Molecular Mechanisms of Ca\(^{2+}\) Signaling in Neurons Induced by the S100A4 Protein

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The S100A4 protein belongs to the S100 family of vertebrate-specific proteins possessing both intra- and extracellular functions. In the nervous system, high levels of S100A4 expression are observed at sites of neurogenesis and lesions, suggesting a role of the protein in neuronal plasticity. Extracellular oligomeric S100A4 is a potent promoter of neurite outgrowth and survival from cultured primary neurons; however, the molecular mechanism of this effect has not been established. Here we demonstrate that oligomeric S100A4 increases the intracellular calcium concentration in primary neurons. We present evidence that both S100A4-induced Ca\(^{2+}\) signaling and neurite extension require activation of a cascade including a heterotrimeric G protein(s), phosphoinositide-specific phospholipase C, and diacylglycerol-lipase, resulting in Ca\(^{2+}\) entry via nonselective cation channels and via T- and L-type voltage-gated Ca\(^{2+}\) channels. We demonstrate that S100A4-induced neurite outgrowth is not mediated by the receptor for advanced glycation end products, a known target for other extracellular S100 proteins. However, S100A4-induced signaling depends on interactions with heparan sulfate proteoglycans at the cell surface. Thus, glycosaminoglycans may act as coreceptors of S100 proteins in neurons. This may provide a mechanism by which S100 proteins could locally regulate neuronal plasticity in connection with brain lesions and neurological disorders.

The S100 family is a group of vertebrate-specific Ca\(^{2+}\)-binding proteins with a highly conserved primary structure possessing both intra- and extracellular functions. Most S100 family members, including S100A4, are antiparallelly packed homodimers stabilized by disulfide bridges (reviewed in references 8 and 9). Intracellularly, S100 proteins are involved in a variety of processes, including the regulation of cytoskeletal dynamics, Ca\(^{2+}\) homeostasis, and cell proliferation and differentiation. Importantly, some S100 proteins can also be secreted, form oligomers owing to the nonreducing conditions of the environment, and exert their effects acting at the cell surface (10; 43; reviewed in reference 20). A plasma membrane target for S100B and S100A12, the receptor for advanced glycation end products (RAGE), has been identified on inflammatory and neuronal cells (14). However, RAGE is probably not the sole receptor for members of the S100 family, since the effects of extracellular S100A12 and S100B proteins can be observed in cells lacking RAGE (32), and some of these effects are RAGE independent in cells expressing the receptor (37).

The S100A4 (also termed Mts1) gene was isolated from tumor cells (11, 40), where its expression increased the ability of the tumor to metastasize. S100A4 has also been detected in healthy tissues, particularly in the nervous system. In both the brain and spinal cord, S100A4 expression appears in astrocytes shortly after the start of myelination, with the highest level observed in the areas in which neurogenesis takes place and in regions possessing high plasticity in adults (1). Moreover, in the peripheral nervous system, expression of S100A4 increases after sciatic nerve or dorsal root injury (25). Thus, the release of S100A4 from S100A4-positive astrocytes as a result of either secretion or cell damage might play a role in neuronal plasticity under normal and pathological conditions.

The importance of S100A4 in brain development and/or regeneration is supported by the fact that the protein is a potent promotor of neurite outgrowth in hippocampal neurons in vitro (28). Moreover, S100A4 acts as a neuroprotectant for primary neurons induced to undergo cell death (29). The molecular mechanism of this effect, including a receptor transducing S100A4 signals, has not been identified. However, S100A4-induced neurite outgrowth could be reduced by inhibitors of intracellular Ca\(^{2+}\) homeostasis (28). This indicates that extracellular S100A4 might affect the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), thereby modulating neuronal differentiation.

We show here that the differentiation-promoting oligomeric form of S100A4 increases [Ca\(^{2+}\)]\(_i\), in primary neurons, and we elucidate the molecular mechanism of this effect. We demonstrate that the S100A4-induced neurite outgrowth and [Ca\(^{2+}\)]\(_i\), rise are not mediated by RAGE but depend on an S100A4 interaction with heparan sulfate proteoglycans at the cell surface. Our results suggest that glycosaminoglycans may act as coreceptors of S100 in neurons and/or serve to immobilize S100 proteins in the extracellular matrix. This may provide a mechanism by which S100 proteins could locally regulate neuronal plasticity in connection with brain traumas and neurological disorders.

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MATERIALS AND METHODS

Materials. Tetrodotoxin, the inhibitor of voltage-gated sodium channels, the nonselective cation channel inhibitors fluconanide and spermicane, l-hanks' balanced salt solution (HBSS) were purchased from Sigma-Aldrich AS (Copenhagen, Denmark). A modified HBSS was prepared by adjusting the Ca\(^{2+}\) concentration to 2 mM at pH 7.4. The cyclic AMP (cAMP) antagonist 2',5'-dideoxycadenosine (dd-Ado), the receptor tyrosine kinase inhibitors lavine, genistein, the antagonist of G\(_{\alpha}\) proteins, pertussis toxin, blockers of voltage-operated calcium channels of the T type (flunarizine), L type (nifedipine), M type (omega-conotoxin MVIIA), and P/Q type (omega-conotoxin TG), and carboc elor were all obtained from Calbiochem (Merck, Darmstadt, Germany). The phospholipase C (PLC) inhibitor U-73122 and the diacylglycerol (DAG)-lipase inhibitor RHC-80267 were purchased from Biomol (Butler Pike, PA). A T-type Ca\(^{2+}\) channel inhibitor, mibebradil (dihydrochloride salt), was a gift from Hoff mann La Roche (Basel, Switzerland). Recombinant S100A4 and S100A12 were cloned and produced as described previously (39).

