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Chondroitin Sulfate Proteoglycan Elevates Cytoplasmic Calcium in DRG Neurons

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Proteoglycans have been implicated in neuronal pathfinding during development, yet related second messenger and signaling systems are unknown. We have used the calcium indicator fura-2/AM to monitor cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$) in chick dorsal root ganglion (DRG) neuronal growth cones elongating on laminin during contact with chondroitin sulfate proteoglycan (CSPG): (1) to determine whether there is a change in $[Ca^{2+}]_i$ in neurons that contact CSPG, and (2) to determine whether changes in $[Ca^{2+}]_i$ are necessary for inhibition of growth cone migration. The majority of DRG neurons responded to CSPG contact with a transient rise in $[Ca^{2+}]_i$ (mean $\Delta[Ca^{2+}]_i$ above resting level was 554 ± 109 n*M*; $P < 0.0001$). The effect of CSPG contact was concentration dependent and required the carbohydrate moiety of CSPG. Addition of soluble CSPG did not elevate $[Ca^{2+}]$. Treatment with reagents that blocked plasma membrane calcium channels, or that perturbed intracellular Ca^{2+} stores, indicated that extracellular Ca^{2+} was the major source of the $[Ca^{2+}]_i$ elevation, and that Ca^{2+} entry occurred through non-voltage-gated calcium channels. Although general Ca^{2+} channel blockers abolished the CSPG-induced $[Ca^{2+}]_i$ rise, they did not abolish growth cone avoidance of surface-bound CSPG in these assays. We conclude: (1) that DRG neurons elevate $[Ca^{2+}]_i$ in response to CSPG contact to levels that can modify cytoskeletal mechanisms of growth cone migration, and (2) that avoidance of substratum-bound CSPG may not be dependent upon elevated $[Ca^{2+}]_i$. © 1994 Academic Press, Inc.

INTRODUCTION

The development of the nervous system is dependent upon interactions between neurons and the extracellular matrix (ECM). Proteoglycans (PGs) are one class of molecule abundantly expressed in the ECM. PGs are structurally diverse (Herndon and Lander, 1990), are present in numerous forms and microenvironments (Andres *et al.*, 1989; Nishiyama *et al.*, 1991), interact with a wide variety of molecules (Walicke, 1988; Andres *et al.*, 1992; Grumet *et al.*, 1993), and have a dynamic pattern of expression involving numerous cell types (Margolis *et al.*, 1986; Rauch *et al.*, 1991; Halfter, 1992; Faissner *et al.*, 1994). Functionally, they have been implicated in the regulation of cell morphology (Leppa *et al.*, 1992), cell migration (Perris and Johansson, 1990; Oakley *et al.*, 1994), proliferation (Wright *et al.*, 1989), differentiation (Trautman *et al.*, 1991), cell adhesion (Knox and Wells, 1979; Bidanset *et al.*, 1992; Drake *et al.*, 1992; Faassen *et al.*, 1992; Iida *et al.*, 1992; Minguell *et al.*, 1992), signal transduction (Nishiyama *et al.*, 1991), growth factor presentation (Walicke, 1988, 1989; Turnbull *et al.*, 1992), synapse formation and stabilization (Hockfield *et al.*, 1990; Gordon *et al.*, 1993), regeneration (McKeon *et al.*, 1991; Pindzola *et al.*, 1993), and nervous system pathology (Shioi *et al.*, 1992; Canning *et al.*, 1993).

An important function of PGs in neural development is inhibition of growth cone migration. Certain PGs inhibit neurite elongation *in vitro* (Carbonetto *et al.*, 1983; Verna *et al.*, 1989; Snow *et al.*, 1990a, 1991, 1992, 1993; Cole and McCabe, 1991; Oohira *et al.*, 1991; Brittis *et al.*, 1992; Dou and Levine, 1992; Snow and Letourneau, 1992), and are expressed in regions where axons do not elongate *in vivo* (Snow *et al.*, 1990b, 1991; Tosney and Oakley, 1990; Cole and McCabe, 1991; Oakley and Tosney, 1991). Responses *in vitro* to contact with bound PGs include growth cone turning and/or stopping, also described in non-PG systems (Kapfhammer *et al.*, 1986; Burmeister and Goldberg, 1989; Lohof *et al.*, 1992), reduction of neurite length, or decreased rate of neurite outgrowth. Negative responses to PGs in embryos may contribute to defining axonal pathways (Snow *et al.*, 1990b; Oakley and Tosney, 1991) and during adulthood may reduce or impede axonal outgrowth following CNS injury (McKeon *et al.*, 1991; Pindzola *et al.*, 1993; Levine *et al.*, 1994). Hypotheses for the mechanisms by which PGs may inhibit growth cone migration include repulsion from a substratum by the abundant negative charge of the carbohydrate moiety of PGs, the ability of PGs to bind water, creating nonpermissive spaces, blocking of growth-promoting substrata, or activation of cell-surface receptors for PGs that ultimately lead to reduced adhesion or diminished recognition of growth-promoting molecules

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in the growth cone's environment (Margolis *et al.*, 1986; Rutka *et al.*, 1988; Snow *et al.*, 1990a,b).

Little is known about the second messenger and signaling systems that may mediate the effects of PGs. However, knowledge about the diverse roles for PGs in the nervous system, combined with numerous reports showing correlations between changes in cytoplasmic calcium and growth cone behavior (Goldberg, 1988; Lankford and Letourneau, 1989; Letourneau *et al.*, 1990; Kater and Mills, 1991; Lankford and Letourneau, 1991; Rehder *et al.*, 1991; Davenport and Kater, 1992; Lohof *et al.*, 1992; Rehder and Kater, 1992; Bandtlow *et al.*, 1993; Zheng *et al.*, 1994), suggest that PGs may modulate $[Ca^{2+}]_i$, thereby regulating cytoskeletal dynamics or cellular processes within growth cones to change motile behaviors.

