Identification of a Signaling Pathway Activated Specifically in the Somatodendritic Compartment by a Heparan Sulfate That Regulates Dendrite Growth

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In two earlier reports we demonstrated that natural heparan sulfate, but not dermatan or chondroitin sulfate glycosaminoglycans, stimulate axonal elongation and inhibit dendrite growth *in vitro* (Lafont et al., 1992). The latter specific effect on dendrite elongation was reproduced by chemically synthesized heparan sulfates and by SR 80037A, a purified sulfated and hexanoylated heparin fragment (Lafont et al., 1994). Adding radioactive SR 80037A to purified neurons demonstrated the existence, at the neuronal surface, of heparan sulfate-specific and saturable binding sites, suggesting that SR 80037A activates specific signal transduction pathways. In the present study, using rat or mouse neurons from the embryonic cortex, we show that SR 80037A signaling involves one or several G-coupled receptor or receptors, small GTPases ρ A and/or ρ C,

and one or several PKCs. We also demonstrate that the rapid soma rounding elicited by SR 80037A does not require protein synthesis but that the long-term effect on dendrite initiation requires protein synthesis in a short period after the addition of the heparan sulfate. Finally, by preparing membranes from the somatodendritic or axonal compartments we demonstrate that the identified signaling pathway is activated by SR 80037A primarily in the somatodendritic compartment and is not sensitive to the addition of a dermatan sulfate glycosaminoglycan that does not induce the axonal phenotype by impairing dendrite initiation and elongation.

Key words: neurons in culture; neuronal polarity; glycosaminoglycans; signal transduction; dendrite growth inhibition; cell rounding; cell permeable peptides

Neurons are highly polarized cells with characteristic axons and dendrites. This polarity is established during development through interactions of the neurons with cellular partners, in particular astrocytes (Prochiantz, 1995) or Schwann cells (Lein et al., 1995), and molecular signals (Higgins et al., 1997). Among the latter are growth factors, such as neurotrophins (McAllister et al., 1997) or bone morphogenetic proteins (Lein et al., 1995) and matrix molecules (Chamak and Prochiantz, 1989; Lafont et al., 1992; Prochiantz, 1995).

An important aspect of neuronal polarity is the regional specificity of the interactions taking place between neurons and their environment. Indeed, by coculturing astrocytes and neurons from different regions, it was observed that neurons will develop, or not, their dendritic arbor, depending on the anatomical origin of the astrocytes (Denis-Donini et al., 1984; Chamak et al., 1987; Rousselet et al., 1988, 1990; Qian et al., 1992; Le Roux and Reh, 1994). Recently, using slice cultures, it was further demonstrated that neuronal polarity is regulated by different neurotrophins in layers 4 and 6 of the developing cortex (McAllister et al., 1997).

The influence of the anatomical origin of astrocytes on neuronal polarity led us to investigate the activity of molecules present in astrocyte-conditioned medium and to observe that glycosami-

noglycans (GAGs) regulate neuronal polarity (Lafont et al., 1992). Interestingly, heparan sulfates (HSs) induced an axonal phenotype (long axons and no or short dendrites), whereas dermatan and chondroitin sulfates had a general trophic effect on all neurites or favored dendrite elongation. Because GAGs are composed of a combination of heparan, chondroitin, and dermatan motifs, it appeared that elementary carbohydrate units might participate in the specificity of neuroastroglial interactions revealed by polarity studies.

We, therefore, screened a GAG bank composed of synthetic disaccharides to hexasaccharides for their effects on neuronal polarity (Lafont et al., 1994). We found that, when active, sugars from the HS series, as well as SR 80037A (a purified heparin-derived heparan sulfate) decrease neuron–substratum adhesion, inhibit dendrite outgrowth, and either stimulate or do not affect axonal elongation, whereas synthetic dermatan sulfates (DSs) have the opposite influence on neuronal differentiation. These effects were observed on all embryonic rat or mouse neurons tested, and the sugars were only active in solution. Substratum-bound GAGs lost their ability to regulate polarity. We also observed that neurons express $3\text{--}5 \times 10^5$ HS-binding sites (K_D , 1 μ M) with a clear structural specificity, because only SR 80037A or active HS-like GAGs competed with labeled SR 80037A for binding to neurons, and the DS-derived GAGs were inactive, even at high concentrations.

These results suggested that HS, natural or synthetic, bind specific receptors and activate pathways related to dendrite growth. We now demonstrate that SR 80037A signaling involves G-coupled receptor(s), small ρ GTPases, and one or several PKCs and that the HS signaling pathway, which is only activated

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Figure 1. Effects of Heparan sulfate on neuronal morphology. *A*, E16 rat cortical neurons incubated 21 hr (left) or 48 hr (right) with (SR 80037A) or without (Cdm) 10 $\mu\text{g}/\text{ml}$ SR 80037A were fixed and double-immunostained for MAP2 (red) and τ (green). *B*, Morphometric analysis was performed on 50 neurons per condition (21 hr with or without SR 80037A). Cumulative distributions of the neurons according to spreading index (*B1*), total neurite length or axonal length (*B2*), and dendrite length (*B4*) demonstrate a specific effect of SR 80037A on cell spreading and dendrite growth. This effect is parallel to a clear reduction in the number of primary neurites (*B3*). SR 80037A differs significantly from Cdm for all parameters ($p < 0.001$), except axonal length.

