Augmentation of Ca\textsubscript{v}1 channel current and action potential duration after uptake of S100A1 in sympathetic ganglion neurons

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The S100 proteins, a vertebrate-specific subfamily of small (9–13 kDa) EF-hand Ca\textsuperscript{2+}-binding proteins, regulate a myriad of intracellular processes ranging from motility through gene transcription (19, 49, 54). Presently, 24 genes of the S100 family have been identified (50). S100 proteins are expressed in a cell-specific manner and exert their effects via protein-protein interactions with different targets through both Ca\textsuperscript{2+}-dependent and -independent mechanisms (64, 82). S100A1, the predominant S100 member in cardiac muscle cells, is a regulator of Ca\textsuperscript{2+} homeostasis and cardiac contractility (62), particularly during cardiac hypertrophy (55, 56). In addition to its intracellular functions, S100A1 is secreted into the extracellular space and exerts paracrine functions (19). In particular, the extracellular translocation of S100A1 has been documented to occur after heart ischemia (9). In cultured ventricular cardiomyocytes, S100A1 enhanced Ca\textsuperscript{2+} influx when added to the extracellular media (63).

The sympathetic nervous system regulates heart function through cell-cell signaling between sympathetic neurons and cardiomyocytes. The heart receives sympathetic input initiated from neurons located in the intermediolateral columns of the spinal cord at the T\textsubscript{1}–T\textsubscript{4} levels (30, 36). Axons from these spinal neurons synapse on superior, middle, and inferior (stellate) cervical ganglia neurons, which extend postganglionic axons that reach the heart (4, 69). Sympathetic axons innervate sino-sinoatrial and atrioventricular nodes, along with myocardial muscle fibers, primarily in the ventricles (4, 48, 59). Sympathetic axons release norepinephrine, which, via \(\beta\)-adrenergic signaling, increases heart rate and conduction as well as excitability and contractility of the myocardium (4, 20). Concurrently, signaling from the heart, via the release of neurotrophic factors, is an important regulator for the maintenance of sympathetic synaptic activity, and is especially critical during development (27, 32, 65). Given this reciprocal anatomic and functional interaction between sympathetic ganglion neurons and the heart, and the fact that S100A1 is released to the extracellular media during cardiac distress, we sought to address potential effects of exogenous S100A1 functioning as a signaling molecule in sympathetic neurons.

MATERIALS AND METHODS

Cell Cultures

Mass cultures of superior cervical ganglion neurons. Superior cervical ganglia (SCG) were dissected from 5-wk-old Wistar rats. The animals were killed according to procedures authorized by the Institutional Animal Care and Use Committee of the University of Maryland Baltimore. SCG neurons were dissociated, plated onto poly-d-lysine- and collagen-coated glass coverslip-bottom culture dishes, and maintained as described previously (24, 25, 31). Dulbecco’s modified Eagle’s medium (DMEM) was supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%), and nerve growth factor (NGF, 25 ng/ml; Invitrogen). For some experiments, sympathetic neurons were maintained up to 7 days in culture in growth medium containing cytosine arabinoside (ara-C, 10\(\mu\)M) to eliminate nonneuronal, mitotically active cells. For electrophysiology and Ca\textsuperscript{2+} imaging neurons were established as described previously (24, 25, 31). Dulbecco’s modified Eagle’s medium (DMEM) was supplemented with fetal bovine serum (10%), penicillin-streptomycin (1%), and nerve growth factor (NGF, 25 ng/ml; Invitrogen). For some experiments, sympathetic neurons were maintained up to 7 days in culture in growth medium containing cytosine arabinoside (ara-C, 10\(\mu\)M) to eliminate nonneuronal, mitotically active cells. For electrophysiology and Ca\textsuperscript{2+} imaging neurons were evaluated between 1 and 5 days after plating.

Compartmentalized cultures of SCG neuronal cultures. SCG neurons from 1-day-old rats were isolated and compartmentalized cultures were established as described previously (11, 12). Briefly, a rake made by clamping together 20 insect pins was used to make 20 parallel scratches ~250 \(\mu\)m apart on the poly-d-lysine/collagen-coated plastic culture dishes. To assemble the system, the scratched region was first coated with medium containing methylcellulose (1%; Sigma, St. Louis, MO) before a three-compartment Campenot cham-
ber (Camp10 Teflon dividers; Tyler Research, Edmonton, AB, Canada) was set with silicone vacuum grease (Dow Corning). When assembled properly, this silicone vacuum grease-sealed multicompartmentalized neuronal culture constitutes a virtually fluid-impermeable system between the chambers, thus allowing the control of the media environments of the side compartments independently of the central compartment (11, 12). Cells (50–100 × 10³) were plated only in nonleaky chambers into the central compartment, which contained DMEM, FBS (10%), antibiotics, and NGF (50 ng/ml). Neurons in Campenot chambers were allowed to adhere for 6–12 h, and side compartments were filled with DMEM, FBS (10%), antibiotics, and NGF (100 ng/ml). The integrity of compartment seals was constantly monitored, and leaky chambers were discarded. Fresh medium was added every 48 h, and ara-C was kept in the central compartment until nonneuronal cells were eliminated.

**Overexpression, Purification, and Labeling of Recombinant S100A1 Protein**

Wild-type recombinant S100A1 was overexpressed and purified in *Escherichia coli* [HMS174(DE3) strain] transformed with an expression plasmid (pET-11b; Novagen, Madison, WI) containing the gene for rat S100A1 as previously described (78). Purified S100A1 was labeled with the fluorescent dye Alexa 488 and a standard protein label strategy suggested by the manufacturer (Invitrogen).

**Immunocytochemistry**

Neurons were fixed in phosphate-buffered saline (PBS, pH 7.4) containing 4% (wt/vol) of paraformaldehyde for 10 min and then permeabilized in PBS containing 0.1% (vol/vol) Triton X-100 (Sigma) for 10 min. The neurons were then exposed to PBS containing 8% (vol/vol) of donkey serum for 1 h at 4°C to block nonspecific labeling. Primary and secondary antibodies were diluted in PBS containing 2% (vol/vol) donkey serum. Neurons were incubated with mouse monoclonal antibody against S100A1 (1:400; Affinity Bioreagents, Golden, CO) for 2 h at room temperature and then incubated with the secondary antibody, Alexa 488-conjugated donkey anti-mouse IgG (1:200 dilution; Invitrogen, Molecular Probes, Eugene, OR), for 2 h at room temperature. Dishes were washed thoroughly after each step in PBS containing 2% (vol/vol) donkey serum. For each primary antibody-treated dish, another dish of SCG neurons was treated with the secondary antibody only and used as control.

