalized current traces (control (7 ms) versus S100A1 (3 ms)).
S100A1-mediated Modulation of \(I_{\text{Ca}}\)

**FIGURE 3.** S100A1 translocates into the cell via an endocytotic mechanism. *a,* fluorescence picture of an embryonic cardiomyocyte after loading with rhod-S100A1. *b,* the fluorescence increase after the addition of inactivated rhodamine was instantaneously removed by wash-out, whereas after the addition of rhod-S100A1, increased intracellular fluorescence persisted after another wash-out step. Individual cells are represented by solid lines. The dashed line indicates extracellular fluorescence. Intensities were normalized to maximal background fluorescence and auto-fluorescence. *c,* intracellular accumulation of rhod-S100A1 was monitored by confocal laser-scanning microscopy in an embryonic cardiomyocyte (for details, see “Materials and Methods”). Nuclear exclusion and vesicular-like accumulation of rhod-S100A1 in the three layered images confirmed intracellular localization of rhod-S100A1. Extracellular application of inactivated rhodamine did not alter capacitance (\(d\)), whereas rhod-S100A1 (arrowhead) \(e\) led to a pronounced decrease of cellular capacitance (HP = −80 mV).

S100A1 Does Not Alter Cellular cAMP Levels and Acts Distal of Adenylylcy clase/cAMP—The finding that S100A1 increased \(I_{\text{Ca}}\) density within 20 min after the addition to the extracellular solution suggests a direct action of S100A1 on intracellular signaling mechanisms rather than transcriptional up-regulation of gene expression. As proof, ATP\(\gamma\)S (2 mM) together with forskolin (1 \(\mu\)M) were applied to maximally enhance \(I_{\text{Ca}}\) in embryonic mouse cardiomyocytes (32). Strong stimulation of \(I_{\text{Ca}}\), reaching similar densities was observed in both S100A1-treated (−29.8 ± 2.8 pA/pF, +76 ± 16.3%, \(n = 9, p < 0.001\)) and control cardiomyocytes (−27.6 ± 2.4 pA/pF, +115 ± 19%, \(n = 9, p < 0.001\)) (Fig. 4, \(a–c\)). These data indicate no increase in the functional expression of voltage-dependent \(Ca^{2+}\) channels.

To identify the mechanisms of the S100A1 action on \(I_{\text{Ca}}\), its effect on intracellular signaling components was analyzed. Following \(\beta\)-adrenoceptor-mediated stimulation of \(I_{\text{Ca}}\) by adding submaximal concentrations (22) of ISO (0.1 \(\mu\)M; Fig. 5, \(a\) and \(b\)), a similar stimulation of \(I_{\text{Ca}}\) was observed in S100A1-treated (−22 ± 3 pA/pF versus −14.3 ± 2 pA/pF, +54 ± 8%, \(n = 13, p < 0.04\)) and control (−15 ± 5.5 pA/pF versus −9.5 ± 3.9 pA/pF, +59.9 ± 6.5%, \(n = 8, p < 0.05\)) embryonic mouse cardiomyocytes. We then applied saturating concentrations of ISO (1 \(\mu\)M; Fig. 5c) and found significantly less \((p < 0.02)\) enhancement of \(I_{\text{Ca}}\) in S100A1-treated cells (−15.8 ± 1.6 pA/pF versus −12 ± 1.1 pA/pF, +36.2 ± 13.7%, \(n = 8, p < 0.001\)) as compared with controls (−14 ± 1 pA/pF versus −7.6 ± 0.5 pA/pF, +84.6 ± 13.6%, \(n = 8, p < 0.001\)), indicating that S100A1 acted through the same signaling pathway. Application of the muscarinic agonist carbachol (50 \(\mu\)M), known to cause \(G_{\beta}\)-mediated inhibition of AC activity, led to a slight depression of \(I_{\text{Ca}}\) in both S100A1-treated (−11.3 ± 2 pA/pF versus −14 ± 3 pA/pF, −19.9 ± 1%, \(n = 5\)) and untreated cells (−6.1 ± 1 pA/pF versus −7.2 ± 1 pA/pF, −15.3 ±
S100A1-mediated Modulation of I$_{\text{Ca}}$