Immunohistochemical and experimental evidence indicate that one type of PG, chondroitin sulfate proteoglycan (CSPG), may block growth cone migration during development of axon pathways and following nervous system injury (Carbonetto *et al.*, 1983; Verna *et al.*, 1989; Snow *et al.*, 1990, 1991; Fichard *et al.*, 1991; McKeon *et al.*, 1991; Oakley and Tosney, 1991; Oohira *et al.*, 1991; Brittis *et al.*, 1992; Snow and Letourneau, 1992; Pindzola *et al.*, 1993). There is little evidence to indicate a similar role in axonal guidance for other PGs, or other negatively charged molecules (except keratan sulfate (Snow *et al.*, 1990; Cole and McCabe, 1991), which comprises a portion of certain CSPGs). For this reason, we chose to analyze the effect of CSPG on growth cone $[Ca^{2+}]_i$.

The goals of the present study were to determine whether contact with PGs could result in changes in intracellular signaling systems, and whether such changes may affect growth cone behavior. To test this, we analyzed the affect of CSPG on growth cone $[Ca^{2+}]_i$. These studies were undertaken as part of our ongoing investigations into the guidance of neuronal growth cones by PGs. CSPG was bound to laminin or fibronectin in a striped pattern (Snow *et al.*, 1990a, 1992) or to polystyrene beads. Fluorescence imaging with the calcium indicator fura-2/AM, and time-lapse video microscopy revealed a large transient elevation of $[Ca^{2+}]_i$ when chick dorsal root ganglion (DRG) neurons contacted CSPG, using both the stripe and bead techniques. The magnitude and time course of the changes we observed suggest that changes in $[Ca^{2+}]_i$ provide an important candidate mechanism for CSPG-induced changes in neuronal function.

MATERIALS AND METHODS

Materials

Purified bovine nasal cartilage proteoglycan (CS/KS-PG; contains C-O-S, C-6-S, a small contribution from C-4-S and KS (keratan sulfate), and O- and N-linked oligosaccharides) was provided by L. Culp, Case Western Reserve University (CWRU, Cleveland, OH) (for characterization see Rosenberg *et al.*, 1983); chick limb bud CSPG was provided by D. Carrino and A. Caplan, CWRU (for characterization see Carrino and Caplan, 1985); and bovine aorta CSPG was from Collaborative Biomedical Products (Cat. No. 40252). EHS laminin was supplied by S. Palm, University of Minnesota, Department of Laboratory Medicine and Pathology, or Gibco, Inc. (Grand Island, NY), and fibronectin was supplied by J. McCarthy, University of Minnesota, Department of Laboratory Medicine and Pathology. Chondroitinase ABC and keratanase were purchased from Miles Scientific (Cat. Nos. 32-030 and 32-032, respectively). Hanks' buffered saline solution, DMEM, and F14 medium were purchased from Gibco BRL, Inc.

Cell Culture

Preparation of cells. Day 8–12 chicken embryos were euthanized by decapitation according to AALAC regulations for humane animal sacrifice. The internal organs, vertebral column, and spinal cord were removed. Lumbar DRG neurons were dissected in Hepes-buffered medium containing 10% fetal calf serum, or Hanks' buffered saline solution with 6 mg/ml Hepes and 20 μg/ml gentamycin, using fine forceps. The DRG were cleaned of surrounding tissue and dissociated to single cells by treatment with 0.25% crude bovine trypsin in 0.1 *M* calcium-magnesium-free phosphate-buffered saline (PBS; pH 7.89) for 18 min at 37°C, resuspended and triturated in medium with 5% fetal calf serum, and counted. Approximately 7×10^3 cells for small coverslips (22 mm, round) and 2×10^4 cells for large (24 × 40 mm) coverslips were plated in 200–500 μl of medium. Media used were: (1) 10 m*M* Hepes-buffered F14 medium supplemented with L-glutamate (2 m*M*), sodium selenite (5 ng/ml), sodium pyruvate (200 μg/ml), phosphocreatine (5 m*M*), progesterone (20 n*M*), insulin (5 μg/ml), transferrin (100 μg/ml), and nerve growth factor (NGF, 50 ng/ml), and an antibiotic/antimycotic solution (PSF); (2) DMEM with 10% fetal bovine serum, 20 μg/ml gentamycin, 6 mg/ml Hepes, and 50 ng/ml NGF (7S); or (3) calcium-free medium prepared by adding essential amino acids, vitamins, MgCl, $MgSO_4$, and $NaHCO_3$ to calcium-magnesium-free Hanks' balanced salt solution with 1 mM EGTA, containing calcium-free NGF (30 ng/ml). In addition, lanthanum chloride (1 mM), a general calcium channel blocker, was prepared in media lacking carbonate, sulfate, and phosphate to prevent precipitation of polyvalent cations. Cells were incubated in a 5% CO_2 incubator or in a humidified, ambient chamber (Hepes-buffered) at 40°C for at least 6 hr.

Preparation of substrata. Glass coverslips (24 × 40 mm or 22 mm^2) were acid-washed overnight or washed in 2%

MICRO solution for 20 min, rinsed with distilled water, and oven-dried at 100°C. The coverslips were mounted over holes drilled in either 35- or 60-mm Petri dishes (Falcon), using Dow-Corning vacuum grease or a mixture of petroleum, bee's wax, and paraffin (1:1:1). All dishes were UV-treated for 30 min and subsequently treated as sterile. Small coverslips were treated with poly-L-lysine (0.25 mg/ml) overnight, rinsed thoroughly in distilled water, and air-dried. All coverslips were coated with 10–25 μg/ml filter-sterilized laminin or fibronectin for at least 3 hr at room temperature or at 37°C. Prior to use, laminin- or fibronectin-coated coverslips were repeatedly rinsed in PBS followed by rinses with sterile distilled water and then either covered with medium and stored until use or air-dried and adsorbed with PG, as described below. Note: The remainder of this report will refer only to those results using laminin, since experiments using CSPG bound to laminin were indistinguishable from experiments in which CSPG was bound to fibronectin, in these assays.