Primary cultures of rat hippocampal neurons and analysis of neurite outgrowth. Hippocampal and cerebellar neurons were prepared from E19 rat embryos and P3 rat pups as previously described (26, 29, 38). Cultures were seeded at a density of 10,000 cells/cm\(^2\) in eight-well LabTek tissue culture chambers with neurobasal media. Under these conditions, unstimulated cells extended few neurites after 24 h in vitro; however, \(>90\%\) of the neurons remained alive (unpublished data) and maintained physiological levels of cytoplasmic Ca\(^{2+}\) (unpublished data). Hippocampal cultures of primary neurons were seeded in eight-well poly-l-lysine-coated LabTek coverglass slides (Nunc) at a density of 50,000 cells/well and grown for 7 to 10 days. Cytose arabinofuranoside (final concentration, 5 \(\mu\)M; Sigma) was added after 2 days in vitro to inhibit glial cell proliferation. To test whether RAGE was involved in 100 protein-induced neuritogenesis, hippocampal neurons were preincubated with different dilutions of inhibitory antibodies to RAGE and subsequently stimulated with S100A4 and S100A12 (1 \(\mu\)M each) or plated on an amphotlin substrate (100 nmol/cm\(^2\)). The time of preincubation was 1 h for S100A4 and S100A12 and 15 min for the amphotelin substrate. To test the effect of the SA4BP peptide on S100A4 and S100A4-induced neurite outgrowth, recombinant human S100A4 and S100A4 (1 \(\mu\)M each) were pretreated with the SA4BP peptide (molar ratios from 1:20 to 1:100) for 1 h and further applied to the cultures of hippocampal neurons. Analysis of neurite outgrowth was performed as previously described (38).

Fluorescent Ca\(^{2+}\) measurements. Dye loading and experiments were performed in modified HBSS at room temperature (22 to 24°C). Since S100A4 is known to bind Ca\(^{2+}\) with a \(K_d\) of \(-100\) \(\mu\)M (with binding affecting functional properties of the protein), solutions with nominally zero calcium could not be used in the experiments. Instead, low-calcium HBSS was used, which was obtained by decreasing the Ca\(^{2+}\) concentration to 200 \(\mu\)M and adding C\(_{6}\)Cl\(_4\) to the solution (final concentration of 200 \(\mu\)M). The Ca\(^{2+}\) concentration in the neurons was measured in eight-well poly-l-lysine-coated LabTek coverglass slides (Nunc) at a density of 50,000 cells/well and grown for 12 to 14 days. Attached cells were loaded with the Ca\(^{2+}\)-sensitive dye fura-2 AM (5 \(\mu\)M; Molecular Probes, Eugene, Ore.) or fluo-4 AM (2 \(\mu\)M; Molecular Probes). To eliminate the effect of synaptically induced Ca\(^{2+}\) influx, all experiments were performed in the presence of tetrodotoxin (1 \(\mu\)M). The regions of interest were positioned on somata of the neurons, which were identified by their morphological characteristics. Data were collected from at least three sets of cultures obtained from different animals; each experiment provided simultaneous measurements for up to eight neurons.

Transmembrane potential measurements. The transmembrane potential of neurons was determined using oxonol-6 (diBAC\(_{4}(3)\)) (4), a fluorescent membrane potential-sensitive dye (1 \(\mu\)M; Molecular Probes), according to the manufacturer's instructions. Dye loading and experiments were performed in modified HBSS at room temperature (22 to 24°C). Fluorescence intensity traces from individual neurons were obtained by monitoring the average overall intensity of the Ca\(^{2+}\)-sensitive dye fura-2 AM (5 \(\mu\)M; Molecular Probes) before and after the addition of the Ca\(^{2+}\)-specific dye fura-2 AM (5 \(\mu\)M; Molecular Probes) to the medium. Transmembrane potential changes of neurons were determined using Imaging Workbench software (Axon, Foster City, CA). Ratio images (340/380-nm excitation; 510-nm LP emission) were collected after background subtraction at 2 to 5 s intervals. The calibration constants and the estimated [Ca\(^{2+}\)]\(_{i}\) were calculated from a fluorescence ratio, \(R\), as described previously (33).

In experiments employing fluo-4 AM, a confocal laser scanning system (equipped with a Radiance 2000 argon laser (Bio-Rad, NJ)) connected to a Nikon Eclipse TE 200 microscope (Tokyo, Japan) (1.3 numerical aperture, \(>60\) oil immersion objective) and the Lasersharp 2000 software package (Bio-Rad) were used for image acquisition and processing. Fluo-4 was excited using a 488-nm line of a 14-mW argon-ion laser; emitted fluorescence was detected at 510 ± 15 nm at 2-s intervals. The laser light intensity was reduced to 1 to 3% of the nominal intensity by using neutral filters to avoid photodamage of the cells and dye bleaching. Fluorescence intensity traces from individual neurons were obtained and processed as described previously (18).

SPP analysis. Experiments employing surface plasmon resonance (SPR) were performed using a BIAcore-X or BIAcore 2000 instrument (Biacore, Uppsala, Sweden). Two methods of ligand immobilization were used, yielding similar results. In the first method, 3,500 resonance units (RU) of a recombinant human RAGE/Fc chimera (extracellular domain; R&D Systems, Minneapolis, Minn.) was immobilized covalently on a CM5 sensor chip (BIAcore) according to the manufacturer's instructions. In the second method, 3,500 RU of the same RAGE/Fc chimera was immobilized with biotinamidoacrapo G\(_{\alpha}\)-hydrosucinimide ester (Sigma) was immobilized on an SA sensor chip (BIAcore) with preimmobilized streptavidin. For the analysis of competitive binding of S100A4 and S100A4 to RAGE, 1,200 RU of RAGE was immobilized covalently on a CM5 sensor chip. To study S100A4 binding to S100A4, 1,400 RU of oligomeric S100A4 was immobilized covalently on the sensor chip. Binding of recombinant amphotelin (0.025 to 1.0 \(\mu\)M; R&D Systems), S100A4 (0.2 to 7.5 \(\mu\)M), or S100A12 (0.3 to 9.2 \(\mu\)M) to RAGE and data analysis were performed as described previously (38). Three independent experiments were performed. To analyze binding of heparin to S100 proteins, heparin (1 mg/ml) was biotinylated and immobilized on an SA sensor chip with preimmobilized streptavidin (BIAcore) (see reference 23 for details).