FIGURE 4. S100A1 does not alter the functional expression of voltage-dependent Ca$^{2+}$ channels. a and b, time course of I$_{\text{Ca}}$ shows that dialysis with ATP-γS (2 mM) and superfusion with forskolin (1 μM) led to a significant augmentation of I$_{\text{Ca}}$ density in a representative control (a) and S100A1-treated (b) embryonic cardiomyocyte. The insets show original current traces. c, statistical analysis proved that coapplication of ATP-γS and forskolin stimulated I$_{\text{Ca}}$ to similar densities. S100A1 (1 μM) was applied for 48 h, step potential 0 mV.

4%, p > 0.05). This observation was confirmed by experiments with the nonselective phosphodiesterase inhibitor IBMX. Phosphodiesterases are known to be functionally up-regulated in embryonic mouse cardiomyocytes, probably because of increased cAMP levels (23). The IBMX effect on I$_{\text{Ca}}$ density did not significantly differ between untreated (n = 5) and S100A1-treated (n = 5) cardiomyocytes, suggesting similar cAMP levels (Fig. 5a). These findings were further substantiated by cAMP measurements in homogenates obtained from embryonic ventricular cardiomyocytes, where control (279 ± 51 fmol/liter, n = 5) and S100A1-treated (274 ± 37 fmol/liter, n = 8) lysates (1 μM, 20 min) yielded almost identical values (Fig. 5a). When ISO (1 μM) and IBMX (200 μM) were used as positive controls to maximally enhance cAMP levels, similar increases of the cAMP concentration to 904 ± 47 fmol (control, n = 4) versus 919 ± 76 fmol (S100A1, n = 4) were found. Similar qualitative results were obtained 48 h after the addition of S100A1 (n = 6, data not shown). These findings indicate that S100A1 acts distal of the adenylyl cyclase/cAMP pathway.

PKA Activity—To study whether the S100A1-induced increase of I$_{\text{Ca}}$ depends on the activation of PKA, I$_{\text{Ca}}$ was measured in the presence of the selective PKA inhibitor PKI (10 μM), added via the patch pipette. Since S100A1-mediated augmentation of PKA activity was expected to result in an accelerated decay of I$_{\text{Ca}}$, in the presence of PKI, the current was measured immediately after establishing the whole-cell configuration and 4 min later in embryonic mouse cardiomyocytes. In S100A1-treated cardiomyocytes, a more pronounced decay (−7 ± 1.5 pA/pF versus −15.6 ± 1 pA/pF, −55.3 ± 9.5%, n = 10, p < 0.001) of I$_{\text{Ca}}$ was observed than in controls (−9.2 ± 1.1 pA/pF versus −10.7 ± 0.8 pA/pF, −13.5 ± 10.4%, n = 9, p > 0.05), suggesting a S100A1-associated augmentation of PKA activity (Fig. 6, a–c). In a next step, embryonic cardiomyocytes were prestimulated with the cell membrane-permeable analogon of PKI and inhibitor of PKA, myristoylated PKI (1 μM, 30 min; Fig. 6d) (33). Under these conditions, S100A1 (1 μM, 20 min) failed to stimulate I$_{\text{Ca}}$ (−8 ± 1 pA/pF, n = 11), and accordingly ISO (1 μM) was almost without effect on I$_{\text{Ca}}$ (−9.8 ± 1.2 pA/pF, +12.2 ± 5%, p ≤ 0.04, n = 10) compared with controls (−8.2 ± 1.3 pA/pF, n = 11) (Fig. 6d).

To evaluate biochemically whether S100A1 led to an increase of PKA activity in cardiomyocytes, we performed PKA assays on cardiomyocyte homogenates. As depicted in Fig. 7a, a marked increase of PKA-specific substrate phosphorylation was observed 20 min after S100A1 (1 μM) application (+50.3 ± 7%, n = 8) and after 48 h (+53.7 ± 7.4%, n = 4) as compared with controls.