Table 1. Presentations of morphological parameters investigated in Figures 1–5, 8, and 9

		Primary neurites	Dendrite length (μm)	Axon length (μm)
Figure 1	Cdm	2.4 ± 0.065	45.2 ± 1.8	50 ± 1.2
	SR 80037A	$0.99 \pm 0.075^*$	$17.4 \pm 1.4^*$	$45.4 \pm 1.4^{**}$
Figure 2	Cdm	3.5 ± 0.15	64.0 ± 4.0	48.9 ± 2.7
	SR 80037A	$2 \pm 0.18^*$	$34.5 \pm 3.7^*$	$70.5 \pm 4.0^*$
	SR 80037A-FITC	$2 \pm 0.15^*$	$34.7 \pm 3.0^*$	57.8 ± 3.5
Figure 3B	Cdm	2.6 ± 0.11	55 ± 3.8	66.4 ± 3.8
	SR 80037A	$1.7 \pm 0.12^*$	$34.3 \pm 4.6^{**}$	$92.6 \pm 6^*$
	Chx	2.6 ± 0.13	52.6 ± 4.7	58.2 ± 3.3
	Chx + SR 80037A	$1.7 \pm 0.15^*$	48.2 ± 3.6	$74.5 \pm 5^*$
Figure 4A	Cdm	2.8 ± 0.17	62.9 ± 3.7	49.6 ± 2.8
	SR 80037A	$1.6 \pm 0.16^*$	$34 \pm 4.5^*$	62.9 ± 4.4
	EGFR1068Y-P	2.5 ± 0.18	56.8 ± 4.4	45.5 ± 2.3
	EGFR1068Y-P/SR 80037A	1.6 ± 0.2	54.5 ± 2.8	$63.4 \pm 3.7^{**}$
Figure 4B	Cdm	2.9 ± 0.13	49.9 ± 3.9	28 ± 0.8
	SR 80037A	$1.2 \pm 0.1^*$	$20.2 \pm 1.9^*$	28.7 ± 0.9
Figure 4C	Cdm	2.3 ± 0.08	50.5 ± 2.3	60.8 ± 2.4
	SR 80037A	$1.4 \pm 0.08^*$	$30.8 \pm 2.4^*$	51.3 ± 2.6
	PTX	1.9 ± 0.8	42.7 ± 2	51.6 ± 2.2
	PTX + SR 80037A	2 ± 0.1	42.9 ± 2.8	55 ± 3.2
Figure 5A	Cdm	3.4 ± 0.16	65.2 ± 3.6	62.9 ± 3.9
	SR 80037A	$2.1 \pm 0.18^*$	$42.2 \pm 3.8^{**}$	72.6 ± 5.3
	V-PKCi	3.2 ± 0.15	56.6 ± 5.2	55.1 ± 2.3
	V-PKCi/SR 80037A	3.3 ± 0.19	70.8 ± 5.6	58 ± 4
Figure 8	Cdm	2.7 ± 0.09	58.4 ± 2.4	55.8 ± 2.3
	SR 80037A	$2.1 \pm 0.09^*$	$45.5 \pm 2^*$	63.2 ± 2.3
	C3 toxin	$2 \pm 0.08^*$	$41.0 \pm 2.3^*$	68.4 ± 2.3
Figure 9	Cdm	3 ± 0.14	59.3 ± 3.1	69.1 ± 5.6
	SR 80037A	$1.7 \pm 0.09^*$	$43.7 \pm 3.6^{**}$	$95.3 \pm 3.7^*$
	DSbm	$2.4 \pm 0.13^{**}$	70.7 ± 3.7	65.9 ± 3.7

Figure 1: Cdm versus SR 80037A; Figure 2: Cdm versus SR 80037A, Cdm versus SR 80037A-FITC; Figure 3: Cdm versus SR 80037A, CHX versus CHX-SR80037A; Figure 4A: Cdm versus SR 80037A, EGF versus EGF-SR80037A; Figure 4B: Cdm versus SR 80037A; Figure 4C: Cdm versus SR 80037A, PTX versus PTX-SR 80037A; Figure 5A: Cdm versus SR 80037A, V-PKCi versus V-PKCi-SR 80037A; Figure 8: Cdm versus SR 80037A, Cdm versus C3; Figure 9: Cdm versus SR 80037A, Cdm versus DSbm.

* $p < 0.0005$.

** $p < 0.05$.

in the somatodendritic compartment, does not respond to a DS GAG that does not block dendrite growth.

MATERIALS AND METHODS

Cortical neuron cultures. Pure neuronal cultures were prepared according to Lafont et al. (1994). In brief, dissociated cortical cells from 16-d-old [embryonic day 16 (E16)] rat embryos (IFFA Credo, France) or from transgenic mice were seeded on polyornithin-coated (40 kDa, Sigma, St. Louis, MO) 16-mm-diameter culture wells (1.5 $\mu\text{g}/\text{ml}$) or glass coverslips (15 $\mu\text{g}/\text{ml}$) at a density of 25×10^3 cells/ cm^2 (immunocytochemistry) or 12×10^3 cells/ cm^2 (morphological analysis). Chemically defined medium (Cdm) consisted of DMEM-F-12 (1:1, Life Technologies, Gaithersburg, MD) complemented with a mixture of hormones, proteins, and salts (Rousselet et al., 1988). Peptides (concentrations indicated in the text), cycloheximide (1 μM), or GAGs [SR 80037A, SR 80037A-FITC, dermatan sulfate from bovine mucosa (DSbm, Sigma) at a concentration of 10 $\mu\text{g}/\text{ml}$] were added 30 min after seeding. SR 80037A was prepared as described earlier (Petitou et al., 1992; Petitou and van Boeckel, 1993). Collagen from one rat tail was removed sterilely, dissolved overnight at 4°C in 100 ml of acetic acid (0.1%), centrifuged at low speed, and dialyzed against 2×10 volumes of DMEM 1:10 adjusted to pH 4 with HCl. Collagen gels were obtained by adding 75% rat tail collagen to the culture medium (in replacement of water) and by adjusting the pH to 7.2 with NaOH.

Immunocytochemistry. Cells fixed for 20 min at room temperature

(RT) with 4% paraformaldehyde (PAF) in PBS were permeabilized in 0.1% Triton X-100 for 5 min, incubated (1 hr, 37°C) with polyclonal anti-MAP2 antibody (1:400, gift of Dr. A. Fellous), washed three times, and further incubated for 1 hr at 37°C with monoclonal anti- τ antibody (1:50, Boehringer Mannheim, Indianapolis, IN). After three washes, cells were incubated (1 hr, 37°C) with CY3-conjugated anti-rabbit Igs (1:200), washed three times, incubated (1 hr, 37°C) with biotinylated anti-mouse Igs (1:200), washed three times, incubated (1 hr, 37°C) with FITC-conjugated streptavidin (1:200), washed three times in PBS and once in water, and mounted in Vectashield. All dilutions and washes were in PBS, and all incubations were in PBS plus 10% fetal calf serum. Immunocytochemical staining of E16 rat embryo cortical explants cultured in collagen gels was achieved as above but with longer incubation periods. Cells and explants were observed on an inverted fluorescence microscope (Leitz) or a confocal microscope (Zeiss, Molecular Dynamics system).