Immunoblotting assay. Hippocampal neurons were seeded in 60-mm tissue culture dishes (10\(^5\) cells) and grown for 4 h before treatment. Immunoblotting was performed as described previously (24). Rabbit anti-phospho-PLC\(_1\) antibody (diluted 1:500; Cell Signaling), rabbit anti-phospho-GAP-43 antibodies (Ser41, diluted 1:1,000; Cell Signaling), or rabbit anti-phospho-ERK1,2 antibodies (Tyr204, diluted 1:1,000; Cell Signaling) were used. Protein bands were visualized using the enhanced chemiluminescence substrate Western Dura (Pierce Biotech, Rockford, IL) and processed with the GenTools software package (Syngene, Cambridge, United Kingdom). To estimate the total amount of PLC\(_1\), PLC\(_2\), or GAP-43 antibodies on the cell membrane, 50 cells in each sample were counted and multiplied by the number of cells in the sample. 

Statistics and graphical presentation. Statistics and graphical presentations were carried out using the Origin, version 6.0, software package (OriginLab, Northampton, NY). Statistical evaluations were performed using two-sided Student's t test. The results are given as means ± standard errors of the means. Unless stated otherwise, asterisks in figures indicate statistical significance compared to the controls, as follows: *; \(p < 0.05\); **; \(p < 0.01\); and ***; \(p < 0.001\).

RESULTS

Rapid neurite induction by S100A4 involves GAP-43 phosphorylation. It has previously been shown (28) that oligomeric but not dimeric S100A4 induces the differentiation of primary hippocampal neurons. We determined the time-response relationship of the neurotrophic effect of S100A4. Cells stimulated with dimeric S100A4 remained morphologically quiescent even after 4 hours of treatment (Fig. 1A). In contrast, oligomeric S100A4 had already induced lamellipodium formation 5 minutes after application (Fig. 1B). Fifteen minutes after application, the total area of lamellipodia had increased, and solitary

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Filopodia appeared (Fig. 1C). After 4 hours, the formation of primary neurites was observed (Fig. 1D). The induction of neurite outgrowth by S100A4 was associated with phosphorylation of growth-associated protein 43 (GAP-43) by activated protein kinase C (PKC) on a specific site, Ser41, where phosphorylation is known to be essential for neurite elongation in primary neurons (5). In Fig. 1E, it can be seen that S100A4 had already induced phosphorylation of GAP-43 30 min after the start of treatment; the increase in the amount of the phosphorylated form was comparable to that induced by the PKC agonist phorbol 12-myristate 13-acetate and by an activator of PLC/H9252-mediated signaling, carbachol, which activates PKC downstream.

S100A4-induced neurite outgrowth is mediated by a cell surface receptor(s) different from RAGE. Inside the cell, S100A4 is known to bind various target proteins affecting numerous signaling pathways (8). We therefore investigated whether S100A4 enters the cytoplasm after addition to the culture medium. In 7-day in vitro neuronal cultures not treated with S100A4, double immunostaining for GAP-43/S100A4 demonstrated the presence of a few (<0.5% of the total population) S100A4-positive cells that were negative for the neuronal marker GAP-43, probably representing contaminating astrocytes, which are known to express S100A4. Conversely, neurons that were distinctly stained for GAP-43 at the plasma membrane exhibited no trace of S100A4 in the cytoplasm (Fig. 1F). Similarly, no intraneuronal staining for S100A4 was observed in S100A4-treated cultures either 10 min (Fig. 1G) or 30 min (Fig. 1H) after the start of treatment. However, a thin layer or small “blocks” of S100A4 were detected on the surface of the culture dish and on cell membranes, apparently corresponding to extracellularly bound S100A4 (Fig. 1G and H, vertical sections). The intensity of surface staining for S100A4 increased the longer the treatment lasted (compare vertical sections in Fig. 1G and H). Interestingly, the fraction of S100A4-positive cells was significantly higher for S100A4-treated than untreated cultures (2% versus 0.5%, respectively), indicating that some glial cells might take up S100A4 when exposed to the protein.

The same pattern of immunostaining was observed when 6-hour or 7-day in vitro cultures were treated with S100A4 for 5 hours.
FIG. 2. Role of RAGE in S100A4-stimulated neurite outgrowth. (A) Expression of RAGE in primary hippocampal (HN) and cerebellar (CN) neurons, PC12E2 cells, and VMR cells (mouse adenocarcinoma cell line). (B) SPR analysis of binding of immobilized RAGE with recombinant amphoterin, S100A4, S100A12 (1 μM [all]), or S100A2 (10 μM). RU, resonance units. (C) Effect of inhibitory antibodies to RAGE on S100A4-, S100A4-, S100A12, and S100A2 (10 μM). RU, resonance units.
min, 20 min, 1 h, 4 h, or 24 h (data not shown). Taken together, these data suggest the existence of a plasma membrane target for S100A4. This target rapidly initiates intracellular signaling, resulting in distinct morphological changes within minutes.

To date, only one plasma membrane receptor, RAGE, for S100 proteins (S100B, S100A1, and S100A12) has been identified in neuronal cells (14). RAGE was found to be expressed in cultures of primary hippocampal and cerebellar neurons (Fig. 2A). We therefore examined whether S100A4 is also able to interact directly with RAGE employing SPR analysis. The established RAGE ligands, amphoterin and S100A12, were used as positive controls. As a negative control, we used the S100A2 protein, which in our experiments had no effect on neurite extension from primary hippocampal neurons (not shown). Earlier, radioligand-binding studies showed RAGE to bind amphoterin and S100A12, with \( K_d \) of 6.4 ± 1.0 nM and 91 ± 29 nM, respectively (14, 15). In accordance with these observations, we found that both amphoterin and S100A12 interacted with RAGE, with \( K_d \) of 6.5 ± 1.9 nM and 79 ± 19 nM, respectively. S100A4 also bound RAGE, although with a lower affinity (\( K_d = 138 \pm 28 \) nM), whereas binding of S100A2 to RAGE was negligible (Fig. 2B).