To elucidate possible mechanisms through which S100A1 enhanced PKA activity, reconstitution experiments using highly purified PKA subunits in a radioactive PKA assay with histone H1 as a substrate were performed. Fig. 7b shows that the regulatory subunit (R) was effectively inhibiting the activity of the catalytic subunit (C) (RC, left lane). Application of C alone resulted in a 7.3 ± 1.5-fold higher amount of 32P-labeled histone H1 (n = 8) compared with RC. cAMP stimulated RC to similar levels (i.e. 6.5 ± 1-fold increase of basal RC activity) (n = 8). A similar effect (7.3 ± 1.3-fold, n = 8) was seen when S100A1 (1 μM) was preincubated with R (right lane). The S100A1-induced stimulation of the enzymatic activity of PKA was concentration-dependent, yielding maximal effects at 10$^{-8}$ M (Fig. 7c). Incubation with purified S100A1 protein alone did not result in phosphorylation of histone H1. In addition, S100A1 and cAMP did not further enhance the enzymatic activity of C (data not shown).

DISCUSSION

S100A1, a member of the EF-hand Ca$^{2+}$-binding protein family, is a novel regulator of cardiac contractility (8), in particular during the course of myocardial hypertrophy (5, 6). This assumption is corrobor-
rated by transgenic mouse models where deletion or overexpression of S100A1 results in deterioration or improvement of cardiac contractility, respectively (9, 10). Since this protein has been shown to represent an important regulator of Ca\(^{2+}\)/H\(_{11001}\) homeostasis, we have analyzed whether extracellular S100A1 modulates voltage-dependent Ca\(^{2+}\)/H\(_{11001}\) channels, key determinants of cardiac contractility.

We show that extracellular application of S100A1 markedly enhanced peak \(I_{Ca}\) and Ca\(^{2+}\)/H\(_{11001}\) transients. S100A1 shifted the \(I/V\) relationship of \(I_{Ca}\) to negative potentials and accelerated fast inactivation kinetics of the Ca\(^{2+}\)/H\(_{11001}\) currents. This effect is similar to \(\beta\)-adrenergic stimulation of \(I_{Ca}\) and ensuing Ca\(^{2+}\)-induced Ca\(^{2+}\) release (31). Interestingly, this was more prominent in neonatal rat cardiomyocytes than in embryonic mouse cardiomyocytes, suggesting differences in L-type Ca\(^{2+}\) channel regulation and the amount of Ca\(^{2+}\)-induced Ca\(^{2+}\) release during embryonic development.\(^4\) This hypothesis is further validated by the finding that ISO (1 \(\mu M\)) shifts the \(I/V\) curve in late stage but not early stage embryonic mouse cardiomyocytes (data not shown). The additional enhancement of \(I_{Ca}\) after ISO application illustrates a submaximal activation of \(I_{Ca}\) by S100A1. Interestingly, the acceleration of \(I_{Ca}\) inactivation was clearly visible at 20 min but not at 48 h following S100A1 application, although PKA activity measurements in cell homogenates showed a sustained PKA activation by S100A1 after 48 h. Although at this point, we do not know the underlying mechanism(s), this finding may be of a compensatory nature. In fact, despite unchanged PKA activity, the function of phosphatase type 1 and 2A could be significantly

\(^4\) P. Sasse, M. Reppel, and B. K. Fleischmann, unpublished results.
enhanced, leading to an elevated dephosphorylation of L-type Ca\(^{2+}\) channels as shown earlier in a rat model of long term \(\beta\)-adrenergic stimulation (34).

The effect of S100A1 on \(I_{Ca}\) occurred at step potentials from \(-10\) to \(+20\) mV, hence within the physiological voltage range of contracting ventricular cardiomyocytes. The internalization of S100A1 was proven by laser-scanning and fluorescence microscopy. Membrane capacitance measurements clearly suggest, in line with an earlier report (35), that uptake of S100A1 occurs through an endocytotic process.