PG Presentation to Neurons

Two paradigms were used to examine DRG neuron contact with CSPG. In the first paradigm, CSPG was adsorbed to the surface of polystyrene beads. Polystyrene microspheres (Polysciences, Inc.; 3 μm, 300-μl volume) were washed three times in 0.1 *M* PBS. A 400-μl solution of beads was incubated overnight at 4°C in 1 mg/ml CSPG. CSPG-coated beads were stored at 4°C in 50-μl aliquots until use. Immunocytochemistry was used (Snow *et al.*, 1990b) to determine that the CSPG bound to the beads. Binding of CSPG to beads was quantitated using ^{35}S-labeled chick limb bud CSPG (generously provided by D. Carrino and A. Caplan), and indicated that 5–10% of the CSPG in the incubation solution bound to the beads. This corresponds to an approximate surface density of CSPG binding to beads of 0.0005 pg/μm^2, which is on the order of the surface density of CSPG in the stripe assay (0.003 pg/μm^2). The CSPG-coated beads were presented to neurons, as neurites elongated on laminin, by means of a micropipet moved by a hydraulic micromanipulator (Narishige MO-103). Specifically, calcium measurements were taken prior to CSPG contact, and then beads were touched to the cells and pulled away while calcium measurements were taken at the moment of contact. Calcium was then measured at approximately 30 sec- to 1 min-intervals following CSPG contact. Phase-contrast images were recorded simultaneously with calcium measurements in many cases.

In the second paradigm, 1 mg/ml bovine nasal cartilage CSPG, chick limb bud CSPG, or bovine aorta CSPG, was mixed with 5% rhodamine-labeled isothiocyanate (RITC) as a marker and adsorbed to air-dried laminin-coated coverslips, three stripes per dish (transfer = approx 5 min.), using cellulose paper strips (350 μm × 10–20 mm). Following one wash with Hepes-buffered F14, dissociated DRGs were grown on the patterned substrata (see cell culture details above). Cytoplasmic calcium levels were measured as described above as DRG growth cones migrated on laminin and contacted the CSPG-adsorbed substratum.

A general criterion was established in order to determine the specificity of CSPG-induced calcium rises. Since simply touching neurons can result in rises on the order of 10 to 20 n*M*, depending upon cell type, we set a criterion level for scoring positively an induced rise in intracellular calcium at a minimum of 50 n*M*. In all cases, CSPG alone was nearly an order of magnitude higher than this criterion, and in most cases controls proved to be nearly an order of magnitude lower than this criterion.

As controls, cell bodies, axons, and growth cones elongating on laminin were touched with a glass micropipet alone, 3-μm polystyrene beads incubated in PBS alone, or CSPG-coated beads that were treated with 0.1 Unit/ml keratanase or endo-β-galactosidase in 0.1 *M* PBS for 1 hr at 37°C and 0.1 Unit/ml chondroitinase ABC in 0.1 *M* Tris-acetate for 1 hr at 37°C and washed in PBS. Enzyme treatment digests the carbohydrate moieties, but leaves the protein core intact (Oike *et al.*, 1980; Snow *et al.*, 1990a). As a final control, a blind study was conducted in which the assayer was unaware of whether beads were incubated in PBS alone or CSPG.

Microscopy

Phase-contrast microscopy. In order to analyze the behavior of DRG growth cones as they encounter stripes of CSPG, cells were observed with an inverted microscope (IM 35; Carl Zeiss, Inc., Thornwood, NY) warmed to 40°C with an air curtain incubator (ASI 400; Carl Zeiss, Inc.). Growth cone behavior was monitored by phase-contrast optics using a Newvicon video camera (NC-65; Dage-MTI, Inc., Michigan City, IN) and image analysis software (Image 1; Universal Imaging, Inc., West Chester, PA), which was run on a 486/33 computer system (Gateway 2000, North Sioux City, SD). Images were viewed with a monitor (Trinitron; Sony Corp. of America, New York, NY) and recorded every 10–60 sec with an optical disc video recorder (TQ-2026F; Panasonic Industrial Comp., Secaucus, NJ).

Imaging of cytoplasmic calcium. Neurons were incubated in media containing 2–5 μ*M* fura-2/AM (Molecular Probes, Inc.) for 30–40 min. Cells were washed several times and incubated 30 min at 37°C in medium alone to allow for deesterification of fura-2 before calcium levels were measured. Experiments were done in 10 m*M* Hepes-buffered F14, or Hepes-buffered DMEM medium warmed to 40°C with an air curtain, or in a tem-

perature-maintained chamber. Images were viewed with either a 40×, 63×, or 100× fluor/phase-contrast oil immersion objective and appropriate neutral density filters to ensure minimum UV exposure to the cells. For most experiments, fura-2 fluorescence imaging was done using an intensified CCD camera (PaulTek) and the Image-1 fluorescence analysis system (Universal Imaging, Inc.) in combination with the Panasonic optical disc recorder. For some experiments, phase-contrast and fluorescence emission images were acquired using a cooled CCD camera (Photometrics), and images of fluorescence emission in response to 350 and 380 nm excitation were ratioed on a MacIntosh IIfx computer. Images were captured every 10–30 sec. Quantitative calcium values were calculated according to the calcium equation by Grynkiewicz *et al.* (1985), K_d = 224 n*M*, in a system calibrated *in vitro* or *in situ* (in living cells).