Morphological analysis. Cells fixed with PAF for 20 min at RT were washed in PBS, stained with toluidine blue (0.2% in 1% Na_2CO_3), rinsed in water, and air-dried. Each experiment was done three times. Fifty to 100 neurons per condition were digitalized and analyzed with morphological analysis software (Neurolab France). Scheffe's test (5%) was used to determine whether treated and control cells were statistically different. A one-tailed p value < 0.05 was considered significant.

Preparation of plasma membranes. Cortical tissues (E16 rat embryos) homogenized with a Dounce homogenizer (10 strokes) were passaged

Figure 2. One hour of exposure to SR 80037A induces the phenotype. *A*, E16 cortical cells were incubated with SR 80037A-FITC (10 μ g/ml) for 1 hr, briefly washed (SR 80037A W, third panel) or not (SR 80037A, first panel) with 0.5 M NaCl, fixed, and analyzed by confocal microscopy. SR 80037A accumulation at the cell surface (bright rim) was lost after washing (compare with control cells not incubated with SR 80037A-FITC, second panel). *B*, E16 cortical cells were treated with 10 μ g/ml SR 80037A or SR 80037A-FITC for 1 hr, washed with 0.5 M NaCl, and let to differentiate for 21 hr in Cdm (without NaCl). Cumulative distributions of the neurons according to spreading index (left) and dendrite length (right) demonstrate that SR 80037A-FITC is as active as SR 80037A and that the two aspects of the phenotype are induced within 1 hr of SR 80037A presentation. $p < 0.0001$ for SR 80037A versus Cdm (spreading index); $p < 0.002$ for SR 80037A-FITC versus Cdm (spreading index). For all other parameters, see statistics in Table 1.

(three times) through a 26 gauge needle in cold buffer A (10 mM Tris, pH 7.4, 100 μ M EDTA, and 0.25 M sucrose) plus protease inhibitors (1 mM Pefablock, 1 μ M leupeptin, 1 μ M pepstatin, and 0.3 μ M aprotinin). The homogenate was loaded on sucrose (1.7 M in buffer A) and centrifuged (150,000 $\times$ g, 30 min, 4°C) in an SW41 rotor (Beckman). The membranes collected at the 1.7 M/0.25 M interface were loaded on a second 1.7 M sucrose cushion, centrifuged (150,000 $\times$ g, 30 min, 4°C), collected, resuspended in 10 mM Tris, pH 7.4, 100 μ M EDTA, and 0.25 M sucrose, pelleted (15,000 $\times$ g, 15 min, 4°C), and resuspended in buffer A plus protease inhibitors. In the case of explant cultures, before membrane preparation (as above), the somatodendritic and axonal regions of the explant were cut off the gel, and the axons were rid of collagen by a brief incubation (10 μ g/ml, 30 min at 37°C) with collagenase (Sigma). ADP-ribosylation of axonal and dendritic membranes was performed as indicated below using 10 μ g of proteins. Proteins were quantified using the micro BCA protein assay reagent kit (Pierce, Rockford, IL).

ADP-ribosylation. ADP-ribosylation with pertussis toxin (PTX, Sigma) or C3 (a kind gift of Dr. P. Boquet) was carried out according to Brabet et al. (1990). The membranes (23–30 μ g) were preincubated for 1, 15, or 30 min with or without SR 80037A (or DSbm) at 37°C, in (in mM) 70 Tris-HCl, pH 7.5, 25 dithiothreitol, 20% glycerol, 1 EDTA, 0.1 MgCl₂, 1 ATP, 100 thymidine, and 10 nicotinamide. Incubation was in a final volume of 100 μ l for 1 hr at 37°C, with 0.5 μ M nicotinamide adenine dinucleotide (NAD), 1 μ Ci ³²P-NAD (DuPont NEN, Boston, MA) and PTX (2.5 μ g) or C3 (the appropriate dilution of C3 was established for each batch of enzyme). Samples precipitated in 10% TCA were separated by SDS-PAGE and analyzed by phosphorimaging (Fuji).

Immunoprecipitation. ADP-ribosylated membranes were boiled for 5 min in 10% SDS, and four volumes of 190 mM NaCl, 50 mM Tris, pH 7.4,

6 mM EDTA, and 2.5% Triton X-100 were added. After 30 min on ice the samples were centrifuged (10,000 rpm, 5 min, 4°C). Supernatants were added to 50 μ l of protein-A-Sepharose (in buffer A) previously incubated (or not) with the appropriate anti-G_{ai} or -G_{ao} antibody and incubated for 1 hr at 37°C. After centrifugation (14,000 rpm, 10 min, 4°C), the pellets were washed five times in 150 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 0.05% SDS, and 0.1% Triton X-100, and once in 150 mM NaCl, 10 mM Tris, pH 7.4, and 5 mM EDTA. The pellets were resuspended in Laemmli buffer, heated for 5 min at 100°C and centrifuged (14,000 rpm, 10 min, RT). Supernatants were analyzed by SDS-PAGE. Affinity-purified antibodies against C-terminal decapeptides of G_{ao} and G_{ai-3} (which also recognizes the C-terminal domains of G_{ai1} and G_{ai2}) were from Santa Cruz Biotechnology, Santa Cruz, CA.

Western blot analysis. Membranes from E16 rat cortices were prepared as indicated above, and the proteins separated by SDS-PAGE were transferred on Immobilon. Filters were incubated with anti- ρ (Santa-Cruz, 1:100), anti-G_{ao} (1:1000), or anti-G_{ai-3} (1:1000) overnight at 4°C, washed, further incubated for 2 hr at RT with peroxidase-conjugated anti-rabbit whole antibody, washed, and revealed with ECL (Amersham, Arlington Heights, IL).