Since RAGE was expressed in primary neurons and interacted with S100A4, we next checked whether RAGE was involved in S100A4-induced neurite outgrowth. However, in our experiments, inhibitory antibodies specific for the extracellular part of RAGE did not affect S100A4-induced neurite outgrowth, whereas they markedly inhibited neurite extension stimulated by either S100A12 or amphoterin (Fig. 2C). Thus, the extracellular part of RAGE seemed to be required for S100A2- or not for S100A4-induced neurite outgrowth.

In order to further characterize the S100A4-induced neurite outgrowth, we used the peptide SA4BP (HSLRSWDVSPNT GGC), identified by a phage display library approach, which specifically bound S100A4, as confirmed by a phage capture assay (J. Klingelhofer et al., unpublished data). When S100A4 or S100A12 was preincubated with SA4BP, the S100A4- and not S100A12-induced neuritogenic response was inhibited in a dose-dependent manner (Fig. 2D). This suggested that the peptide either specifically bound to and blocked the site in S100A4 responsible for the interaction with the receptor transducing the neurite outgrowth signal or changed the conformation of S100A4 so that the protein lost the ability to interact with its receptor. In both cases, if this receptor were RAGE, one would expect that S100A4-RAGE binding would also be affected by SA4BP. To test this, binding of S100A4 or S100A12 to RAGE was compared in the absence and presence of SA4BP (molar ratio of 100 to SA4BP, 1:100 to 1:200). However, no effect of the peptide on S100A4 or S100A12 binding to RAGE was observed (Fig. 2E), indicating that the putative neurite-inducing sites of S100A4 probably do not interact with RAGE.

The dimeric form of S100A4 has been shown to be incapable of inducing cellular responses, even when applied at high concentrations. Therefore, we tested whether reduction of the oligomeric S100A4 protein with dithiothreitol, resulting in the formation of dimers and monomers (Fig. 2F, inset), affected the binding to RAGE. As expected, the resonance response to dimeric/monomeric S100A4 was lower than that to oligomeric S100A4 (Fig. 2F), since the molecular mass of the ligand was decreased. However, the \( K_d \) of S100A4 for RAGE was not changed, reflecting similar affinities of reduced and oligomeric S100A4 for RAGE. Thus, the neuritogenically ineffective form of S100A4 was still able to interact with RAGE, further indicating that the neurite-promoting effect of S100A4 is not mediated by RAGE.

It is possible that S100A4 and S100A12 interact with different sites on the RAGE molecule. This might be the reason that the SA4BP peptide and RAGE antibodies have different effects on S100A4- and S100A12-induced neurite outgrowth as well as on the binding of the proteins to RAGE. Therefore, we tested whether S100A4 and S100A12 bind to RAGE in a competitive manner. S100A12 and polymeric S100A4 were applied to a chip with immobilized RAGE either separately (Fig. 2G, A12 and A4) or simultaneously (Fig. 2G, A12 + A4). As shown, the resonance response to the simultaneous addition of the proteins did not differ from the response to the S100A12 protein applied alone. This agrees with the observation that the affinity of S100A12 for RAGE is higher than that of S100A4. Thus, upon simultaneous addition of the two proteins, the majority of binding sites of RAGE are expected to be occupied by molecules of S100A12, resulting in association and dissociation dynamics close to those of S100A12 alone. The experiments performed using dimeric/monomeric S100A4 yielded similar results (not shown). In control experiments, S100A12 did not bind S100A4 (not shown). Thus, S100A12 and S100A4 interacted with RAGE competitively, probably sharing the binding site on the RAGE molecule. To confirm this, we tested whether the interaction between S100A4 and immobilized RAGE was affected by prior binding of S100A12 to RAGE. As shown in Fig. 2H, a robust resonance response to S100A4 was observed in the absence of S100A12 (A4ctl). When the same concentration of S100A4 was applied during injection of S100A12 (Fig. 2H, A12+ A4), no additional response was detected compared to that induced by injection of S100A12 alone (Fig. 2H, A12ctl). Thus, it can be assumed that...
S100A12 and S100A4 interacted with the same or significantly overlapping regions of RAGE and that “saturation” of immobilized RAGE by S100A12 decreased the number of binding sites available for subsequent interaction with S100A4.

Oligomeric but not dimeric S100A4 induces a rise of $[\text{Ca}^{2+}]_i$ involving voltage-dependent Ca$^{2+}$ channels (VDCC) and affects intracellular Ca$^{2+}$ stores. The rapid and pronounced morphological changes induced by oligomeric compared to dimeric S100A4 in hippocampal (Fig. 3A and B) and cerebellar (Fig. 3D and E) neurons may be partially accounted for by a change of the cytoplasmic Ca$^{2+}$ level. Indeed, we found that oligomeric S100A4 elevated $[\text{Ca}^{2+}]_i$ in hippocampal neurons grown for either 6 h or 7 to 14 days in vitro (Fig. 3C) as well as in cerebellar neurons grown for 3 to 7 days in vitro (Fig. 3F). The Ca$^{2+}$ response was induced 1 to 2 seconds after protein application, strengthening the contention that S100A4 was acting extracellularly. Interestingly, no Ca$^{2+}$ response to dimeric S100A4 was observed (Fig. 3C and F), indicating that the S100A4-induced $[\text{Ca}^{2+}]_i$ rise correlated with the capability for neurite induction of the protein. In addition, the time course of the S100A4-induced Ca$^{2+}$ responses correlated with that of the S100A4-induced phosphorylation of ERK1 and -2.
N-type VDCC, which can be selectively blocked by increased Ca$_{2+}$, evoked the rise in intracellular Ca$_{2+}$ transient could be induced by these agonists before overloading the stores (not shown). After being overcharged, neurons acquired the ability to release Ca$_{2+}$ from the lumen of the ER, suggesting that Ca$_{2+}$ transient could be induced by these agonists before overloading the stores (not shown).