Determination of the Source of Calcium Elevation Following CSPG Contact

$[Ca^{2+}]_i$ is regulated by a variety of channels and pumps in the plasma membrane and by release and uptake from internal calcium stores (Fox *et al.*, 1987; Tsien and Tsien, 1990). Voltage-gated calcium channels respond to a number of selective reagents typically used to test the presence and efficiency of these channels (Bean, 1989; Boland *et al.*, 1994): dihyropyridines (verapamil, nifedipine, and diltiazem), which block T- or L-type channels; ω-conotoxin, which blocks the N-type channel selectively; or general calcium channel blockers, such as nickel chloride, or lanthanum chloride (Miledi, 1971). Dantrolene blocks the ryanodine-sensitive calcium channel (Palade *et al.*, 1989), and thapsigargin (TG) (Takemura *et al.*, 1989; Thastrup *et al.*, 1990) and cyclopiazonic acid (CPA) (Holzapfel, 1968) inhibit the Ca^{+2}-ATPase pump on ER, disallowing the refilling of intracellular calcium stores leading to a net Ca^{2+} release. Few reagents are well-characterized that perturb IP_3-sensitive calcium release (Berridge, 1993).

To determine the source of the $[Ca^{2+}]_i$ rise in DRGs that contact CSPG, cells were grown on glass coverslips, as described above, for at least 6 hr. Following incubation, Hepes-buffered F14, or calcium-free medium (see above), was added to the dishes with or without the addition of calcium channel blockers. L- and T-channel blockers [verapamil (10 μ*M*), nifedipine (100 n*M*), diltiazem (10 μ*M*)], an N-channel blocker [ω-conotoxin (5 μg/ml)], and global calcium channel blockers [nickel chloride (100 μ*M*–10 m*M*) and lanthanum chloride (1 m*M*)] were used. To perturb intracellular calcium stores, dantrolene (40 μ*M*), TG (1 μ*M*), and CPA (1 μ*M*), were also used.

Behavioral Experiments

For most behavioral experiments, in which calcium imaging was not performed, and, therefore, the highest optical resolution was not needed, a simplified preparation using petri dishes was employed. CSPG was bound in a striped pattern to a nitrocellulose substrata according to Lagenaur and Lemmon (1987). Briefly, petri dishes (60 mm) were evenly coated with 0.5 ml of a mixture of 5-cm^2 nitrocellulose (Schleicher & Schuell, Type BA 85) dissolved in 6 ml methanol and allowed to air-dry in a laminar flow hood. Cellulose strips (Whatman No. 1; 350 μm × 15–20 mm) were soaked in 20 μl of 1 mg/ml CSPG mixed with laminin (10–25 μg/ml) and 5% RITC and transferred to the nitrocellulose-coated dish in stripes. After removing the strips (approx 5 min), a solution of 20 μg/ml laminin (Gibco, Inc.) was spread evenly across the dish with a bent glass Pasteur pipet. The laminin bound to the remaining nitrocellulose, resulting in alternating lanes of CSPG and laminin. The boundaries between the lanes were sharply defined. Cells were incubated at 40°C in medium alone or nickel chloride (10 μ*M*–10 m*M*), lanthanum chloride (1 m*M*), dantrolene (40 μ*M*), TG (1 μ*M*), CPA (1 μ*M*), or calcium-free media. After 6–18 hr, the cells were fixed with 4% paraformaldehyde/0.1% glutaraldehyde for 1 hr and then coverslipped, photographed, and qualitatively analyzed using a 20× phase objective. Each experiment was repeated three or more times. In an additional experiment, each of the above reagents was added just prior to filopodial contact with CSPG, and growth cone behavior was recorded by time-lapse video microscopy using a 63× oil immersion objective.

RESULTS

Our initial experiments determined whether CSPG could produce a reproducible change in $[Ca^{2+}]_i$ in DRG cell bodies. Figure 1A demonstrates the paradigm used to present CSPG-coated beads to DRG cell bodies. Three or more CSPG beads were touched to DRG cell bodies elongating on laminin. The dose-response relationship between the number of CSPG-coated beads and elevation of $[Ca^{2+}]_i$ in DRG neurons was threshold, requiring three or more beads, before a response that met our criteria of >50 μ*M* increase was observed. DRG neurons responded to CSPG contact (86.4%) by elevation of $[Ca^{2+}]_i$. CSPG contact induced a rise in $[Ca^{2+}]_i$ in DRG cell bodies with the mean $\Delta[Ca^{2+}]_i$ = 517 ± 115 n*M* (n = 22; $P < 0.0001$) (Fig. 1B), where baseline $[Ca^{2+}]_i$ averaged 160 ± 8.9 n*M*. Following removal of the beads, there was a reduction of $[Ca^{2+}]_i$ toward baseline.

Interestingly, there were no obvious differences in the response to CSPG contacts with cell bodies in comparison to contacts with growth cones, since contact of DRG growth cones with CSPG resulted in rises of the same magnitude as for cell bodies (Fig. 2A). Rises induced by the same kind of contact as used for DRG cell bodies but in growth cones produced a mean $\Delta[Ca^{2+}]_i$ = 548 ± 66 n*M*

($n = 22$; $P < 0.0001$), compared to a mean $\Delta[Ca^{2+}]_i$ for cell bodies above of 517 ± 115 n*M*. Note: Our further characterization of the effects of CSPG coated beads on $[Ca^{2+}]_i$ combines results of stimulation to cell bodies and growth cones of DRG neurons.

In controls, DRGs were touched with: (1) a glass micropipet alone (mean $\Delta[Ca^{2+}]_i = 25.5 \pm 1.5$ n*M*; $n = 6$; $P > 0.1$), or (2) beads incubated in PBS [PBS is the diluent for CSPG; mean $\Delta[Ca^{2+}]_i = 78 \pm 2.5$ n*M*; $n = 4$; $P > 0.05$; multiple beads (three or more) were used in all bead controls]. Further, to rule out bias, we performed a blind study where the experimenter did not know whether the beads were CSPG-coated or incubated in PBS only (mean $\Delta[Ca^{2+}]_i = 1500$ n*M* with CSPG-coated beads; mean $\Delta[Ca^{2+}]_i = 82$ n*M* with PBS-coated beads; $n = 3$; $P > 0.1$). In all control experiments, treatments did not result in elevation of $[Ca^{2+}]_i$ in DRG neurons. A further control to test specificity used enzyme degradation of the carbohydrate moiety and is described below.