Antibody-labeled neurons were imaged on an Olympus Fluoview 500 confocal system built on an Olympus IX71 inverted microscope. Neurons were viewed with a UPlan Apo ×60 NA 1.20 water-immersion objective lens using 488-nm excitation (Multiline argon laser) for Alexa 488 and emission detected with a 505-nm long-pass filter. Photobleaching and photodamage were reduced by operating the lasers at the lowest possible power that still provided images with satisfactory signal-to-noise ratio. Confocal images were processed with custom-written software in IDL (RSI, Boulder, CO) and ImageJ (National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

**Western Blot and Lysate Preparation**

Ten mass culture plates containing the SCG neurons from 8–10 dissociated ganglia provided a sufficient amount of cellular material for Western blot experiments. Briefly, cultured SCG neurons were lysed with a mammalian protein extraction reagent (M-PER, Pierce), and the homogenates were supplemented with protease inhibitor cocktail and pooled together (Complete-Mini EDTA-free, Roche Diagnostics). The homogenates were subjected to centrifugation at 10,000 rpm for 10 min at 4°C. The supernatant was extracted and stored. Protein content was determined with the Bradford assay (Bio-Rad, Hercules, CA). Samples for electrophoresis were not boiled but were solubilized at 70°C for 10 min. Approximately 60–300 μg of protein sample was fractionated by 4–12% SDS-PAGE and transferred to nitrocellulose membrane at 20 V for 3 h at 4°C with the Xcell II blot module (Invitrogen). Blots were then processed and probed with antibodies against S100A1 and GAPDH (Sigma). The immunoreactive bands were visualized with ECL Western Blotting Detection Reagents (Amersham). ImageJ was used to analyze data.

**Fluorescent Probes Used to Study Endocytosis and Labeling of Organelles**

In some experiments neurons were simultaneously or sequentially labeled with S100A1-Alexa 488 and different fluorescent probes to stain plasma membrane, evaluate endocytosis, and label organelles (Invitrogen, Molecular Probes). FM4-64 was used to stain plasma membrane and to follow bulk membrane internalization and transport (76). Dextran-TMR (molecular mass 10,000 Da; 500 μg/ml for 12 h) was used as a fluid-phase endocytotic marker (21, 35). Cholera toxin subunit B-Alexa 594 (5 μg/ml for 12 h) was used as a marker for lipid raft-mediated endocytosis and to label Golgi apparatus (58). Labeling of cells with transferrin-Alexa 568 (40 μg/ml for 18 h) was used as a marker for clathrin-dependent endocytosis and to label early/recycling endosomes (28, 80). Labeling of lysosomes was done by incubating cells with LysoTracker (LysoRed DND-99; 5 μg/ml for 40 min). Labeling was conducted on living cells maintained at 37°C at 5% CO2 in DMEM with 10% FBS. After incubation, cells were washed three times in L-15 medium at room temperature and then imaged with a confocal microscope. Colocalization analysis was conducted with a compilation of general colocalization indicators (ImageJ plugin JACoP; Ref. 6).

**Solutions**

Neurons were locally perfused extracellularly during recording (1–2 ml/min) with mammalian Ringer solution [containing (in mM) 160 NaCl, 3 KCl, 2 CaCl₂, 8 glucose, 1 MgCl₂, 10 HEPES, pH 7.4 with NaOH] or with an external solution designed to isolate whole cell Ca²⁺-channel currents [containing (in mM) 162.5 TEA-Cl, 2 CaCl₂, 8 glucose, 1 MgCl₂, 0.001 TTX, 10 HEPES, pH adjusted to 7.4 with TEA-Cl]. In some experiments CaCl₂ was replaced with BaCl₂ (1–2 mM). Where noted, nifedipine (5 μM; to selectively block L-type Ca²⁺ channels), α-conotoxin-GVIA (α-CTX, 1 μM; to selectively block N-type Ca²⁺ channels), and CoCl₂ (0.2 mM; a nonspecific Ca²⁺ channel blocker) were added to the external solution. For whole cell voltage-clamp experiments the pipette solution (internal solution) contained (in mM) 140 CsCl, 0.2 EGTA, 4 MgCl₂, 14 creatine phosphate, 4 Na₂ATP, 0.3 Na₂GTP, 0.1 leupeptin, and 10 HEPES, pH adjusted to 7.4 with CsOH. Current-clamp experiments were carried out with Ringer solution and an intracellular pipette solution containing (in mM) 150 KCl, 10 NaCl, 4 MgCl₂, 4 Na₂ATP, 14 phosphocreatine-Na₂, 0.3 Na₂GTP, and 10 HEPES, pH adjusted to 7.4 with KOH. For intracellular uptake and axonal transport experiments as well as for Ca²⁺ imaging, neurons were assayed 2–4 h after extracellular addition of S100A1. Where indicated, we used either Alexa 488-labeled or unlabeled S100A1 added to the extracellular solution or intracellularly via the patch pipette.

**Electrophysiology and Data Analysis**

Membrane current and voltage measurements were performed in the whole cell configuration of the patch-clamp technique (29) with a EPC-10 plus amplifier (HEKA Instruments) and pipettes of borosilicate glass (Kimax-51) with resistance of 1.2–1.8 MΩ when filled with the internal solution. For voltage-clamp experiments capacitance compensation was adjusted to largely cancel linear capacity transients, and series resistance was compensated to >60% and periodically monitored. Linear capacitive and leak currents were routinely subtracted by a P/4 protocol from a subholding potential of ~120 mV. Care was taken to select neurons with no evident neuronal process to improve space clamp. Neurons with signs of qualitative clamp error were rejected from the analysis. Measurements were started ~3 min after
breakthrough into whole cell configuration. Because the magnitude of the Ca\(^{2+}\)-channel currents was dependent on cell size, aggregate current data are presented as current density normalized to cell capacitance, which was estimated with the capacitance cancellation circuitry of the patch-clamp amplifier.

After establishment of the whole cell current-clamp configuration, we waited for the membrane potential to stabilize. Action potential (AP) height was defined as the peak relative to the most negative voltage reached during the afterhyperpolarization. AP width was measured as the full width at half-maximal spike height. Data were excluded from analysis if the AP height was <70 mV. APs were initiated by 20-ms suprathreshold depolarizing current pulses. To test the effect of S100A1, we adjusted the membrane potential to −60 mV by using the amplifier in the low-frequency voltage-clamp mode, a modified current-clamp mode that works like a current clamp for fast signals and like a voltage clamp for low-frequency signals.

Data were acquired, digitized, stored, and analyzed with commercial software (Patchmaster and Fitmaster; HEKA Instruments). For further analysis we used Origin 8 software (OriginLab, Northampton, MA). Command pulses were delivered at 10-s intervals from a holding potential of −80 mV, unless otherwise indicated. Electrical signals were typically low-pass filtered at 3–10 kHz (3-pole Bessel filter). The sampling frequency for Ca\(^{2+}\)-channel currents and for membrane potentials was 50 and 10 kHz, respectively.

To measure and quantify inactivation, we divided current amplitude at the end (640 ms) of the test pulse (\(I_{\text{end}}\)) by peak current value (\(I_{\text{peak}}\); Ref. 25). The fractional inactivation was then calculated as 1 − (\(I_{\text{end}}/I_{\text{peak}}\)). We also used a double-exponential function plus one constant to quantify the fast and slow components of inactivation during 640-ms voltage steps. The amplitude of each inactivating component was divided by the total amplitude, which was the sum of the two inactivating and one noninactivating component.