To investigate whether the application of S100A4 evoked a Ca$_{2+}$ release from the ER stores. Cultured hippocampal neurons have been reported to possess overlapping Ca$_{2+}$- and inositoltrisphosphate (IP3)-sensitive stores which are functionally emptied at rest (17). Nevertheless, raising [Ca$_{2+}$]$_{i}$ for a period of time enhances Ca$_{2+}$ uptake into the stores so that more Ca$_{2+}$ eventually becomes available for release (12). In our experiments, the stores were overcharged by depolarization with 60 mM KCl at an external Ca$_{2+}$ concentration of 2 mM for 3 min. After being overcharged, neurons acquired the ability to respond to activators of Ca$_{2+}$- and IP3-induced calcium release (caffeine and carbachol, respectively), whereas no Ca$_{2+}$ transient could be induced by these agonists before overloading the stores (not shown). To estimate the S100A4-induced Ca$_{2+}$ release from the ER, the overcharged stores were challenged with S100A4 in a low-Ca$_{2+}$ medium (see Materials and Methods) ca. 1 min after termination of the charge. A long-lasting, low-amplitude [Ca$_{2+}$]$_{i}$ transient was observed (Fig. 5A, left panel). After the S100A4-induced response, caffeine was still able to release Ca$_{2+}$ from the lumen of the ER, suggesting that S100A4 did not empty the caffeine-sensitive stores (Fig. 5A, left panel). When caffeine was applied before S100A4, thereby

![FIG. 4. Effects of different blockers of VDCC on S100A4-induced [Ca$_{2+}$]$^	ext{2-}$ rise (A) and neurite outgrowth (B) in primary hippocampal neurons.](Image)
emptying the stores, the cells were no longer able to mount a Ca$^{2+}$ response to S100A4 (Fig. 5A, right panel), indicating that S100A4 did indeed induce Ca$^{2+}$ release from the ER. In the fluorometric experiments, we did not attempt to determine whether S100A4 triggered Ca$^{2+}$ release via an IP3- or Ca$^{2+}$-induced mechanism (ICR and CICR, respectively) or both. However, in the neurite outgrowth assay, xestospongin, which blocks ICR, but not ryanodine, which at high concentrations blocks CICR, inhibited S100A4-induced neurite elongation (Fig. 5B). This suggests the involvement of ICR, and thus a phosphoinositide-specific PLC isoform(s), in S100A4-induced intracellular signaling.

**S100A4-induced $[Ca^{2+}]_i$ rise involves nonselective cation channels and requires activation of the PLC–DAG–DAG-lipase signaling pathway.** We next checked whether other types of ion channels besides T- and L-type VDCC were involved in the S100A4-induced $[Ca^{2+}]_i$ rise. To test this, the $[Ca^{2+}]_i$ and plasma membrane potential of individual cells stimulated with S100A4 were monitored at a physiological or low level of extracellular Ca$^{2+}$ (Fig. 6A and B, respectively). As shown, oligomeric S100A4 evoked a pronounced $[Ca^{2+}]_i$ rise (Fig. 6A) and plasma membrane depolarization (Fig. 6B) in full Ca$^{2+}$ medium, whereas the dimeric form of the protein failed to affect these parameters. In low-Ca$^{2+}$ medium, the S100A4-induced Ca$^{2+}$ responses were inhibited ca. 95% compared to those in full Ca$^{2+}$ medium (Fig. 6A); however, the S100-triggered change of the membrane potential was only partially decreased (Fig. 6B). This suggested that ion channels permeable for ions other than Ca$^{2+}$ opened in response to S100A4 and that those might mediate the initial depolarization, subsequently resulting in the opening of T- and L-type VDCC.

We thus tested whether nonselective cation channels (NSCC) (reviewed in reference 13) were involved in S100A4-induced signaling. Indeed, two different inhibitors of nonselective Ca$^{2+}$ entry, Loe-908 and SKF-96562, decreased the S100A4-induced Ca$^{2+}$ response (Fig. 6C and D), as well as neurite elongation (Fig. 6E), in a dose-dependent manner. In addition, S100A4-induced cellular effects were sensitive to spermine and flufenamic acid, other known inhibitors of NSCC, particularly of transient receptor potential (TRP) family channels (not shown). This indicated that the S100A4-evoked nonselective Ca$^{2+}$ entry, presumably alongside with entry via T-type VDCC, contributed to the initial plasma membrane depolarization, resulting in the opening of the L-type VDCC and a $[Ca^{2+}]_i$ rise in the bulk of the cytoplasm.

In many cell types, activation of NSCC is dependent on a phosphoinositide-specific PLC-associated signaling cascade. In this mechanism, the activation of PLC stimulates hydrolisis of phosphatidylinositol 4,5-bisphosphate to IP3 and DAG, which can be further hydrolyzed by DAG-lipase to 2-arachidonoyl-glycerol (2-AG) and, subsequently, arachidonic acid (AA). All of these messengers activate NSCC (13). We thus checked whether PLC was involved in the cytoplasmic Ca$^{2+}$ elevation induced by S100A4. Indeed, pretreatment with a low dose (1 μM) of the phosphoinositide-specific PLC inhibitor U-73122 almost completely blocked the Ca$^{2+}$ response to S100A4 (Fig. 6F). Moreover, blocking DAG-lipase with the specific inhibitor RHC-80267 led to a dose-dependent inhibition of S100A4-triggered Ca$^{2+}$ transients (Fig. 6G) and neurite outgrowth (Fig. 6H). This ruled out a direct role of DAG in the induction of the S100A4-triggered Ca$^{2+}$ response and, together with our previous findings, indicated that S100A4-triggered signaling required phosphoinositide-specific PLC and that the hydrolysis of DAG to 2-AG and/or AA was necessary for the induction of the cellular response to S100A4.