Characteristics of CSPG-Induced Elevation of $[Ca^{2+}]_i$

CSPG-induced elevation of $[Ca^{2+}]_i$ requires the carbohydrate component of CSPG. The inhibitory response of growth cone migration to CSPG *in vitro* is dependent upon the carbohydrate moiety of CSPG (Snow *et al.*, 1990a, 1991). Therefore, CSPG-coated beads were treated with a mixture of keratanase, chondroitinase, and endo-β-galactosidase to remove all carbohydrate moieties from CSPG and then touched to DRG neurons (Fig. 3A). The digestion of carbohydrate from CSPG-beads resulted in a change from a mean $\Delta[Ca^{2+}]_i$ for untreated CSPG-coated beads equal to 517 ± 115 n*M* to a mean $\Delta[Ca^{2+}]_i$ for enzyme-treated beads equal to 18.5 ± 5.5 n*M* ($n = 4$; $P > 0.5$) (Fig. 3B). Thus, as for growth cone guidance, the carbohydrate component of CSPG is required for the elevation of $[Ca^{2+}]_i$.

CSPG-induced elevation of $[Ca^{2+}]_i$ is related to the number of CSPG-coated beads. Inhibition of growth cone migration by a CSPG-adsorbed stripe is dependent on the density of CSPG binding (Snow *et al.*, 1990a). In the CSPG bead assays, contact with one or two beads was not sufficient to elicit a $[Ca^{2+}]_i$ rise in most cases, while three or more beads induced elevation of $[Ca^{2+}]_i$ to commonly obtained levels (Fig. 4). The approximate surface density of CSPG on the beads was 0.0005 pg/μm^2, which is on the order of the surface density of CSPG in the stripe assay (0.003 pg/μm^2). Because of the curved surfaces of 3-μm beads, a single filopodium may contact only a limited region of the CSPG-containing surface on a bead at one time, whereas it could interact with more CSPG on a flat substratum. In addition, several filopodia of a growth cone may contact CSPG on a stripe at the same time. The requirement of three or more beads to reach a threshold to elevate $[Ca^{2+}]_i$ suggests that the stimulus to induce elevation of $[Ca^{2+}]_i$ in DRG neurons requires a sufficient surface area of growth cone contact by CSPG-coated beads or, possibly, that multiple discrete stimulus sites are required.

CSPG in solution does not elevate $[Ca^{2+}]_i$ in DRG neurons. Studies from our laboratory indicate that CSPG added in solution does not inhibit neurite outgrowth (unpublished observations). However, some studies have shown that soluble PGs, or their carbohydrate side chains (glycosaminoglycans) in solution, can affect growth cone behavior (Fichard *et al.*, 1991; Oohira *et al.*, 1991; LaFont *et al.*, 1992; Bovalenta *et al.*, 1993). While CSPG added to the culture medium at the time of seeding DRG neurons reduces adhesion proportional to the CSPG concentration (D.M.S. and P.C.L., in preparation), we found that 1–1000 μg/ml CSPG added in solution failed to reach our criteria levels for significant elevation of $[Ca^{2+}]_i$ (Fig. 5; mean $\Delta[Ca^{2+}]_i$ over all concentrations = 14.4 n*M*, experiment performed three times, each experiment tested the range of 1–1000 μg/ml CSPG; $P > 0.05$).

CSPG-induced elevation of $[Ca^{2+}]_i$ results in two types of recovery phases. Three types of responses occurred for DRG neurons that contacted CSPG, in either the stripe or bead assays: (1) no elevation of $[Ca^{2+}]_i$ (17%); (2) elevation of $[Ca^{2+}]_i$ where recovery was fast (<5 min; mean = 3 min 43 sec; $n = 8$) and rapidly returned to the original baseline (36.8%; $n = 19$) (Fig. 6A); and (3) elevation of $[Ca^{2+}]_i$ where recovery was slow (>10 min; mean = 23 min 20 sec; $n = 3$) and returned to a level that was higher than the original baseline (63.2%, $n = 19$) (Fig. 6B). Calcium transients (Tsien and Tsien, 1990) often occurred during the slow recovery phase (Fig. 6B). These data may reflect the heterogeneity of DRGs, the past history of individual cells, or the amplitude of initial calcium rise.

Calcium Responses of Growth Cones Encountering CSPG in a Guidance Assay

Results presented above show that $[Ca^{2+}]_i$ was significantly elevated when beads were touched to either cell bodies or growth cones (Figs. 1B, 2A, and 2B). Since growth cones respond behaviorally in the guidance assay in which stripes of CSPG bound to a laminin-coated substratum are encountered by advancing growth cones, we used this same paradigm to investigate changes in $[Ca^{2+}]_i$ in growth cones.

Two approaches were used to examine changes in $[Ca^{2+}]_i$ when DRG growth cones contacted bound CSPG in the stripe assay. First, we measured changes in $[Ca^{2+}]_i$ for individual growth cone contacts as they occurred. Second, we analyzed static images of many DRG neurons at various stages of contact with CSPG by measuring at low magnification the $[Ca^{2+}]_i$ of growth cones at a CSPG stripe.