**NMR Spectroscopy**

Sample preparation. A synthetic peptide derived from the cAMP-dependent regulatory subunit IB of protein kinase A (PKA) was chemically synthesized and prepared for NMR in a manner previously described (78). Recombinant \(^{15}\)N-labeled rat S100A1 protein was purified as previously described (78). NMR samples contained (in mM) 15 Tris, pH 7.2, 15 dithiothreitol, 10 CaCl\(_2\), 0.34 NaN\(_3\), 20 NaCl, and 0–3 PKA peptide, with 10% D\(_2\)O and 500 \(\mu\)M S100A1 protein.

NMR spectroscopy and chemical shift assignments. NMR spectra were collected at 37°C with a Bruker AVANCE 800 NMR spectrometer (800.27 MHz for protons) equipped with four frequency channels and a 5-mm triple-resonance z-axis gradient cryogenic probe head. A series of heteronuclear single-quantum coherence (HSQC) spectra were run with increasing amounts of PKA peptide to S100A1 protein (0, 0.5×, 1×, 3×, and 6× molar ratios). Generally, HSQC peaks could be followed on titration of the PKA peptide into Ca\(^{2+}\)-S100A1. However, three-dimensional (3D) \(^{15}\)N-edited nuclear Overhauser enhancement spectroscopy (NOESY) and 3D \(^{13}\)N-edited heteronuclear multiple-quantum coherence (HMQC)-NOESY-HSQC (2) experiments were collected on the final PKA-S100A1 titration point in order to verify the sequence-specific assignments of the HSQC spectra.

To avoid one source of systematic bias, experimental and control measurements were alternated whenever possible and concurrent controls were always performed. All recordings were obtained at room temperature (19–22°C). Where appropriate, data are expressed as means ± SE, with the number of observations (n) in parentheses. Statistical significance was determined by using an unpaired Student’s t-test. Data were considered significant when \(P < 0.05\).

**RESULTS**

**S100A1 Is Not Expressed in SCG Neurons**

Previous reports have shown that S100A1 is expressed in satellite and glial cells of the SCG but not in principal neurons (15, 68). In agreement with these previous reports, no detectable immunofluorescent signal was seen when using confocal microscopy and antibodies directed against S100A1 in principal SCG neurons (Fig. 1A). As a positive control, this same antibody gave a strong immunofluorescent signal when tested in fast skeletal muscle fibers, a cell type in which S100A1 is expressed (data not shown; see Ref. 61). Further evidence of
the absence of S100 in SCG neurons came from Western blot experiments. Lysates from principal SCG neurons (cultures were exposed to ara-C to remove nonneuronal cells; see MATERIALS AND METHODS) demonstrated no immunoreactivity to S100A1 antibodies (Fig. 1B). Purified S100A1 was used as a positive control. These results indicate that principal neurons from SCG contain no detectable endogenous S100A1.

**Intracellular Translocation of Exogenous S100A1 in Sympathetic Neuron Cultures**

In some cells, specific S100 members are released into the extracellular space and exert paracrine functions on the neighboring cells (19). In particular, the extracellular release of S100A1 occurs after heart ischemia (9), and cultured ventricular cardiomyocytes internalized S100A1 when it was added to the culture medium (63). Therefore, we asked whether cultured sympathetic neurons were able to internalize extracellular S100A1. Neurons were incubated for 30 min to 24 h with Alexa 488-labeled S100A1 protein at a final concentration of 5–10 μM. Cellular uptake was minimal after 30 min and slightly evident after 2 h of incubation (data not shown). Over the next 12–24 h, some aggregates were observed and dramatically increasing numbers of much smaller particles of fluorescence were observed on the cell surface, within cell bodies, and along neuronal projections of virtually all the imaged neurons. Figure 2 shows representative images of live neurons incubated for 24 h with S100A1-Alexa 488 (5 μM, Fig. 2A) and neurons with IgG-Alexa 488 (Fig. 2B). It should be noted that the neurons incubated with S100A1-Alexa 488 exhibited a fluorescent punctate pattern that was persistent even after the washout of the extracellular solution containing the labeled S100A1 protein. Neurons incubated with IgG-Alexa 488 did show a weak intracellular fluorescent signal (likely the result of autofluorescence), and the removal of the extracellular solution resulted in a complete washout of the extracellular labeled IgG (Fig. 2B), indicating no translocation of immunoglobulin after 24 h. After 3–5 days, intracellular S100A1 particles were still readily apparent (data not shown). Figure 3A, a–c, shows representative images at different focal planes of a neuron incubated with S100A1-Alexa 488 (10 μM) for 24 h. It should be noted that the S100A1-Alexa 488 signal is distributed across the cytoplasm and is present in neuronal projections (Fig. 3Ac). Figure 3B shows x-y images at higher magnification of another neuron incubated with S100A1-Alexa 488 for 24 h and costained with FM4-64 (a plasma membrane dye) and DAPI (to stain the nucleus). The S100A1-Alexa 488 image appears as a clear punctate pattern distributed across the cytoplasm (Fig. 3Ba). No S100A1-Alexa 488 signal was detected in the nuclei (Fig. 3B, ROI1). FM4-64 labels membrane structures, including membrane vesicles, and was used to follow bulk membrane internalization (76). Figure 3B, ROI2, shows codistribution of S100A1-Alexa 488 and FM4-64, suggesting uptake of S100A1 via an endocytotic mechanism.

**Uptake of S100A1 Involves More Than One Endocytotic Mechanism**

To identify the endocytotic mechanism(s), as well as the subcellular compartments that accumulate and transport exogenous S100A1-Alexa 488, coantibody and organelle markers were performed. Cultured neurons were exposed to extracellular S100A1-Alexa 488 (5 μM) and also labeled with fluorescent 10-kDa dextran (Fig. 4A), a marker of fluid-phase endocytosis (21, 35); cholera toxin subunit B (Fig. 4B), a marker of lipid rafts and Golgi apparatus (58); fluorescent transferrin (Fig. 4C), a tool to evaluate clathrin-dependent endocytosis as well as a marker of early/recy-
cling endosomes (28, 80); and LysoTracker (Fig. 4D), a fluorescent acidic organelle marker, to label lysosomes. Figure 4 shows representative confocal images of neurons treated with extracellular S100A1-Alexa 488 (10 μM) for 24 h. S100A1-Alexa 488 fluorescence is located in the cytoplasm and present in the neuronal processes (arrows in c) but not in the nucleus. Scale bars, 10 μm. B: representative confocal images of another neuron treated with extracellular S100A1-Alexa 488 for 24 h (a) and colabeled with DAPI (b) to stain the nucleus and FM4-64 (c) to stain plasma membrane and to follow bulk membrane internalization; d shows the merge of a–c. ROI1 and ROI2 are enlarged views of the outlined regions of interest in Bd, demonstrating clear S100A1 exclusion from the nucleus and association with membranous structures.