Electrophysiological and biochemical experiments were performed to identify intracellular target(s) of S100A1 involved in its action on \(I_{Ca}\). The open probability of voltage-dependent Ca\(^{2+}\) channels is critically dependent on PKA activity. Thus, an increase of cAMP-dependent signaling could eventually also lead to stimulation of peak \(I_{Ca}\) via activation of PKA. Maximal concentrations of ISO led to a reduced enhancement of \(I_{Ca}\) density in S100A1-treated cells compared with control cells prior to PKI application; a double asterisk reveals statistical significance of treated cells before and after PKI application. 

In contrast, rundown was small in control cardiomyocytes without PKI in the pipette (open circles). c, the results were confirmed by statistical analysis. A single asterisk denotes statistical significance of S100A1-treated cells compared with control cells prior to PKI application; a double asterisk reveals statistical significance of treated cells before and after PKI application. d, prestimulation with myristoylated PKI significantly reduced the effect of ISO (see also Fig. 5c) on \(I_{Ca}\) and prevented the increase of \(I_{Ca}\) by S100A1.

Interestingly, a recent report investigating S100A1 action on phospholamban phosphorylation after adenoviral overexpression could not detect S100A1-induced modulation of PKA activity (8). However, enhanced Ca\(^{2+}\) cycling observed in S100A1-overexpressing cardiomyocytes was shown to predominantly involve sarcoplasmic reticulum Ca\(^{2+}\)-fluxes, whereas endocytosed S100A1 was supposed to alter intra-

![FIGURE 6. Effect of protein kinase A inhibition. a and b, time course of \(I_{Ca}\) density, showing that dialysis with the protein kinase inhibitor (PKI (10 \(\mu\)M); filled circles) resulted in a pronounced decay of \(I_{Ca}\) after S100A1 treatment (48 h) (b) versus control (a). In contrast, rundown was small in control cardiomyocytes without PKI in the pipette (open circles). c, the results were confirmed by statistical analysis. A single asterisk denotes statistical significance of S100A1-treated cells compared with control cells prior to PKI application; a double asterisk reveals statistical significance of treated cells before and after PKI application. d, prestimulation with myristoylated PKI significantly reduced the effect of ISO (see also Fig. 5c) on \(I_{Ca}\) and prevented the increase of \(I_{Ca}\) by S100A1.](http://www.jbc.org/content/journal/jbc/280/43/3626/F1.large.jpg)
cellular Ca\(^{2+}\)-turnover through sarcosomal modulation (35). Thus, subsarcolemmal compartmentation of S100A1 might be a possible explanation for the apparent discrepancies (9, 35).

During heart injury cardiac proteins are known to be locally released resulting in an increase in serum concentration. In fact, serum concentrations of troponin I and T, also belonging to the EF-hand protein family, are currently used as sensitive indicators of cardiac injury (38). For S100A1 a correlation between the extent of myocardial damage and concentrations of troponin I and T, also belonging to the EF-hand protein family, are currently used as sensitive indicators of cardiac injury (38).

Taken together, we demonstrate that S100A1 enhances \(I_{Ca}\) and Ca\(^{2+}\) transients in ventricular cardiomyocytes via activation of PKA after endocytotic uptake. We currently envision a complex action of S100A1 and enhanced Ca\(^{2+}\)-induced Ca\(^{2+}\) release; this is supported by the observed increase of the fast component of \(I_{Ca}\) inactivation. Second, since a colocalization of S100A1 with sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and phospholamban (40) and its interaction with the ryanodine receptor 2 have been reported previously (9), S100A1 may also induce PKA-mediated phosphorylation of the ryanodine receptor 2 and phospholamban, thereby further increasing sarcoplasmic reticulum Ca\(^{2+}\) release and Ca\(^{2+}\) reuptake. In line with this hypothesis, we and others (35) found besides increased systolic also decreased diastolic Ca\(^{2+}\) levels. Thus, S100A1 is a novel, interesting modulator of PKA signaling and cardiac contractility.

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