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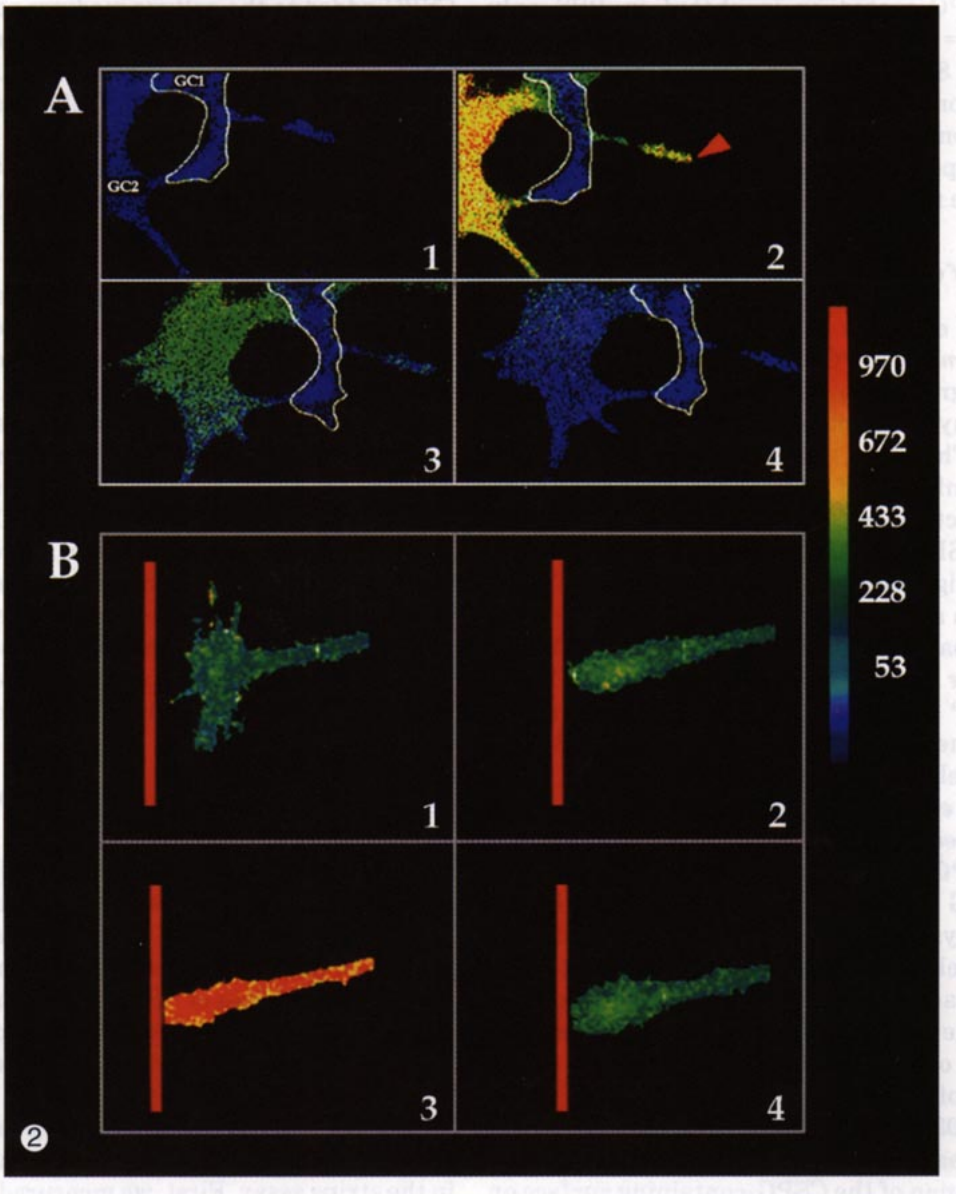
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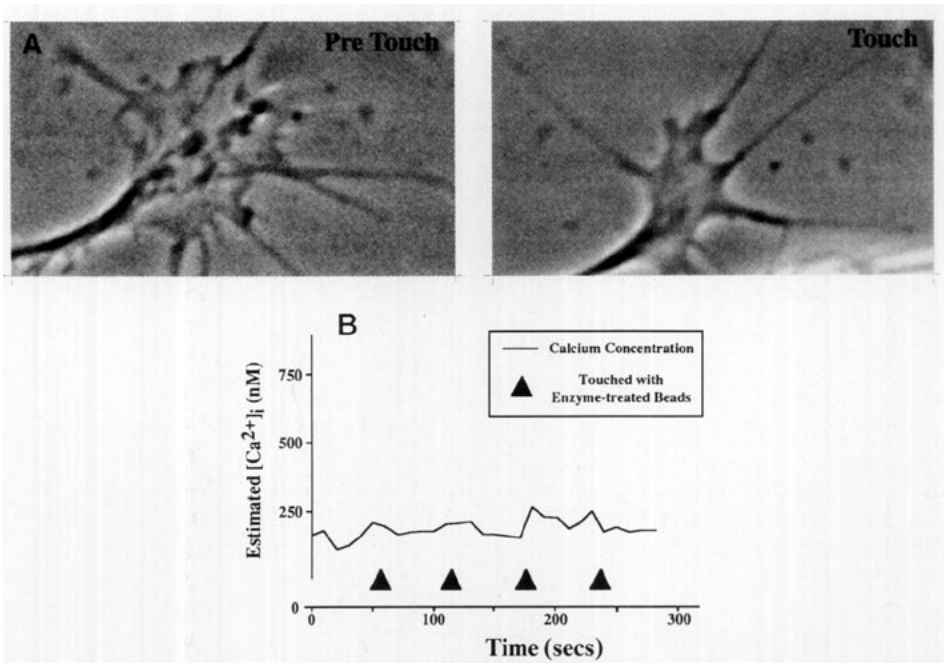

FIG. 3. CSPG-induced elevation of $[Ca^{2+}]_i$ requires the carbohydrate component of CSPG. DRG growth cones and cell bodies were touched with CSPG-coated beads treated with enzymes that completely remove the carbohydrate moieties of CSPG. (A) Phase-contrast micrographs illustrate growth cone contact with enzyme-treated beads. Filopodia remain extended and active. (B) $[Ca^{2+}]_i$ is not elevated by contact with enzyme treated beads (mean $\Delta[Ca^{2+}]_i = 18.5 \pm 5.5$ n*M*; $n = 4$; $P > 0.5$). DRG growth cone is approximately 10 μm.