Fig. 3. Intracellular distribution of exogenous S100A1. A: confocal x-y images at different z-axial planes (a, top; b, middle; c, bottom) from a neuron treated with extracellular S100A1-Alexa 488 (10 μM) for 24 h. S100A1-Alexa 488 fluorescence is located in the cytoplasm and present in the neuronal processes (arrows in c) but not in the nucleus. Scale bars, 10 μm. B: representative confocal images of another neuron treated with extracellular S100A1-Alexa 488 for 24 h (a) and colabeled with DAPI (b) to stain the nucleus and FM4-64 (c) to stain plasma membrane and to follow bulk membrane internalization; d shows the merge of a–c. ROI1 and ROI2 are enlarged views of the outlined regions of interest in Bd, demonstrating clear S100A1 exclusion from the nucleus and association with membranous structures.

S100A1 ENHANCES CAV1 CHANNEL CURRENTS IN NEURONS

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S100A1 Is Transported into SCG Neuron Somas Via Axonal Retrograde Transport

Frequently, in mass cultures of SCG neurons, S100A1-Alexa 488 punctate fluorescent signals within cell bodies and axons were transported in both an anterograde and a retrograde fashion. Time-lapse x-y confocal images (see Supplemental Video S1) of the labeled cells revealed movement (0.4 μm/s)
of small fluorescent particles within cell bodies and along neuronal processes.1 A fraction of the postganglionic distal axons that reach the heart originate at the SCGs. The extracellular translocation of S100A1 has been documented to occur after heart ischemia (9). Therefore we sought to evaluate whether S100A1 can be transported from distal axons to the cell bodies of neurons. To this end we used a compartmentalized neuronal culture, a model that allows physical separation between cell somas and axonal projections (see MATERIALS AND METHODS; Ref. 11). In this compartmentalized culture, evidence for the operation of different mechanisms of both anterograde and retrograde signaling has been provided (67, 73). Figure 5A, c and d, shows representative transmitted light images of cell bodies located in the central compartment and their axonal projections on the side compartment of a Campenot chamber. Neurons plated into the central chamber tend to reorganize to produce small clusters of somas; axonal projections from these neurons usually grow toward the partitions separating the central and side chambers. We assessed uptake and retrograde transport of S100A1 when the length of axons in the side compartment reached 200–400 μm. Figure 5 also shows representative confocal images of center and side compartments of control (Fig. 5A, a and b) and S100A1-treated (Fig. 5B, a and b) compartmented SCG cultures. Side compartments shown in Fig. 5, A and B, were exposed to Alexa 488-IgG and S100A1-Alexa 488 (5 μM), respectively. After 24 h chambers were rinsed twice with Ringer solution and images were obtained. In control (Alexa 488-IgG treated) neurons no fluorescence was detected (Fig. 5A, a and b). However, S100A1-Alexa 488-treated neurons located in the center compartment displayed a punctalike fluorescent pattern, indicating that S100A1 was effectively transported from axons toward the cell bodies and stained in punctate structures (Fig. 5B, a and b). This result indicates that S100A1 undergoes retrograde transport from distal axons toward the somas when S100A1 is present in the extracellular space.

**Whole Cell Ca$^{2+}$ Channel Current Enhancement on Extracellular Application of S100A1**

The above results detailing the uptake, localization, and axonal retrograde transport of exogenous S100A1, coupled with the fact that previous studies have shown that S100 family

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1 The online version of this manuscript contains supplemental material.
members enhance Ca\(^{2+}\) signals (44, 63), prompted us to look for effects of S100A1 at the level of Ca\(^{2+}\) signals, particularly at the level of Ca\(^{2+}\) influx. We first measured whole cell Ca\(^{2+}\) currents, using 2 mM Cu\(^{2+}\) as a charge carrier. Figure 6 shows representative Ca\(^{2+}\) current traces from a control SCG neuron cultured for 24 h (Fig. 6A, left). Current was elicited by 50-ms step depolarizations from a holding potential of −100 mV to the voltage values indicated close to each trace. The pooled current density vs. test potential (I-V relationship) from control neurons in Fig. 6B shows that the currents activated at potentials positive to −20 mV, reached a maximum inward amplitude near 0 mV, and reversed at +40 mV.

Figure 6 also shows representative Ca\(^{2+}\) current traces from another neuron treated with extracellular S100A1 (10 μM for 24 h; Fig. 6A, right). S100A1 was added 30 min after the neurons were plated. The extracellular application of S100A1 induced a ~20–50% enhancement of the peak inward Ca\(^{2+}\) current density (Fig. 6B). Also note that the inactivation during the test pulse is more pronounced in S100A1-treated neurons (see below).

To evaluate the effect of S100A1 on the voltage dependence of Ca\(^{2+}\) current activation, I-V plots from control and S100A1-treated neurons were fitted to a Boltzmann-ohmic function, described by the following equation (7):

\[
I = G_{\text{max}} \frac{(V - V_{\text{rev}})}{[1 + \exp(-V - V_{h})/k]},
\]

where \(G_{\text{max}}\) is the maximum conductance, \(V\) is the membrane potential, \(V_{\text{rev}}\) is the reversal potential, \(V_{h}\) is the half-activation potential, and \(k\) is a measure of the steepness. The solid lines through the I-V curves in Fig. 6B were least square fits of the data to this modified Boltzmann-ohmic equation. Average parameter values for \(G_{\text{max}}, V_{h},\) and \(k\) for control neurons were 0.32 mS/cm\(^2\), −5.3 mV, and 4.5 mV, respectively. For S100A1-treated neurons \(G_{\text{max}}, V_{h},\) and \(k\) were 0.43 mS/cm\(^2\), −6.1 mV, and 3.6 mV, respectively. These parameters were used to derive the activation curves for control and S100A1-treated cells, as plotted in Fig. 6C. S100A1 dialysis had little or no effect on the voltage dependence of current activation, as shown by Fig. 6C, inset. Therefore, judging by the lack of effect on voltage dependence of the Ca\(^{2+}\) channel activation we conclude that the enhancement of whole cell Ca\(^{2+}\) current can be classified as voltage independent.