Two-dimensional gel electrophoresis. ADP-ribosylated samples were lyophilized and resuspended in 20 μ l of 9.5 M urea, 10% NP-40, 5% β -mercaptoethanol, 1.6% biolyte 5:7 (Bio-Rad, Hercules, CA), 0.4% biolyte 3:10 (Bio-Rad) and mixed with 20 μ l of 9 M urea, 0.8% biolyte 5:7, 0.2% biolyte 3:10, and 0.0025% bromophenol blue. First dimension was in 9.2 M urea, 4% acrylamide, 2% NP-40, 1.6% Biolyte 5:7, and 0.4% biolyte 3:10. Second dimension was achieved on a 7–18% acrylamide gradient (SDS-PAGE).

Figure 3. Effect of protein synthesis inhibition on early and late phenotypes. *A*, E16 cortical neurons treated for 1 hr with SR 80037A in the presence of cycloheximide (CHX) at $1 \mu\text{M}$ (scheme) and fixed still adopt the rounded phenotype. The *left panel* is without CHX (for control). $p < 0.0001$ (CHX vs CHX-SR 80037A and control vs SR 80037A). *B*, Cycloheximide was removed at the same time as SR 80037A, allowing protein synthesis to resume rapidly (scheme), and the cells were allowed to grow for another 19 hr. The *left panel* is without CHX (for control). The decrease in cell spreading was preserved ($p < 0.0001$; CHX vs CHX-SR 80037A), but the effect on dendrite length was lost ($p < 1.0$ CHX vs CHX-SR 80037A). For other parameters, see Table 1.

Table 2. Effects of various inhibitors on SR 80037A activity

Drugs	Targets	Soma	Dendrite length
Genestein	Tyrosine kinase R	No	No
EGFR ¹⁰⁶⁸ Y-P	SH2 domain of Grb2 (Williams et al., 1997; Fig. 4)	No	No
FGFR ⁷⁶⁶ Y-P	SH2 domain of the PLC γ (Hall et al., 1996)	No	No
Transgenic FGF-R1 animals	FGF-R (Fig. 4)	No	No
Pertussis toxin (PTX)	G $_i$ and G $_o$ (Fig. 4)	Yes	Yes
V-PKCi peptide	PKC (PKN) (Theodore et al., 1995; Fig. 5)	Yes	Yes
Staurosporine	PKC/PKN	Yes	Yes

This table summarizes the results obtained with SR 80037A and various compounds (genestein at 100 μ M, EGF¹⁰⁶⁸Y-P at 5 μ g/ml, FGFR⁷⁶⁶Y-P at 1 μ g/ml, PTX at 0.5 μ g/ml, V-PKCi at 0.5 μ M, and staurosporine at 0.1 μ M). No and Yes mean no inhibition or inhibition, respectively, of SR 80037A effect on soma spreading and dendrite elongation.

RESULTS

SR 80037A decreases cell spreading and inhibits dendrite outgrowth

Figure 1*A* illustrates the influence of SR 80037A on the shape and polarity of E16 rat cortical neurons 21 and 48 hr after seeding. In control conditions (Cdm), neurons exhibit a clear polarized phenotype with several MAP2-positive short extensions and a long τ -positive neurite. This specific distribution of the axonal (τ) and dendritic (MAP2) markers (for review, see Craig and Banker, 1994) is already present after 21 hr and allowed us to establish that, in our culture conditions, the longest neurite is always the axon. Figure 1*A* also illustrates that SR 80037A decreases the apparent diameter of the soma and the number of primary dendrites. This is quantified in Figure 1*B* and Table 1; in the presence of SR 80037A the mean number of primary neurites, soma spreading index (in fact the maximal cross-sectional area, corresponding to the surface occupied by the soma on the culture dish after fixation), total neurite length, and dendrite length decrease significantly, whereas axonal length is not modified. The main effect of SR 80037A is, thus, an inhibition of soma spreading and of dendrite initiation and elongation.

In all experiments presented in this study, axonal length was either increased or not modified after SR 80037A addition, whereas the number of primary neurites and dendrite development were always reduced. Soma spreading estimated by spreading index was also often reduced by the addition of SR 80037A. For each experiment, results concerning the number of primary neurites, dendrite length, and axonal length are presented in Table 1. Finally, the same effect on cell rounding and dendrite elongation was observed for rat or mouse neurons from all tested brain regions (cerebellum, spinal cord, and cortex; data not shown).

One hour exposure to SR 80037A is sufficient to induce cell response

SR 80037A, added to neurons for 1 hr, was washed by a brief rinse in Cdm supplemented with 0.5 M NaCl. The medium was then changed for Cdm, and the culture was pursued for an additional 19 hr before morphological analysis. To verify that NaCl washing

is efficient in removing the sugar, we used fluoresceinated SR 80037A (SR 80037A-FITC) (Lafont et al., 1994). Figure 2*A* illustrates that all SR 80037A-FITC is removed by the brief NaCl rinse. Figure 2*B* and Table 1 demonstrate that FITC conjugation does not modify the activity of the sugar and that only 1 hr of incubation with SR 80037A or SR 80037A-FITC is sufficient to induce and maintain cell rounding and dendrite inhibition for at least 19 hr.

To evaluate the importance of protein synthesis in the induced phenotype, we used cycloheximide (CHX), a drug that specifically and reversibly blocks protein synthesis. CHX was added 30 min before SR 80037A. After 1 hr exposure to SR 80037A, the cells were either fixed (Fig. 3*A*) or washed with 0.5 M NaCl to remove SR 80037A and CHX and let to differentiate in Cdm for 19 hr (Fig. 3*B*). Cell body rounding occurs within 1 hr (Fig. 3*A*) and in the absence of protein synthesis. When the cells were analyzed 19 hr after removal of CHX and SR 80037A, the effects of the HS on cell rounding and on the number of primary neurites were maintained (Fig. 3*B*, Table 1), but the effect on total dendrite length was lost. Interestingly, axonal elongation was strongly stimulated, suggesting that the neurites (axons and dendrites) that are able to initiate despite the presence of SR 80037A grow at a very rapid pace after CHX and sugar removal. The same result was obtained if CHX was left for another 3 hr after SR 80037A removal (data not shown).