In the first approach, growth cones of neurites extended from DRGs were observed in the CSPG stripe assay as they approached the border between laminin and CSPG at approximately right angles (Fig. 2B). When growth cones contacted CSPG, two changes were commonly observed: (1) some filopodia transiently retracted (partially or completely); and (2) $[Ca^{2+}]_i$ was transiently elevated. Elevation of $[Ca^{2+}]_i$ occurred in 83.3% ($n = 22$) of the growth cones that contacted CSPG-adsorbed stripes (mean $\Delta[Ca^{2+}]_i = 594 \pm 102$ n*M*; $P < 0.0001$). Growth cone $[Ca^{2+}]_i$ returned toward baseline, following CSPG contact. In all cases, controls in which growth cones elongated from laminin onto laminin-adsorbed stripes without CSPG did not result in elevation of $[Ca^{2+}]_i$, nor did growth cones display changes in behavior (Gomez and Letourneau, in press).

In the second approach, $[Ca^{2+}]_i$ was measured for growth cones either: (1) on laminin alone, or (2) at a border between laminin and CSPG at an undetermined time after initial contact with CSPG. On laminin alone, 18 of 19 DRG neurons were at baseline $[Ca^{2+}]_i$ (160 ± 8.9 n*M*). In comparison, of the 41 neurons observed at the laminin/CSPG border, 50% were at baseline $[Ca^{2+}]_i$, 18% had a moderate elevation in $[Ca^{2+}]_i$ (<450 n*M*), and 32.0% had a high $[Ca^{2+}]_i$ (>700 n*M*). These data support our previous observations that high $[Ca^{2+}]_i$ is not sustained following an initial increase, but $[Ca^{2+}]_i$ fluctuates and may return toward baseline over time. If this were not

FIG. 1. DRGs respond to CSPG by elevation of $[Ca^{2+}]_i$. (Top) Phase-contrast microscopy demonstrating the method of presentation of CSPG-coated beads to DRG cell bodies. (A) DRG cell bodies on laminin, (B) cell body touched with CSPG-coated beads, (C) cell body immediately after, and (D) 5–20 min after CSPG-coated beads are pulled away. (Bottom) Fluorescence microscopy of the calcium indicator fura-2/AM to image $[Ca^{2+}]_i$ in cell bodies that contact CSPG. A DRG cell body on laminin (Pre), elevates $[Ca^{2+}]_i$ in response to contact with CSPG-coated beads (Touch). CSPG contact induced a rise in $[Ca^{2+}]_i$ in 86.4% of DRG cell bodies with the mean $\Delta[Ca^{2+}]_i = 517 \pm 115$ n*M*; $n = 24$; $P < 0.0001$. Within 10 min, the DRG cell body recovered toward baseline. DRG cell bodies are approximately 10 μm.

FIG. 2. Fluorescence microscopy with the fluorescent calcium indicator fura-2/AM to image $[Ca^{2+}]_i$ in growth cones. (A) Two overlying growth cones prior to CSPG contact (1). A filopodium of growth cone 2 (GC2) is touched with CSPG beads (red arrowhead in 2) and simultaneously elevates $[Ca^{2+}]_i$. Within 3 min, calcium levels recover toward baseline (3) and have returned to baseline by 3 min (4). CSPG contact with GC2 does not induce elevation of $[Ca^{2+}]_i$ in the overlying growth cone (GC1). (B) A DRG growth cone elongating on laminin (1) first contacts a CSPG-adsorbed stripe (2). Although the growth cone temporarily withdraws many filopodia in this case, it does not collapse and retract as described by Kapfhammer and Raper (1987). When the growth cone makes greater contact with CSPG, i.e., a greater amount of surface area of the growth cone comes in contact with CSPG, $[Ca^{2+}]_i$ is greatly elevated (3; also see Fig. 6B for time course). Elevation of $[Ca^{2+}]_i$ occurred in 83.3% ($n = 22$) of the growth cones that contacted CSPG-adsorbed stripes (mean $\Delta[Ca^{2+}]_i = 594 \pm 102$ n*M*; $P < 0.0001$). This growth cone exhibits a slow recovery toward baseline (4; about 45 min following peak elevation of $[Ca^{2+}]_i$). See Results for analysis of recovery phases.

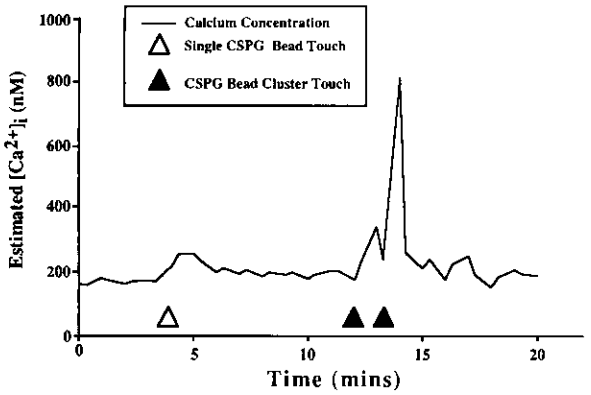

FIG. 4. CSPG-induced elevation of $[Ca^{2+}]_i$ is dependent on a threshold number of beads contacting the growth cone. Contact between growth cones growing on laminin and a single CSPG-coated bead (white arrowhead) does not usually result in the elevation of $[Ca^{2+}]_i$, while contact with a cluster of CSPG-coated beads (three or more; black arrowheads) results in a large increase in $[Ca^{2+}]_i$.