**Extracellular S100A1 Modifies Kinetics of Whole Cell Ca\(^{2+}\) Current Inactivation**

The observation that inactivation kinetics seemed faster in neurons treated with S100A1 prompted us to characterize this process. In Fig. 7A Ca\(^{2+}\) current traces recorded during a voltage step from −80 to 0 mV are displayed. Over the course of this 640-ms depolarization, the current partially inactivated to 43% of its peak value in control neurons vs. 28% of its peak value observed in S100A1-treated neurons (Fig. 7, A and B). The inactivation process could be fit well with the sum of two exponentials plus one constant as shown in Fig. 7C. Mean fast and slow amplitudes of 0.16 ± 0.1 and 0.44 ± 0.2, respectively, were calculated for control neurons vs. 0.17 ± 0.1 and 0.45 ± 0.2 for S100A1-treated neurons (not significant, \(n = 8;\) data not shown). Amplitude was measured as the fraction relative to total amplitude, which is the sum of both inactivating and noninactivating components. In general, the amplitude of the fast component was ~0.3 that of the slow component, and this ratio varied little over recording times up to 10 min. In control neurons the mean time constant for the fast component of inactivation was 29 ± 1.9 ms, whereas the slow component had a time constant of 401 ± 13 ms (Fig. 7, D and E). In S100A1-treated neurons the mean time constant for the fast component of inactivation significantly increased to 37 ± 3.2 ms, whereas the time constant of the slow component significantly decreased to 244 ± 11 ms (Fig. 7, D and E). The above results indicate that S100A1 treatment did not modify the relative amplitude of either the fast or the slow component.
However, S100A1 treatment significantly slowed the fast phase of inactivation and considerably accelerated the slow phase of inactivation (n = 8; P < 0.05 for both). These findings may indicate that S100A1 is affecting the inactivation kinetics of one or more population(s) of voltage-dependent Ca\(^{2+}\) channels.

S100A1 Modulates L-type (Ca\(_{L}\)) Component of Total Ca\(^{2+}\) Current in Cultured SCG Neurons

Previous studies have demonstrated that in cultured SCG sympathetic neurons most of the Ca\(^{2+}\) current flows through N-type (Ca\(_{N}\); 80–90%) and L-type (Ca\(_{L}\), predominantly Ca\(_{L,3}\); 10–...
20%) Ca\(^{2+}\) channels, with minimal R-type (Ca\(_{2.3}\)) and P/Q (Ca\(_{2.1}\)) components (47, 53, 60). Here, Ca\(_{1.1}\), Ca\(_{2.2}\), and Ca\(_{2.3}\) channel current components are identified as the fraction of the total current [the difference current obtained by subtraction of currents recorded under the channel blocker from that measured immediately before the channel blocker(s) was applied] sensitive to 5 \(\mu\)M nifedipine and 1 \(\mu\)M \(\omega\)-ctx in the presence of nifedipine and to 0.5 mM Co\(^{2+}\) added in the presence of nifedipine and \(\omega\)-ctx, respectively (24, 25). Figure 8A shows that the sequential addition of nifedipine, \(\omega\)-ctx, and CoCl\(_2\) progressively resulted in reduced Ca\(^{2+}\) currents, allowing the estimation of the proportion of current carried through Ca\(_{1.1}\), Ca\(_{2.2}\), and Ca\(_{2.3}\) channels, respectively. We found that in 24-h-cultured control neurons ~8 ± 2% of whole cell Ca\(^{2+}\) channel current was sensitive to nifedipine, ~80 ± 5% was sensitive to \(\omega\)-ctx, and 10 ± 2% was sensitive to Co\(^{2+}\) (Fig. 8, A and C). However, in neurons exposed to extracellular S100A1 for 24 h the percentage of the whole cell Ca\(^{2+}\) current sensitive to nifedipine was increased to ~40% and only ~60% was then sensitive to \(\omega\)-ctx and Co\(^{2+}\) (Fig. 8, B and C). It should be noted that the amplitudes of the \(\omega\)-ctx- and Co\(^{2+}\)-sensitive components (N and R types) are not changed by S100A1 treatment. The amplitude of the N-type component in control neurons was 7.5 ± 0.8 pA/pF versus 8.2 ± 2.5 pA/pF in S100A1-treated neurons \((P > 0.05; \text{Fig. } 8C)\). The amplitude of the R-type component in control neurons was 1.0 ± 0.2 pA/pF versus 1.1 ± 0.4 pA/pF in S100A1-treated neurons \((P > 0.05; \text{Fig. } 8C)\).

**Fig. 8.** Twenty-four-hour extracellular S100A1 treatment increases L-type Ca\(^{2+}\) currents recorded under the channel blocker from that measured immediately before the channel blocker(s) was applied sensitive to 5 \(\mu\)M nifedipine and 1 \(\mu\)M \(\omega\)-ctx in the presence of nifedipine and to 0.5 mM Co\(^{2+}\) added in the presence of nifedipine and \(\omega\)-ctx, respectively. We found that in 24-h-cultured control neurons (~8 ± 2% of whole cell Ca\(^{2+}\) channel current was sensitive to nifedipine, ~80 ± 5% was sensitive to \(\omega\)-ctx, and 10 ± 2% was sensitive to Co\(^{2+}\) (Fig. 8, A and C). However, in neurons exposed to extracellular S100A1 for 24 h the percentage of the whole cell Ca\(^{2+}\) current sensitive to nifedipine was increased to ~40% and only ~60% was then sensitive to \(\omega\)-ctx and Co\(^{2+}\) (Fig. 8, B and C). It should be noted that the amplitudes of the \(\omega\)-ctx- and Co\(^{2+}\)-sensitive components (N and R types) are not changed by S100A1 treatment. The amplitude of the N-type component in control neurons was 7.5 ± 0.8 pA/pF versus 8.2 ± 2.5 pA/pF in S100A1-treated neurons \((P > 0.05; \text{Fig. } 8C)\). The amplitude of the R-type component in control neurons was 1.0 ± 0.2 pA/pF versus 1.1 ± 0.4 pA/pF in S100A1-treated neurons \((P > 0.05; \text{Fig. } 8C)\).

**Intracellular Dialysis of S100A1 Mimics Effect of Extracellularly Added S100A1**

We next asked which signaling mechanism(s) underlies the S100A1-mediated augmentation of Ca\(^{2+}\) channel current. Is S100A1 binding to cell surface receptors sufficient for the activation of a signaling cascade? Or alternatively, is S100A1 being internalized and then released to the cytosol? There is evidence that some S100 members interact with cell surface receptors that transduce their effects (19). For example, S100B and S100A12 interact with the receptor for advanced glycation end-products (RAGE; Ref. 18). To evaluate whether S100A1 affects Ca\(^{2+}\) channel currents through a plasma membrane receptor-mediated cascade we applied S100A1 intracellularly via the patch pipette. If S100A1 binding to a cell surface receptor is required to modulate Ca\(_{1.1}\), then intracellular dialysis with S100A1 should have no effect on the Ca\(^{2+}\) current. For the following study, we preferred the use of Ba\(^{2+}\) (1 mM) as a charge carrier instead of Ca\(^{2+}\) to increase the stability of the Ca\(^{2+}\) channel current recordings (3). When Ba\(^{2+}\) replaces Ca\(^{2+}\) the I-V plot is left shifted in the voltage axis (3, 7, 25). In our case this shift was ~10 mV, so that the maximum inward current carried by Ca\(^{2+}\) channels occurred at ~10 mV. We first assessed changes in barium current \((I_{Ba})\) amplitude during repetitive depolarizing pulses from a holding potential of ~100 mV to a test potential of ~10 mV, applied 10 s apart. Recordings started 3 min after the breakthrough into the whole cell configuration. We systematically use this time to allow equilibration of the internal solution within the cell. As seen in Fig. 9D, this time window allows significant diffusion of S100A1 into the cell, and, in addition, this method allowed us to address the effects of S100A1 using individual neurons as their own controls (see Supplemental Fig. 2). Figure 9A shows a representative family of 20 overlapped \(I_{Ba}\) traces obtained with this protocol in a control neuron dialyzed with the standard internal solution (see MATERIALS AND METHODS). The comparison of the first trace with the last trace, obtained within a time window of 180-s duration, illustrates the stability of the \(I_{Ba}\) recordings for control neurons. Figure 9C shows pooled data for control neurons \((n = 8)\). In this figure \(I_{Ba}\) amplitude for each trace was normalized to the amplitude of the first trace obtained at time 0. The amplitude of \(I_{Ba}\), recorded in this manner, was minimally reduced (<5%) over the time course of a typical experiment, indicating a minimal or absent current rundown or run-up.