Analysis of SR 80037A signaling pathway

To analyze the SR 80037A signaling pathway involved in the morphological phenotype we used the pharmacological reagents listed in Table 2. Many of these reagents are commercially available, e.g., staurosporin and PTX, but others have been developed in our laboratories (for review, see Derossi et al., 1998). The “homemade” dominant-negative inhibitors are peptides or phosphopeptides that act by interacting with active sites, as in the case of the PKC inhibitor (PKCi) (Derossi et al., 1998) or with the SH2 domain of PLC γ or Grb2 (Hall et al., 1996; Williams et al., 1997). These peptides were made cell-permeable thanks to their linkage to another polypeptide (penetratin-1) that translocates across biological membranes and accumulates in the cytoplasm and nucleus of cells in culture (Derossi et al., 1994, 1996). Because the activity of cell-permeable peptides has been described earlier (Theodore et al., 1995; Hall et al., 1996; Williams et al., 1997), we simply summarized their effects in Table 2. In the same table we report the use of neurons from a transgenic mouse expressing a truncated FGF receptor under the control of the neural specific enolase promoter. This truncated receptor behaves as a dominant negative membrane protein for all FGF receptors (Safell et al., 1997).

The results are summarized in Table 2 and are either not shown or quantified in Figures 4 and 5 and in Table 1. Because 1 hr exposure to SR 80037A was enough to induce the phenotype, chemical inhibitors were added for 30 min before SR 80037A, and the cells were further incubated for 1 hr before replacement of SR 80037A and inhibitors with Cdm for 20 hr. In summary, of all tested compounds, the only active SR 80037A antagonists are pertussis toxin and PKC inhibitors. Figure 4*A* and Table 1 illustrate the absence of inhibition of SR 80037A effects by a peptide that antagonizes EGF signaling through Grb2. Figure 4*B* and Table 1 demonstrate that neurons that do not respond to FGFs are still capable of responding to SR 80037A. In contrast, Figures 4*C* and 5*A* illustrate that PTX (Fig. 4*C*, Table 1) and penetratin-1-PKCi (V-PKCi), the PKC peptidic inhibitor (Fig. 5*A*, Table 1),

Figure 4. SR 80037A effects do not require active EGF and FGF receptors and are inhibited by pertussis toxin. Cumulative distributions according to spreading index and dendrite length of E16 rat (*A*, *C*) and mouse (*B*) neurons incubated with or without SR 80037A in different conditions. *A*, EGF¹⁰⁶⁸Y-P does not affect SR 80037A activity. Spreading index and dendrite length are shown in the presence or absence of the inhibitor. $p < 0.0001$, EGF¹⁰⁶⁸Y-P versus EGF¹⁰⁶⁸Y-P-SR 80037A (spreading index); $p < 0.01$, EGF¹⁰⁶⁸Y-P versus EGF¹⁰⁶⁸Y-P-SR 80037A (dendrite length). For other parameters, see statistics in Table 1. *B*, Nonfunctional FGF receptors (neurons from transgenic embryos expressing a FGF-R1 dominant negative receptor) do not affect SR 80037A activity. $p < 0.0001$ (spreading index and dendrite length, Cdm vs SR 80037A). *C*, PTX (0.5 μ g/ml) added 30 min before SR 80037A and removed at the same time as SR 80037A (1 hr exposure to the sugar) inhibited both soma rounding and dendrite inhibition. Note that PTX has an effect per se on spreading index. $p < 1.0$ (PTX vs PTX-SR 80037A, spreading index, and dendrite length). For all other parameters, see statistics in Table 1.

antagonize the effect of SR 80037A on neuronal spreading and dendrite growth. The inhibition of neuronal rounding is clear but partially masked by the per se effect of PTX and V-PKCi inhibitor on this parameter, even in the absence of SR 80037A. To validate

the use of the V-PKCi we showed that stimulating the cells with PMA for 30 min before peptide addition antagonized its effect in a dose-dependent manner (Fig. 5*B*). Similar effects were obtained with the V-PKCi and staurosporine (data not shown).

Figure 5. Protein kinase C is in the SR 80037A pathway. *A*, Cumulative distributions according to spreading index and dendrite length of E16 rat neurons incubated with or without SR 80037A (for 1 hr) with or without pretreatment with the cell-permeable PKC inhibitor (V-PKCi). V-PKCi inhibits both cell rounding and the effect on dendrite growth, $p < 0.001$, Cdm versus SR 80037A (spreading); $p < 0.01$, Cdm versus SR 80037A (dendrite growth); $p < 1$, V-PKCi versus V-PKCi-SR 80037A (spreading); $p < 0.6$, V-PKCi versus V-PKCi-SR 80037A (dendrite growth). For all other parameters, see statistics in Table 1. *B*, Treating the cells with PMA antagonizes the effect of V-PKCi in a dose-dependent manner. Inhibition is partial at 0.1 μM (left panel) and total at 1 μM PMA (right panel). This confirms that the effect of V-PKCi is through PKC inhibition. Left panel, $p < 0.001$, Cdm versus SR 80037A (spreading); $p < 0.008$, Cdm versus SR 80037A (dendrite length); $p < 0.07$, V-PKCi-SR 80037A versus V-PKCi-SR 80037A-PMA 1 μM (spreading); $p < 0.2$, V-PKCi-SR 80037A versus V-PKCi-SR 80037A-PMA 1 μM (dendrite length). Right panel, $p < 0.0001$, Cdm versus SR 80037A (spreading and dendrite length); $p < 0.004$ V-PKCi-SR 80037A versus V-PKCi-SR 80037A-PMA 10 μM (spreading); $p < 0.006$ V-PKCi-SR 80037A versus V-PKCi-SR 80037A-PMA 10 μM (dendrite length).