**Effects of inhibitors of Ca$^{2+}$ homeostasis on spontaneous neurite outgrowth from primary neurons.** To test whether S100A4-induced neurite outgrowth specifically depended on the activation of Ca$^{2+}$-regulating mechanisms by the protein, we evaluated the effects of inhibitors of these mechanisms on spontaneous neurite outgrowth from primary neurons. To induce spontaneous neurite extension, cells were plated on monolayers of 3T3 fibroblasts, a physiological neuritogenic cue for primary neurons. As can be seen in Fig. 7, blocking of T-
and L-type VDCC (pimozide and nifedipine, respectively), NSCC (SKF-96365), and DAG-lipase (RHC-80362) all reduced spontaneous neurite outgrowth. However, the effects of the inhibitors on spontaneous neurite extension were 30 to 50% lower than their effects on S100A4-stimulated neuritogenesis (compare to Fig. 4B and 6E and H). Thus, mechanisms of Ca\(^{2+}\)/H\(_{11001}\) homeostasis activated by S100A4 might specifically contribute to S100A4-triggered neurite outgrowth.

**S100A4-induced intracellular signaling does not occur via activation of receptor tyrosine kinases (RTKs) or PLC\(_{\gamma}\) but depends on the activation of a heterotrimeric G protein-coupled pathway.** The inhibitor of phosphoinositide-specific PLC, U-73122, employed in our study has recently been shown to more potently inhibit PLC\(_{\beta}\) than PLC\(_{\gamma}\) (42), indicating that PLC\(_{\gamma}\) might play a minor role, if any, in the transmission of the S100A4-induced signal. We therefore checked whether either of the two known PLC\(_{\gamma}\) isoforms was activated by S100A4. Since PLC\(_{\gamma}2\) expression was not detected in hippocampal or cerebellar neurons (Fig. 8A), only the PLC\(_{\gamma}1\) isoform could, in principle, be activated. Thus, we checked whether PLC\(_{\gamma}1\) was phosphorylated by S100A4 on the Tyr783 residue, since this phosphorylation is known to be both necessary and sufficient for PLC\(_{\gamma}1\) activation (21). However, treatment with S100A4 for 5, 30, or 60 min did not result in phosphorylation of PLC\(_{\gamma}1\), whereas fibroblast growth factor 2 (FGF2), known to activate PLC\(_{\gamma}1\) via its receptor, caused a significant increase in phos-

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**FIG. 6.** PLC, DAG-lipase, and nonselective cation channels participate in S100A4 signaling. (A and B) Effects of S100A4 on [Ca\(^{2+}\)]\(_i\) (A) and transmembrane potential (B) at physiological and low levels of extracellular Ca\(^{2+}\). (C to E) Effects of two different inhibitors of nonselective cation entry, SKF-96565 and Loe908, on S100A4-evoked [Ca\(^{2+}\)]\(_r\), rise (C and D, respectively) and neurite elongation (E). (F) Effects of a blocker of phosphoinositide-specific PLC, U17352, on S100A4-induced [Ca\(^{2+}\)]\(_i\) elevation. (G and H) Dose-dependent inhibition of S100A4-triggered Ca\(^{2+}\) response (G) and neurite outgrowth (H) by a blocker of DAG-lipase, RHC-80267. In all fluorometric experiments, \(n = 20\) to 40.
phorylation of the enzyme (Fig. 8B). Thus, PLCγ did not participate in signaling initiated by S100A4.

Receptors capable of activating phosphoinositide-specific PLCs are mainly associated with RTKs or heterotrimeric G proteins (31). A nonspecific antagonist of RTKs, lavendustin A, was not capable of blocking S100A4-induced neurite outgrowth or Ca²⁺ entry (Fig. 8C and D, respectively). In control experiments, pretreatment with lavendustin A completely prevented the [Ca²⁺]i rise induced by synthetic ligands of the neural adhesion molecule NCAM, which are known to activate RTK-associated signaling pathways (not shown). Genistein, an inhibitor of a broad range of protein tyrosine kinases, including RTKs, did not have any effect on S100A4-induced neurite outgrowth either (Fig. 8D). However, an inhibitor of G protein-mediated signaling, suramin, completely abrogated the S100A4-triggered neuritogenesis (Fig. 8C) and Ca²⁺ rise (Fig. 8D). Suramin is not a specific G protein antagonist, since it not only uncouples G proteins from receptors but also inhibits the binding of growth factors at the cell surface (41) and decreases the activity of protein tyrosine phosphatases (PTPs) (27). However, as mentioned above, our data indicated that RTKs were not activated by S100A4. In addition, a broad-range inhibitor of PTP activity, sodium orthovanadate, had only a moderate effect on S100A4-induced neurite outgrowth and no effect on the S100A4-triggered Ca²⁺ responses (not shown). Thus, even though PTPases might be involved in S100A4-induced neuritogenesis, they seem to have no role in the Ca²⁺ signaling induced by the protein. Therefore, the blocking effect of suramin on Ca²⁺ responses could be attributed, at least in part, to the inhibition of G protein activity. Neither the G12,13- nor Gq/-associated signaling pathway participated in S100A4-triggered signaling, since the respective inhibitors of these pathways (pertussis

FIG. 7. Effects of inhibitors of Ca²⁺ homeostasis on neurite extension from hippocampal neurons grown on top of monolayers of 3T3 fibroblasts for 24 h. A value of 100% indicates neurite outgrowth in the absence of inhibitors. Pim, pimoside; SKF, SKF-90365; Nif, nifedipine; RHC, RHC-80362.