We then tested the effect of S100A1 on the time course of \(I_{Ba}\) amplitude. One striking distinction was that intracellular dialysis of S100A1 via the patch pipette induced a marked increase of the whole cell \(I_{Ba}\) amplitude. Figure 9B shows a representative family of 20 overlapped traces from a neuron in which S100A1 (10 \(\mu\)M) was included in the standard internal solution. The comparison of the first trace with the last trace, obtained at a time window of 180 s apart, illustrates the enhancement of \(I_{Ba}\). Figure 9C shows pooled data for S100A1-dialyzed neurons \((n = 12)\). Intracellular dialysis of S100A1 significantly enhanced \(I_{Ba}\) by >10% in all neurons tested, with a mean enhancement of 23 ± 6% \((P < 0.05 \text{ vs. control})\). Therefore, judging by the similarity of the effects of S100A1 on Ca\(^{2+}\) current when added either extracellularly or intracellularly we conclude that S100A1-
mediated enhancement of whole cell Ca$^{2+}$ current does not require the activation of an extracellular plasma membrane receptor. In another series of experiments we added a L-type Ca$^{2+}$ channel blocker to the extracellular solution and measured the amplitude of Ca$^{2+}$ current in S100A1-dialyzed neurons. The extracellular preapplication of nifedipine (5 μM) to the recording solution of S100A1-dialyzed neurons prevented the increase in the Ca$^{2+}$ current (see Supplementary Fig. S1). In the presence of nifedipine, six of eight S100A1-dialyzed neurons showed no increase of Ca$^{2+}$ channel current density. Additionally, S100A1-treated neurons showed no changes in kinetics or voltage dependence of activation in the presence of nifedipine. These results further suggest that L-type (Ca$_v$1) channels are the major target for modulation by extracellular S100A1 in SCG neurons.

PKA Inhibition Occludes Effect of S100A1

We next asked which intracellular signaling mechanism(s) may underlie the enhancement of the L-type Ca$^{2+}$ current. The preceding study suggested that S100A1 does not require a membrane receptor to augment Ca$^{2+}$ channel currents. Does intracellular S100A1 bind to an effector molecule and indirectly modulate the activity of Ca$^{2+}$ channels? Or does S100A1 bind to and directly interact with Ca$_v$ channels, thereby altering their contribution to Ca$^{2+}$ current? In different cell types Ca$_v$1 currents are often augmented by phosphorylation (14, 17, 38). The δ-subunits of both skeletal and cardiac Ca$_v$1 channels possess sites for phosphorylation by cAMP-dependent PKA (16, 71). PKA modulates Ca$_v$1 channel activation and inactivation, and has been implicated in the augmentation of Ca$_v$1 channels (17). In addition, in cultured...
cardiac myocytes Ca\textsubscript{v1} channel currents are enhanced by S100A1 in a PKA-dependent manner (63). These observations and previous findings led us to the hypothesis that PKA activity is required for the S100A1-induced increase of Ca\textsubscript{v1} currents. To test this hypothesis, SCG neurons were preincubated for 1 h with a specific and cell membrane-permeant PKA inhibitor [14–22 amide (PKAiP), PKA inhibitor; 5 \mu M] and then S100A1 (10 \mu M) was added to the culture medium for 12–18 h. The preincubation with the PKA inhibitor prevented the whole cell Ca\textsuperscript{2+} current augmentation mediated by S100A1 (Fig. 10A). Average peak inward current density values (evaluated at 0 mV from \textit{I-V} relationships) for PKAiP-preincubated and S100A1-treated neurons were 10.6 \pm 2.4 pA/pF (n = 12) versus 18.1 \pm 2.5 pA/pF observed in S100A1-treated neurons (n = 12) (\textit{P} < 0.05, Fig. 10B). A similar result was observed in experiments in which neurons were preincubated with the inhibitory peptide but S100A1 was added via the patch pipette (data not shown). The above results indicate that exogenous S100A1 enhances neuronal Ca\textsubscript{v1} channels via a PKA-dependent mechanism.

### S100A1 Directly Interacts with PKA

To test whether S100A1 could bind directly to PKA, a peptide derived from the cAMP regulatory subunit of PKA type II\beta (SERLKVVDDVGTK) was slowly titrated into Ca\textsuperscript{2+}-loaded, \textsuperscript{15}N-labeled rat S100A1 and an HSQC spectra of S100A1 was collected. This titration was continued until the addition of more peptide no longer produced chemical shift changes in the S100A1 HSQC spectra, at which point the complex was considered saturated. As shown in Fig. 11A, several S100A1 residues shift substantially (>170 Hz) on the addition of this PKA peptide, while other residues do not. Residues that experience the most dramatic chemical shift perturbations are generally located in the hinge region and helix 4 of S100A1. These residues include Ser42, Ser43, Leu45, Asp46, Val47, Glu60, Gln72, Glu73, Val75, Ala79, Ala80, Thr82, Cys85, and Phe89, which are close together in 3D space (Fig. 11B) and comprise a large part of the well-characterized calcium-dependent hydrophobic protein binding cleft of S100A1 (61, 78, 79). In the absence of Ca\textsuperscript{2+}, addition of large amounts of the PKA peptide resulted in no chemical shift perturbations. Together, these data indicate that S100A1 can bind to a region of PKA regulatory subunit in a Ca\textsuperscript{2+} dependent manner, and that this binding event is mediated via the previously characterized hydrophobic pocket of S100A1, as seen in other systems (49, 61, 64, 79).

### S100A1 Enhances Ca\textsuperscript{2+} Transients in SCG Neurons

S100A1 has been shown to participate in Ca\textsuperscript{2+} homeostasis and intracellular Ca\textsuperscript{2+} release in cardiac muscle cells (55, 56), and our group has recently reported (61) modulatory effects of S100A1 on AP-induced Ca\textsuperscript{2+} transients via interaction of S100A1 with Ca\textsuperscript{2+}-release channels in mammalian skeletal muscle cells. Given the role of S100A1 in regulating Ca\textsuperscript{2+} signaling in other cell types, we sought to address the functional significance of the Ca\textsuperscript{2+} influx enhancement induced by S100A1. First, we evaluated the effect of S100A1 on resting Ca\textsuperscript{2+} levels in SCG neurons. We compared indo-1 ratio values of control neurons to those of neurons exposed to extracellular S100A1 (10 \mu M) for 24 h. No significant changes in resting indo-1 ratio values (control 1.106 \pm 0.01 vs. S100A1-treated 1.105 \pm 0.08; \textit{P} > 0.05, n = 25 neurons per each group) were detected (data not shown). This result indicates that S100A1 did not modify intracellular resting Ca\textsuperscript{2+} levels.