SR 80037A activates G_i but inhibits Rho pathways

PTX specifically incorporates ADP through ADP ribosylation of the C-terminal domain of the α subunits of G_o and G_i , two heterotrimeric GTPases (Neer et al., 1984). After heterotrimeric G-protein activation, the α subunits dissociate from the complex and become less accessible to PTX (Li, 1992). A decrease in ADP ribosylation provoked by a pharmacological agent therefore demonstrates that this agent is acting through a pathway involving either G_o or G_i activation. To verify that SR 80037A activity involves a heterotrimeric G-protein, membranes from the cortex of E16 rat embryos were incubated with the sugar, pelleted to remove the unbound ligand, and proceeded for ADP ribosylation of the G-proteins by PTX. As shown in Figure 6*A* (top panel), SR 80037A inhibits the ADP ribosylation of a protein with an apparent molecular weight (MW) of 40 kDa. This inhibition was rapid and almost total after 30 min of incubation with the sugar (Fig. 6*A*, quantification in bottom panel). Western blot analysis of the membrane extracts demonstrates that $G_{o\alpha}$ and $G_{i\alpha}$ are expressed at this stage of development, with a larger amount of $G_{i\alpha}$ (prob-

ably $G_{i\alpha1}$ and $G_{i\alpha2}$ because $G_{i\alpha3}$ is not expressed in neurons; Fig. 6*B*). The same Western blot demonstrates that the addition of SR 80037A does not modify the amount of G-protein present in the membranes and, therefore, that the effect of the sugar on the level of ADP ribosylation cannot be attributed to a degradation of the toxin substrates. Immunoprecipitations with anti- $G_{o\alpha}$ and anti- $G_{i\alpha}$ antibodies (Fig. 6*C*) confirmed that SR 80037A inhibits PTX-stimulated ADP ribosylation of both proteins and, thus, places the heterotrimeric G, in particular G_i , in the SR 80037A signaling pathway.

The activation of G_i and the rapid and CHX-resistant cell rounding that follows SR 80037A addition suggested a role for the actin cytoskeleton and, as a consequence, a possible involvement of ρ GTPase (Machesky and Hall, 1996; Imamura et al., 1997; Hall, 1998). Therefore, we analyzed the effect of an exposure of the cortical membranes to SR 80037A on the ADP ribosylation of ρ by C3, a ρ -specific toxin (Li, 1992). Figure 7*A* illustrates and quantifies the kinetic of ρ ADP ribosylation after exposure to SR 80037A and demonstrates that the sugar rapidly inhibits ρ ADP

Figure 6. Heterotrimeric G-proteins are in the SR 80037A pathway. *A*, ADP ribosylation by PTX of a 40 kDa protein in neuronal membranes pretreated with (+) or without (–) SR 80037A during 1, 15, or 30 min and quantification by phosphorimager (*bottom panel*). This experiment is representative of three independent experiments in which SR 80037A reduced ADP ribosylation by 63, 70, and 90% after 30 min. *B*, Western blot analysis of E16 cortical neurons using $G_{i\alpha}$ and $G_{o\alpha}$ antibodies. Incubation with SR 80037A (+) does not modify the membrane content in G_i or G_o . *C*, Immunoprecipitation, with $G_{i\alpha}$ or $G_{o\alpha}$ antibodies, of ADP-ribosylated G-proteins from E16 neuronal membranes pretreated (+) or not (–) with SR 80037A.

ribosylation. Separation of the ρ proteins by two-dimensional electrophoresis (Fig. 6*B*, *top panel*) further demonstrates that ρ_A and ρ_C but not ρ_B are in the sugar-signaling pathway (Fig. 7*B*, quantification *bottom panel*). In the yeast, PKC is downstream of ρ_1 (Nonaka, 1995). Experiments demonstrating that staurosporine does not antagonize the effect of SR 80037A on the ADP ribosylation of ρ by C3 (data not shown) also places PKC downstream of ρ in the SR 80037A transduction pathway.

The decrease in ρ ADP ribosylation suggests that the addition of SR 80037A decreases the accessibility of Asn⁴¹ (the ADP ribosylation site) to the toxin (Jalink et al., 1994), possibly by

promoting an interaction of ρ with a downstream partner. However, this does not say whether this partner inhibits or, on the contrary, enhances basal ρ biological activity. To investigate this point we compared, in our biological model, the effect of SR 80037A with that of C3, a known inhibitor of ρ . As shown in Figure 8 and Table 1, C3 and SR 80037A have the same effects on cell spreading, the number of primary neurites and dendrite growth, and the same absence of effect on axonal elongation. This experiment, which reproduces the results of Threadgill et al. (1997), strongly suggests that SR 80037A provokes the association of ρ with a downstream inhibitor.

HS signaling pathway is primarily activated in the somatodendritic compartment and does not respond to DS

We had shown previously that DSbm, a commercial dermatan sulfate from bovine mucosa, does not provoke cell rounding and does not decrease dendrite growth (Lafont et al., 1994; Fig. 9*A*, Table 1). Accordingly, DSbm does not displace radioactive SR 80037A from its binding sites at the neuronal surface (Lafont et al., 1994). We, thus, wanted to compare the respective effects of DSbm and SR 80037A on the ADP ribosylation of G_i and ρ in the axonal and somatodendritic compartments. To this end, small cortical explants were cultured in three-dimensional collagen gels, and the axons were allowed to invade the gels for 100–120 hr. Double staining of the explants with anti-MAP2 and anti- τ antibodies (Fig. 9*B*) illustrates that the axons (*green*) invade the collagen gel, whereas cell bodies and dendrites remain within the explant, with some axons. This experimental design permitted separation of the axonal and the somatodendritic compartments and preparation of the corresponding membranes. Figure 9*C* illustrates that, for the same amount of protein, the somatodendritic compartment is highly enriched with G_i and ρ and that the addition of SR 80037A does not modify the amount of protein, for example, through proteolysis. Finally, Figure 9*D* demonstrates that, for an equal amount of membrane proteins, the response to the addition of SR 80037A was much higher on membranes derived from the somatodendritic compartment, strongly suggesting a polarized distribution of the signaling pathway mediating the HS polarizing effect. In addition, the effects of DSbm on G_i or ρ ADP ribosylation were absent or small compared with those of SR 80037A.

The latter experiments suggested that SR 80037A acts at the level of the dendrites, inhibiting their initiation when added very early after seeding and possibly, as suggested by the ADP ribosylation of G_i and ρ in the dendrite-derived membranes, their elongation when added on already differentiated dendrites. To test the effect of SR 80037A on the growth of already initiated dendrites, the cells were plated and cultured for 24 hr before the addition (or not) of SR 80037A and a further 24 hr culture period (Fig. 10*A*). Figure 10*B* illustrates that the addition of SR 80037A after 24 hr in Cdm provokes cell rounding and reduces dendrite elongation in the following 24 hr but does not modify the rate of axonal growth or the number of primary neurites. These results are in accordance with a specific effect of SR 80037A on the soma and at the level of dendritic growth cones.