FIG. 8. S100A4-induced cellular responses do not depend on PLCγ/RTK but probably depend on a G protein-associated signaling cascade. (A) Expression of PLCγ2 in primary hippocampal (HN) and cerebellar (CN) neurons and PC12E2 cells. (B) Primary hippocampal neurons were treated with 5 μM S100A4 or 10 nM FGF2 for the indicated lengths of time and further immunoblotted for Tyr783-phosphorylated PLCγ1. Representative immunoblots of two to four individual experiments are shown. (C and D) Effects of inhibitors of RTKs (genistein and lavendustin A), a nonspecific blocker of G protein-associated signaling (suramin), and a specific blocker of Gq protein-associated signaling (GP-2A) on S100A4-induced neurite extension (C) and Ca²⁺ responses (D). Lav A, lavendustin A; Sur, suramin; Gq inh, GP-2A. The concentrations of the inhibitors in fluorometric experiments were as follows: lavendustin A, 20 μM; suramin, 100 μM, GP-2A, 10 μM.
toxin for G\textsubscript{\alpha}\textsubscript{q}–associated signaling and H-89 or 2',5'-dd-Ado for G\textsubscript{\alpha}-adenylate cyclase-PKA-associated signaling [35]) did not affect S100A4-induced cellular responses (not shown). However, both S100A4-induced neurite outgrowth and Ca\textsuperscript{2+} responses were inhibited by a competitive blocker of G\textsubscript{\alpha}\textsubscript{q}-associated signaling, GP-2A (Fig. 8C and D, respectively). Thus, S100A4 most probably triggered a cascade associated with one or more members of a G\textsubscript{\alpha}\textsubscript{q}/11 class of proteins and a phosphoinositide-specific PLC (presumably PLC\textsubscript{\beta})

S100A4-induced neurite outgrowth is partially dependent on interactions with heparan sulfate proteoglycans. The S100A8 and S100A9 proteins have previously been shown to bind heparin and heparan sulfate glycosaminoglycans on the surfaces of endothelial cells (32). We therefore employed SPR analysis to check whether S100A4 and/or S100A12 was also capable of binding heparin. Indeed, both proteins interacted with heparin immobilized on the surface of a sensor chip, with K\textsubscript{D} of 53 ± 12 nM and 27 ± 5 nM, respectively (Fig. 9A, lines A4 and A12). The observed binding was specific, since it was inhibited by preincubation of S100A4 and S100A12 with sucrose octosulfate (SOS [a chemical analogue of heparin]) as well as with heparin itself (Fig. 9A).

In accordance with the SPR analysis results, both S100A4- and S100A12-induced neuritogenic responses were significantly lower in the presence of heparin (Fig. 9B). Moreover, the Ca\textsuperscript{2+} responses to S100A4 were decreased by heparin in a dose-dependent manner (Fig. 9C). Heparin itself did not affect neurite outgrowth (not shown) and had a minor effect on [Ca\textsuperscript{2+}]\textsubscript{i} at a dose of 3 mg/ml (Fig. 9C). The maximum degree of inhibition of both S100A4-induced neurite outgrowth and Ca\textsuperscript{2+} responses was ca. 50%. In addition, enzymatic removal of heparan sulfates from the cell surface by treatment with heparinase I significantly reduced both S100A12- and S100A4-induced neurite extension (Fig. 9D), emphasizing the importance of these proteoglycans for S100A12 and S100A4 function. Thus, heparan sulfate moieties on the cell surface and/or in the extracellular matrix may function as coreceptors for both S100A4 and S100A12. In particular, S100A12 seems to require the presence of both heparan sulfates and RAGE to induce neuronal differentiation.
DISCUSSION

In this study, we have investigated the mechanism underlying the induction of differentiation and a [Ca^{2+}], rise in primary neurons by S100A4. Interestingly, only the oligomeric form of S100A4 was capable of triggering these responses. Several reasons might account for this. Firstly, the conformation of S100A4 in dimeric form may be different from that of S100A4 in oligomeric form, such that the sites responsible for the binding to its target are not exposed in the dimer. Secondly, S100A4 may activate target proteins by clustering them; in that case, oligomeric S100A4 will be more potent because of a larger number of potential binding sites.

No quantitative data are available on the concentration of S100A4 in the extracellular space under physiological conditions and/or after neural injuries. However, expression of the protein has been demonstrated in a variety of neural cells, and it is strongly increased after brain traumas and nerve lesions (25, 34). Moreover, secretion of S100A4 in vitro has been reported, with the concentration of the secreted protein in the culture medium estimated to be about 10 µM (10), which is comparable with the S100A4 concentrations used in this study.

Thus, given the relatively low volume of the extracellular space, physiological secretion of S100A4 and/or its release from damaged neural cells after neuronal injuries might provide sufficiently high local concentrations of S100A4.

The effect of S100A4 seemed to be mediated by a plasma membrane receptor. Several lines of evidence support this suggestion. Firstly, staining for S100A4 was detected only at the cell surfaces of neurons treated with the protein. Secondly, the effect of S100A4 on [Ca^{2+}] was observed within 2 to 3 seconds, a time span which is too short for the protein to penetrate the plasma membrane via endocytosis or passive diffusion, with the latter being hampered by the large size of the protein (ca. 40 kDa for the tetramer). Thirdly, the induced signaling was abolished by inhibition of PLC, further confining the site of S100A4 action to the membrane. Finally, suramin, known to block the binding of agonists to G protein-associated receptors and RTKs, completely prevented the induction of cellular responses to S100A4.

It has long been known that S100A4, along with other proteins of the S100 family, including S100A1, S100A12, and S100B, serves as a potent glia-derived inducer of neuronal differentiation and as a survival factor. However, the receptor(s) responsible for the S100A4-induced signal transduction has not been identified. A clue came from a study (14) where RAGE was shown to interact with S100A12. The signaling induced by S100A12 was of crucial significance for processes such as chronic inflammation and tissue injury. RAGE was also shown to interact with S100A12. The signaling was abolished by inhibition of PLC, further confining the site of S100A4 action to the membrane. Finally, suramin, known to block the binding of agonists to G protein-associated receptors and RTKs, completely prevented the induction of cellular responses to S100A4.