In mammalian SCG neurons a depolarization-evoked Ca\textsuperscript{2+} transient is mostly the result of Ca\textsuperscript{2+} influx via voltage-dependent Ca\textsuperscript{2+} channels, with little if any contribution from Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (51, 72). Because S100A1 enhanced Ca\textsuperscript{2+} current in voltage-clamped SCG neurons we next evaluated the effect of S100A1 on AP-induced Ca\textsuperscript{2+} transients. Neurons were loaded with fluo-4 AM, a nonratiometric Ca\textsuperscript{2+}- sensitive dye, and the amplitude and temporal properties of Ca\textsuperscript{2+} transients initiated by a single AP were monitored by ultrahigh-speed confocal microscopy. Figure 12A illustrates the average time course of AP-induced Ca\textsuperscript{2+} transients recorded at the periphery of both control and S100A1-treated neurons. The mean peak amplitude of the peripheral \textit{ΔF/F₀} signals in control neurons was 0.25 \pm 0.02 (n = 14). In S100A1-exposed neurons, the mean \textit{ΔF/F₀} amplitude at the peak of the Ca\textsuperscript{2+} transient was 0.37 \pm 0.04 (n = 14). The extracellular addition of S100A1 to the medium thus induced a significant increase in the mean peak amplitude of the peripheral \textit{ΔF/F₀} signals elicited by a single AP (\textit{P} < 0.05; Fig. 12B). S100A1 induced no kinetic modification of the rising phase of the AP-induced Ca\textsuperscript{2+} transient and only a slight, not statistically significant reduction was observed in the rate of the
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Decaying phase of the Ca\(^{2+}\) transient (Fig. 12C). The above results indicate that S100A1, by amplifying Ca\(^{2+}\) entry, increases the amplitude of AP-induced Ca\(^{2+}\) transients.

S100A1 Modulates Action Potential Duration

Changes in intracellular Ca\(^{2+}\) induced by S100A1, via an enhanced Ca\(^{2+}\) influx, could also affect neuronal excitability, in particular the AP. To characterize the action of S100A1 on SCG excitability, we evaluated passive and active membrane potential properties of 5-day-old cultured S100A1-dialyzed and control neurons. SCG neurons from 1- to 2-day-old cultures exhibit repetitive firing, whereas phasic firing patterns predominate in 5-day-old cultured SCG neurons (31). Figure 13, A and B, shows membrane potential recordings in response to subthreshold and suprathreshold current-clamp steps from control and S100A1-treated neurons. Resting membrane potential and AP height were unchanged between control and S100A1-dialyzed neurons [control neurons: resting membrane potential \(-61 \pm 5.0\) mV, AP height \(81 \pm 7.0\) mV \((n = 12)\); S100A1-treated neurons: resting membrane potential \(-60 \pm 5.0\) mV, AP height \(80 \pm 7.6\) mV \((n = 12)\); Fig. 13D] However, S100A1 induced a significant increase in the AP full duration at half-peak [control neurons: \(4.6 \pm 0.8\) ms \((n = 12)\); S100A1-treated neurons: \(6.5 \pm 1.3\) ms \((n = 12)\); \(P < 0.05\), Fig. 13, C and E]. Given the essential role of APs in the release of neurotransmitters, our results suggest that S100A1 may increase sympathetic output at the axon terminals.

DISCUSSION

The Ca\(^{2+}\) binding protein S100A1 displays a high expression level in cardiomyocytes and is considered an important regulator of heart function (55, 56). Compromised or damaged cardiomyocytes secrete S100A1 during ischemic periods (9, 43). It has been reported that exogenous S100A1 enhances Ca\(^{2+}\) entry in cultured cardiomyocytes (63). In this manner, S100A1 may amplify Ca\(^{2+}\) signals, and hence contractility of nonischemic neighbor areas, and function as a signal molecule for adaptive modifications during and after an ischemic episode. Although the effects of exogenous S100A1 have been documented in cardiac cells, it is unclear whether extracellular S100A1 exerts effects on other tissue types (i.e., blood vessels from local cardiac circulation and nerves), which may also be involved in regulatory and homeostatic responses. This work was aimed at investigating the effects of exogenous S100A1 on Ca\(^{2+}\) signals and electrical properties of SCG neurons, a neuronal population whose nerve projections innervate the heart.

Our data indicate that 1) no endogenous S100A1 is expressed in principal SCG neurons; 2) cultured SCG neurons take up S100A1 when it is present in the extracellular media; 3) inside the cell S100A1 localizes in a punctate pattern throughout the cytoplasm but is excluded from the nuclei; 4) both receptor-mediated and non-receptor-mediated endocytosis are involved in S100A1 internalization in SCG neurons; 5) axonal projections are capable of uptake and transport of S100A1 toward the neuronal somas via retrograde transport; 6) exogenous S100A1 enhances voltage-dependent Ca\(_{\text{v}1}\) currents in a PKA-dependent manner, which results in amplified Ca\(^{2+}\) entry and augmented Ca\(^{2+}\) transients; and 7) exogenous S100A1, via this increased Ca\(^{2+}\) influx, produces prolonged APs in cultured SCG neurons.

S100A1 is preferentially abundant in the heart, although the protein is also found in lower amounts in skeletal muscle, brain, and kidney (40, 41, 43). Predominant cardiac expression for S100A1 is also seen in several animal models such as mouse (43) and rat (41), recapitulating the human S100A1 expression profile (56). Our data show no detection of endogenous S100A1 in SCG neurons, supporting previous findings by others (15) and indicating a certain degree of cell specificity for S100A1.

Different S100 members are released or secreted into the extracellular space and exert trophic or proapoptotic effects (19). S100A2 and S100A7 act as chemotactic agents for leukocytes (46, 37). An S100A8/S100A9 complex and S100A12 modulate the proliferation of certain target cells and regulate the activation state of macrophages/microglia and other inflammatory cells (22, 34, 57, 81). S100B, the predominant S100 protein is also found in lower amounts in skeletal muscle, kidney, and heart. The local S100A1 expression profile (56). Our data show no detection of endogenous S100A1 in SCG neurons, supporting previous findings by others (15) and indicating a certain degree of cell specificity for S100A1.
concentration during acute myocardial ischemia is unknown; however, it is possible that locally released S100A1 may reach much higher concentrations (63), similar to that used in the present study. Our data showing S100A1 uptake by neuronal projections and translocation from distal axons to cell bodies suggest the hypothesis that S100A1 released from ventricular myocytes during acute ischemic episodes may exert remote modulatory actions on innervating sympathetic neurons.