DISCUSSION

The synthesis of proteoglycans (PGs) and GAGs during development is highly regulated (for review, see Small et al., 1996). *In vivo* expression patterns suggest that these compounds have important functions, for example, at the border of developmental

Figure 7. ρ A is in the SR 80037A pathway. *A*, ADP ribosylation by C3 of a 21 kDa protein in neuronal membranes pretreated (+) or not (–) with SR 80037A during 1, 15, or 30 min and quantification by phosphorimager (*bottom panel*). This experiment is representative of five independent experiments using two different preparations of toxin in which SR 80037A reduced ρ ADP ribosylation by 70 and 64% (preparation 1) and by 40, 42, and 46% (preparation 2) after 30 min. *B*, Separation by two-dimensional gel electrophoresis of ρ A, ρ B, and ρ C demonstrating that SR 80037A (+) affects ρ A and ρ C ADP ribosylation but not that of ρ B. Gels in *top panel* and quantifications by phosphorimager in *bottom panel*.

Figure 8. C3 and SR 80037A have identical effect on neuronal morphology. Cumulative distributions according to spreading index (*A*), axon length (*B*), and dendrite length (*C*), as well as the number of primary neurites (*D*) demonstrate that SR 80037A and C3 have similar effects on the morphology of E16 rat cortical neurons. $p < 0.0001$, Cdm versus SR 80037A (spreading) and $p < 0.001$ Cdm versus C3 (spreading). For all other parameters, see Table 1.

Figure 9. SR 80037A signaling pathway is specifically activated in the somatodendritic compartment and does not respond to DSbm. *A*, As opposed to SR 80037A, DSbm does not provoke cell rounding (*top panel*) and increases dendrite elongation (*bottom panel*). $p < 0.0001$, SR 80037A/Cdm (spreading); $p < 0.5$, DSbm versus Cdm (spreading). For all other parameters, see Table 1. *B*, Double MAP2/ τ staining of an explant having developed for 100 hr in a collagen gel. Note the separation between axons invading the gel and dendrites that remain within the explant. Dotted lines indicate how somatodendritic and axonal compartments were separated. *C*, Western blot analysis of the axonal (Ax) and somatodendritic (SD) compartments demonstrates that for an equal amount of membrane proteins the SD compartment is highly enriched in G_i and ρ and that the addition of SR 80037A does not induce the degradation of either molecule. *D*, Incubation of equal amounts of somatodendritic (SD) or axonal (Ax) membranes with SR 80037A (HS) or DSbm (DS) followed by ADP ribosylation of $G_{i\alpha}$ (*top panel*) or ρ (*bottom panel*) demonstrates that the signaling pathway is enriched in the somatodendritic compartment and does not respond to DSbm.

territories, as illustrated by the high concentration of a specific chondroitin sulfate at the inter-rhombomeric frontier (Heyman et al., 1995) or around the somatosensory barrels (Faissner and Steindler, 1995). PGs and GAGs have been shown to regulate neurite elongation in vitro and in vivo (Oohira et al., 1991; Lafont et al., 1992; Brittis and Silver, 1994; Faissner et al., 1994; Fernaud-Espinosa et al., 1994) and are upregulated after lesions of the nervous system, suggesting a role during growth and regeneration (Bovolenta et al., 1993; Gates et al., 1996). However, despite the suspected biological importance of PGs and GAGs, very little is known regarding their mode of action, and several hypotheses have been proposed.

A first possibility, illustrated by the direct activation, in the absence of FGF, of FGF receptor 4 by heparin (Gao and Goldfarb, 1995) is that they act as bona fide growth factors with specific receptors and signal transduction pathways. A second

hypothesis well substantiated in the case of TGF- β and FGF-1 or -2 (for review, see Ruoslahti and Yamaguchi, 1991) is that GAGs associate with growth factors, modulate their diffusion, prevent their degradation, and help their presentation to specific receptors (Schlessinger et al., 1995). A third proposed mode of action is through binding to other matrix molecules, for example, laminin (Smalheiser and Kim, 1995), and to cell or substrate adhesion molecules (Hayashi et al., 1992).

Here, we used SR 80037A, an HS with specific and saturable neuronal binding sites (Lafont et al., 1994), to investigate the HS signaling pathway involved in the induction of the axonal phenotype (specific reduction in dendrite number and length) in a well established model of cell polarity (Prochiantz, 1995). We show that SR 80037A acts through a pathway involving heterotrimeric G-proteins, one or several PKCs, and ρ GTPases. In addition, we have localized the SR 80037A-responsive pathway to the soma-

Figure 10. SR 80037A is active on already differentiated neurons. *A*, SR 80037A was added (or not) after 24 hr, and the cells were fixed or culture for another 24 hr. *B1*, Soma spreading increases between 24 and 48 hr in Cdm but decreases in SR 80037A. $p < 0.002$, Cdm 24 hr versus Cdm 48 hr; $p < 0.0001$, Cdm 48 hr versus SR 80037A 24–48 hr; $p < 0.003$, Cdm 24 hr versus SR 80037A 48 hr. *B2*, Dendrite length between 24 and 48 hr increases less in SR 80037A than in Cdm. $p < 0.02$, Cdm 48 hr versus SR 80037A 24–48 hr. *B3*, Axonal elongation is not modified between 24 and 48 hr by the addition of SR 80037A. $p < 0.9$, Cdm 48 hr versus SR 80037A 24–48 hr. *B4*, The number of primary neurites is not modified between 24 and 48 hr by the addition of SR 80036A. $p < 0.1$, Cdm 48 hr versus SR 80037A 24–48 hr.

todendritic compartment and shown that a dermatan sulfate (DSbm), which does not inhibit SR 80037A neuronal binding, does not activate the same pathway. Our results strongly suggest the existence of receptors highly enriched at the surface of soma and dendrites, responsible for the active inhibition of soma spreading and dendrite elongation and activated in the presence of SR 80037A.