S100A12-RAGE and S100A4-RAGE interactions could be competitive but might initiate different signaling events. Accordingly, the SA4BP peptide, which specifically bound to S100A4 and inhibited S100A4-induced neurite outgrowth, did not interfere with S100A4-RAGE binding. In addition, inhibitory antibodies to RAGE were unable to block S100A4-induced neurite outgrowth while significantly inhibiting outgrowth induced by S100A12 and by the RAGE-specific ligand amphoterin (see Results). Finally, the dimeric form of S100A4, which had no effect on neurite outgrowth from primary neurons, bound to RAGE with an affinity similar to that of the oligomeric form. In principle, RAGE-mediated differentiation might require receptor clustering on the cell surface. However, this is not very likely, since in our experiments RAGE-mediated neurite outgrowth was also elicited by S100A12, which was expressed in the dimeric form. Thus, the S100A4-RAGE interaction, though occurring in vitro, is probably not necessary for neurite induction by S100A4. Instead, this interaction may mediate the neuroprotective effect of S100A4 exhibited by both dimeric and oligomeric forms (29).

In our experiments, the threshold concentration of S100A4 inducing an elevation of the bulk [Ca^{2+}], was ca. 5 µM. At this concentration, a low-amplitude Ca^{2+} response lasting for ca. 2 min was observed in the somata of neurons. Interestingly, we also found that S100A4 had already activated ERK1 and 2, the kinases crucial for induction of neurite outgrowth by the protein, after 1 min of treatment (Fig. 3G). This indicates a specific role of Ca^{2+}-regulating mechanisms in S100A4-induced signaling. In addition, the genuine threshold of activation of Ca^{2+} responses might be significantly lower, since low concentrations of S100A4 might evoke a [Ca^{2+}], increase that is not detectable using conventional Ca^{2+} fluorometry, as demonstrated for other neurite-inducing cues (2, 22).

The amplitude of the observed Ca^{2+} response to S100A4 was dependent on how long in vitro a neuronal culture had been grown, probably reflecting the difference in surface density of a putative S100A4 receptor or, more likely, the age-dependent increase in surface density of L-type VDCC (30), which, along with T-type VDCC, are mainly responsible for the depolarization-induced Ca^{2+} influx into neuronal somata. Accordingly, the S100A4-induced [Ca^{2+}], response was crucially dependent on the extracellular calcium concentration and could be significantly inhibited by blockers of both L- and T-type VDCC but not other types of VDCC. Since a blockade of T-type VDCC abolished the S100A4-triggered [Ca^{2+}], rise completely, it seems plausible that low-voltage-activated T-type VDCC open first, resulting in depolarization and a subsequent opening of high-voltage-activated L-type VDCC.

In our experiments, the inhibition of G proteins, but not RTKs or PTPs, abrogated the S100A4-induced Ca^{2+} responses, suggesting that one or more members of the G protein family were involved. However, only the Gq-associated signaling pathway was of importance for the neurite outgrowth/[Ca^{2+}], rise triggered by S100A4. The involvement of a phosphoinositide-specific PLC in S100A4 signaling suggests that S100A4 binding to a receptor on the cell surface might result in activation of the Gq, class proteins associated with PLCβ (summarized in Fig. 10). In this event, Gq, activated PLCβ mediates the formation of DAG, which is hydrolyzed to form two sequential products, 2-AG and AA, with all three binders of the S100A4 signal transduction pathway.
messengers known to increase plasma membrane permeability for Ca\(^{2+}\). In support of this hypothesis, blocking of PLC, DAG-lipase, or NSCC led to an inhibition of the S100A4-evoked neurite outgrowth and [Ca\(^{2+}\)]\(_i\) rise (see Results). These data also indicated that the downstream products of DAG, i.e., 2-AG and AA, were responsible for the induced cellular responses. Recently, intrinsic AA and its metabolites have been shown to activate a noncapacitative Ca\(^{2+}\) entry in a variety of cell types via newly identified arachidonate-regulated Ca\(^{2+}\) channels (reviewed in reference 36) and TRP-family Ca\(^{2+}\)-permeable channels, suggesting that these could be the NSCC mediating the S100A4-induced Ca\(^{2+}\) entry (Fig. 10).

Interestingly, in control experiments, we found that inhibitors of L- and T-type VDCC, NSCC, or DAG-lipase had less effect on neurite extension from neurons grown on monolayers of 3T3 fibroblasts (Fig. 7) than on S100A4-induced neurogenesis. This indicated a specific role of Ca\(^{2+}\)-regulating mechanisms in the S100A4-induced signaling. However, it cannot be ruled out that several neuritogenic cues might influence [Ca\(^{2+}\)]\(_i\) through the same effectors as S100A4, since primary neurons possess a restricted array of Ca\(^{2+}\)-regulating mechanisms.

We also found that heparin specifically bound both S100A4 and S100A12 and influenced the neurite outgrowth and Ca\(^{2+}\) rise induced by these two proteins. Since heparan glycosaminoglycans are abundant both in the extracellular matrix and on cell membranes, they might act as a scaffold to immobilize S100 proteins extracellularly. Due to the high affinity of the S100-heparan sulfate interaction, extracellular S100 proteins would be captured close to the site of their initial release. This might provide a mechanism by which S100 proteins could locally regulate neuronal plasticity in connection with brain trauma and neurological disorders, since the cellular effects of the proteins would be confined to the areas adjacent to the site of damage.

In conclusion, we have shown for the first time that the differentiation-promoting oligomeric form of S100A4 increases the cytoplasmic calcium concentration ([Ca\(^{2+}\)]\(_i\)) in primary neurons. We present evidence that S100A4-induced intracellular signaling involves the activation of a cascade involving a heterotrimeric G protein(s), phosphoinositide-specific PLC, and DAG-lipase, resulting in Ca\(^{2+}\) entry via nonselective cation channels and Ca\(^{2+}\) influx via T- and L-type voltage-gated Ca\(^{2+}\) channels. Moreover, our data indicate that S100A4-induced signaling in primary neurons does not depend on the known target for other extracellular S100 proteins, RAGE, suggesting the existence of other plasma membrane receptors for S100 proteins. To activate these as yet unidentified receptors, S100 proteins might require initial binding to glycosaminoglycans at the cell surface or in the extracellular matrix. Thus, S100 proteins, in particular S100A4, may represent "multireceptor ligands," and their cellular effects may be a combination of outputs of several signaling cascades.

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