Our results using endocytotic and organelle markers suggest that both non-receptor- and receptor-mediated endocytosis are involved in the internalization of S100A1. They also suggest that exogenous S100A1 was internalized and delivered to early and late endosomes, Golgi apparatus, and lysosomes. In this regard, it is possible that much of the internalized S100A1 ended up trapped in the organelles; however, the fact that S100A1 colocalized with dextran, a marker of fluid-phase endocytosis, suggests that S100A1 can enter the cytosol directly from the cell surface, likely through macrophagocytosis from plasma membrane lipid-rich domains and slow leakage into the cytosol from the macropinosomes (70, 77). However, the exact mechanism of internalization remains to be elucidated.

Some S100 members interact with cell surface receptors that transduce their effects (19). A plasma membrane target for S100B and S100A12, RAGE, has been identified on inflammatory and neural cells (18, 34). S100A4-induced signaling depends on interactions with heparan sulfate proteoglycans at the cell surface of hippocampal neurons (44). In our study, the observation that S100A1 enhanced Ca\(^{2+}\) entry when added intracellularly, via the patch pipette to bypass any potential membrane receptor-mediated cascade, suggests that no membrane receptor was required for this effect.

Within cells, S100 proteins are associated with multiple targets that regulate motility, transcription factors, Ca\(^{2+}\) homeostasis, and cell proliferation and differentiation (19). In both cardiac and skeletal muscle cells, S100A1 was shown to directly interact with the ryanodine receptor (56, 61, 79). In cardiomyocytes S100A1 also interacts with sarcoplasmic reticulum Ca\(^{2+}\)-ATPase/phospholamban complex, titin, and the mitochondrial F1-ATP synthase in complex V of the respiratory chain (56).

Interestingly, in this work we find that S100A1 markedly enhanced Ca\(^{2+}\) current density without changing the voltage dependence for activation. In addition we also find that S100A1 modified the kinetics of Ca\(^{2+}\) current inactivation. The use of specific channel blockers allowed us to identify Cav1 channels as the primary target for S100A1 modulation. As far as we know, in SCG neurons Cav1 currents are not augmented by natural neurotransmitters or neuropeptides. Indeed, the predominant effect of neurotransmitters/neuropeptides on Cav1 channels is inhibitory and occurs (in most cases) via G protein-coupled receptors (33, 38). On the other hand, in other cell types Cav1 currents are often augmented by phosphorylation. For example, in both cardiac and skeletal muscle, phosphorylation via cAMP-dependent PKA has been implicated in the augmentation of Ca\(_{\text{L}}\) channel currents (17). The
α-subunit of Cav1 channels has PKA phosphorylation sites (14, 16, 71). Here we show that the preincubation of neurons with a membrane-permeant PKA inhibitor prevented the enhancement of Cav1 current observed in S100A1-treated neurons. This result indicates that Cav1 channel enhancement induced by S100A1 depends on PKA activation. In cardiomyocytes a similar PKA-dependent mechanism of Cav1 channel modulation by S100A1 has been reported (63). When added to the extracellular media S100A1 enhanced Ca^{2+} entry, resulting in elevated cytosolic Ca^{2+} concentrations and enhanced Ca^{2+}-induced Ca^{2+} release via activation of PKA. Reppel et al. (63) concluded that S100A1 uptake resulted in an increment of PKA activity via a possible direct interaction between PKA and S100A1, but without increasing cAMP levels, suggesting the lack of a receptor-mediated effect. It is possible that cardiac and neuronal Cav1 Ca^{2+} channels share a similar mechanism for modulation by S100A1. In this scenario, Cav1 Ca^{2+} channel modulation by S100A1 might occur via an indirect mechanism that uses PKA. Our results of NMR chemical shift perturbation of Ca^{2+}-S100A1 in the presence of a peptide from the regulatory subunit of PKA verify that S100A1 directly interacts with PKA, and that this interaction likely occurs in the hydrophobic binding pocket of Ca^{2+}-S100A1, as seen in other S100 proteins (50, 61, 64, 79). Alternatively, more complex protein-protein interactions between S100A1-PKA complex and Cav1 α-subunit cannot be ruled out. The finding that S100A1 increased Ca^{2+} current density within 5 min after S100A1 dialysis via the patch pipette suggests a direct action of S100A1 on signaling pathways involved in channel modulation or trafficking mechanisms rather than upregulation of gene expression. Possible mechanisms to account for S100A1’s effect on neuronal Cav1 channels could be 1) an increment of the number of functional channels, via the recruitment of previously inserted silent channels that become active (17), 2) an increment of open probability or single-channel conductance, or 3) alternatively through a rapid recruitment of new Cav1 subunits to the plasma membrane. Finally, we find that S100A1 also modified the inactivation kinetics of the whole cell Ca^{2+} current. This, like all other effects of S100A1, was prevented by preapplication of nifedipine to S100A1-dialyzed neurons, which suggests that Cav1 channels are the main target of modulation by S100A1. Cav1 channels inactivate by a dual mechanism: voltage dependent, observable during prolonged depolarization, and calcium dependent, caused by Ca^{2+} ions entering the cell during channel opening (8, 10, 17). Several authors have reported the presence of two kinetic components in the time course of inactivation of Cav1 channels: a fast phase, corresponding to Ca^{2+}-dependent inactivation, and a slow phase, corresponding to voltage-dependent inactivation (23, 26, 39). Our results suggest that S100A1 is slowing Ca^{2+}-dependent inactivation and accelerating the voltage-dependent inactivation mechanism. Further studies are needed to address this issue.

Ca^{2+} is essential for neuronal function; in particular, it is required to trigger transmitter release when an AP arrives at the axon terminal (5, 42). Changes in Ca^{2+} entry modify AP

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Fig. 13. S100A1 prolongs action potential (AP) duration. Representative membrane potential recordings in response to 20-ms subthreshold and suprathreshold current-clamp steps from control (A) and S100A1-treated (B) neurons. C: superimposed AP recordings from a control (dark traces) and S100A1-treated (gray traces) neuron. Note the prolonged AP duration in the S100A1-treated neuron (arrow). D and E: summary of the effects of S100A1 on AP height (D) and full width at half-height (E) compared with control neurons. S100A1 induced a significant increase in AP full width at half-height [control 4.6 ± 0.8 ms (n = 12) vs. S100A1 6.5 ± 1.3 ms (n = 12); *P < 0.05].

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duration (52). Our results showing enhanced somatic Ca\(^{2+}\) entry, larger cytosolic Ca\(^{2+}\) transients, and prolongation of AP duration indicate that S100A1 may lead to an increase of transmitter release at sympathetic axon terminals, which in turn would result in an increase in heart rate and contractility of the myocardium. The preservation of cardiac contractility in the damaged heart through paracrine S100A1’s actions on the sympathetic nervous system may function as an adaptive mechanism protecting against cardiac failure. It is clear that an important goal of future studies will be to resolve the in vivo significance of extracellular S100A1 protein in the context of myocardial damage in more detail.

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