SR 80037A is a heparin-derived GAG of average MW of 6000 Da, devoid of anticoagulant activity. It can be obtained in large quantities, and its purification procedure eliminates all possibilities of contamination by peptides or compounds not related to heparin (Petitou et al., 1992; Petitou and van Boeckel, 1993). More importantly, SR 80037A reproduces the physiological properties of HS GAGs purified from astrocyte-conditioned medium

(Lafont et al., 1992) and of all active HS-like synthetic GAGs (Lafont et al., 1994). Furthermore, SR 80037A binding to the surface of neurons in culture demonstrated the existence of a limited number of binding sites ($3\text{--}5 \times 10^5$ per embryonic neuron) with a K_D of $1 \mu\text{M}$. Finally, SR 80037A binding was displaced by heparin and HS-derived synthetic GAGs but not by DSbm, a dermatan sulfate from bovine mucosa, which, in contrast with HS GAGs, does not inhibit dendrite growth (Lafont et al., 1992, 1994). SR 80037A is, thus, an ideally suited compound to start investigating the transduction pathway involved in HS GAG signaling.

The pharmacological tools used in this study are either classical or prepared in the laboratory by exploiting the capability of permeant peptides and phosphopeptides derived from the third

Figure 11. Hypothetical scheme. *A*, Specific binding of SR 80037A provokes cell rounding and drives the cells into a state in which dendrite initiation is not possible. In contrast, the axon can still initiate and elongate. This suggests that axons differ from dendrites very early in the course of neuronal differentiation. *B*, The addition of SR 80037A 24 hr after the major neurites have started to grow provokes cell rounding and stops dendrite elongation but has no effect on axonal growth. This suggests that initiation and elongation of dendrites are sensitive to the addition of SR 80037A and lends weight to the idea that the soma is already polarized at the time of neurite initiation.

helix of Antennapedia homeodomain to translocate across biological membranes (Derossi et al., 1994, 1996, 1998). The advantage of this procedure is that the peptides show no toxicity, are internalized by 100% of the neurons, and gain direct access to the cytoplasmic compartment. The use of the latter pharmacological tools associated to that of a transgenic mouse in which a dominant negative FGF receptor was expressed in postmitotic neurons under the transcriptional control of the neural specific enolase promoter (Safell et al., 1997) eliminated the possibility that FGF, EGF, or PDGF receptors are involved in the signaling pathway. The absence of serum and the high dilution conditions of the culture make it also very unlikely that SR 80037A signals through a TGF-related receptor. Because NCAM, *N*-cadherin, and L1 signal through FGF-R1 (Safell et al., 1997), we feel that these three transmembrane molecules can also be tentatively eliminated from the list of candidates mediating SR 80037A activity. Our experiments, thus, lead to the conclusion that SR 80037A, after its specific binding to an unidentified binding site, signals through a cascade involving, in this order, heterotrimeric G_i , ρ , and one or several PKC or PKN.

This pathway is reminiscent of that described for lisophospha-

tidic acid (LPA) and leads to a phenotype that also resembles that described on neuronal cell lines incubated with LPA (Jalink et al., 1994; Moolenaar et al., 1997). Indeed, LPA after binding to an unknown receptor activates G_i and ρ , induces the formation of actin stress fibers, and provokes growth cone collapse and cell rounding (Zhang et al., 1997). A striking similarity with SR 80037A is a very rapid cell-rounding phenotype. The results obtained with LPA and SR 80037A are in agreement with the observation that C3, a bacterial toxin that ADP ribosylates ρ , inhibits the formation of dendrites by neocortical neurons (Threadgill et al., 1997).

The effect of SR 80037A on neuronal rounding and dendrite initiation does not require that SR 80037A be present for >1 hr. It is, thus, conceivable that, after SR 80037A addition, a pathway is activated that maintains the cell, at least for 19 hr, in an HS phenotype (cell rounding and no dendrites growing). No protein synthesis is required for the immediate rounding phenotype because it is preserved in the presence of cycloheximide. In contrast, the long-term effect, illustrated by the inhibition of dendrite elongation, requires protein synthesis. This suggests an accumulation of transiently active intracellular signals that acti-

vates the synthesis of downstream effectors. The nature of the signals has not been investigated in detail. We could eliminate NF- κ B because, although the latter factor is present in the cell cytoplasm, the addition of SR 80037A does not induce its nuclear translocation (data not shown; Perona et al., 1997). In contrast, preliminary results suggest that SR 80037A interferes with MAP-K activity (S. Calvet, M. Petitou, and A. Prochiantz, unpublished results).

Finally, we have developed an assay allowing the direct assessment of SR 80037A activity on membranes derived purely from axons or from a combination of somas, dendrites, and some axons. A striking result is that the inhibition of $G_{i\alpha}$ or ρ ADP ribosylation, taken as evidence for SR 80037A signaling, occurs only in somatodendritic membranes, an observation in good accordance with a role of ρ on dendritic remodeling (Threadgill et al., 1997). Furthermore, the inhibition of ρ and $G_{i\alpha}$ ADP ribosylation by GAGs is specific, because it is not observed with a DS-type GAG that does not recognize the neuronal binding sites for SR 80037A (Lafont et al., 1994) and does not inhibit dendrite elongation.

Our results can be discussed in the frame of the model schematized in Figure 11 (for details, see Prochiantz, 1995). In this model, there exists a threshold of spreading below which dendrite growth is inhibited, whereas axonal elongation is still possible. We propose that SR 80037A, by its effect on cell rounding, drives the cell into a no-dendrite state still allowing axonal elongation (Fig. 11A). Because the cells are put in culture at the neuroblast stage (E16 rat or mouse embryos), this suggests that the conditions necessary for axonal elongation differ already at the neuroblast stage from those necessary for dendritic elongation and, therefore, that neuronal polarity preexists well before it is materialized under the form of true axons and dendrites. This proposal is supported by the addition of SR 80037A on cells that have already differentiated for 24 hr (Fig. 11B), suggesting that the sugar has a similar stop-growth effect on the initiation of dendrites (Fig. 11A) and on their elongation (Fig. 11B). The physiological consequences of these observations might be of importance, because they suggest that PGs or GAGs could act as regulators of dendrite growth and that borders might exist that would specifically restrict dendritic development but could be ignored by elongating axons.

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