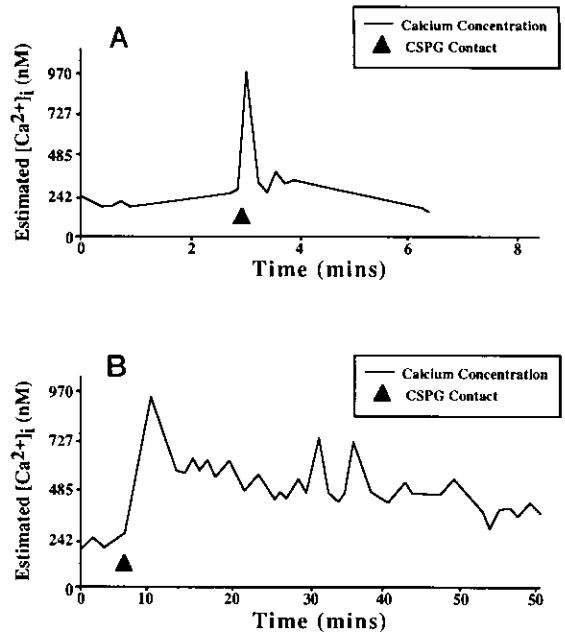

FIG. 6. CSPG-induced elevation of $[Ca^{2+}]_i$ results in two types of recovery phases. Elevation of $[Ca^{2+}]_i$ occurs immediately upon contact with CSPG. However, the recovery phase for these cells is variable. (A) In some cases, recovery is fast (<5 min; mean = 3 min 43 sec; n = 8) where $[Ca^{2+}]_i$ returns to the original baseline quickly and is maintained at that level (36.8%; n = 19). (B) In other cases, recovery is slow (>10 min; mean = 23 min 20 sec; n = 3), and $[Ca^{2+}]_i$ returns to a baseline that is higher than the original (63.2%, n = 19). Intermittent Ca^{2+} transients often occurred during the slow recovery phase. Note: (B) is a graphic representation of the growth cone shown in Fig. 2B using the CSPG stripe technique).

the case, we would have expected that greater than 80% of the growth cones at a CSPG border would express high $[Ca^{2+}]_i$. Since growth cones routinely touch and pull away from a CSPG surface, we have not determined whether growth cones that remain in contact with CSPG repeatedly elevate $[Ca^{2+}]_i$.

Source of $[Ca^{2+}]_i$ Rise of CSPG-Induced Calcium Rises in DRG Neurons

Since elevation of $[Ca^{2+}]_i$ that occurs when a growth cone encounters a stripe of CSPG is sufficient to cause major changes in growth cone behavior, it also becomes important to ask whether elevation of $[Ca^{2+}]_i$ is necessary for the guidance provided by the CSPG. The most straightforward test of this condition is to block the rise in $[Ca^{2+}]_i$. We designed a series of experiments to identify the source(s) of Ca^{2+} that lead to the elevation of $[Ca^{2+}]_i$.

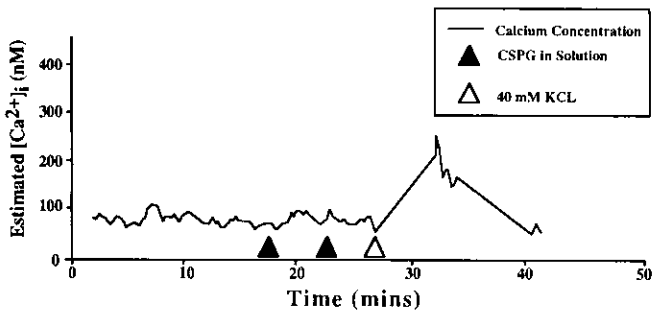

FIG. 5. CSPG in solution does not induce a rise in $[Ca^{2+}]_i$. The addition of 1-1000 μg/ml CSPG to the media, added sequentially to each culture of DRG neurons, does not inhibit normal migration, nor does $[Ca^{2+}]_i$ become elevated. In this example, the black arrowheads denote the addition of the largest concentrations of CSPG: 100 μg/ml (left) and 1000 μg/ml. The addition of 40 m*M* KCl demonstrates that the cells are competent to increase $[Ca^{2+}]_i$.

Conditions and blockers, which have been extensively characterized in this, and other, systems (Fox *et al.*, 1987; Thastrup *et al.*, 1990; Tsien and Tsien, 1990; Foskett and Wong, 1992) and shown to alter the ability of cells to elicit changes in $[Ca^{2+}]_i$ were used in this study. Potential sources of Ca^{2+} can be divided into two groups: those derived from influx of Ca^{2+} from the extracellular milieu and those derived from intracellular calcium stores. Table 1 summarizes the results of these studies.

Influx of Ca^{2+} from extracellular sources appears to be required for the rise induced by CSPG. This influx is not likely to occur through conventional voltage-dependent channels. The ability of added nickel chloride, or lanthanum chloride, or removal of extracellular calcium, to negate the CSPG-induced calcium rise, does, however, demonstrate the importance of extracellular Ca^{2+} and, potentially, provide important tools for determining whether rises in calcium are necessary for providing guidance.

Involvement of intracellular calcium stores were also examined using dantrolene, which blocks the ryanodine-sensitive calcium channel (Palade *et al.*, 1989), and in-

TABLE 1

TREATMENTS USED TO BLOCK ELEVATION OF $[Ca^{2+}]_i$ IN RESPONSE TO CSPG FROM EITHER THE EXTRACELLULAR ENVIRONMENT OR FROM INTRACELLULAR STORES

Source of Ca^{2+} (reagent or method)	Mean $\Delta[Ca^{2+}]_i$ (n*M*)	*N*
Control (2 m*M* Ca^{2+})	+458 ± 153	3
Extracellular		
Voltage-gated channel blockers (diltiazem, nifedipine, verapamil, ω-conotoxin)	+498 ± 48	9
General calcium channel blockers		
Nickel chloride	+16 ± 4	9
Lanthanum chloride	+29 ± 3	3
Zero calcium medium	+23 ± 10	5
Intracellular stores		
Dantrolene	+509 ± 55	5
Thapsigargin	+410 ± 58	3
Cyclopiazonic acid	+490 ± 71	3

hibitors of the microsomal Ca^{2+}-ATPase pump, TG (irreversible) (Thastrup *et al.*, 1990) and CPA (reversible) (Holzapfel, 1968). Addition of these reagents to DRGs in calcium-containing media led to a large release of intracellular calcium that did not occur subsequently if cells were bathed in calcium-free media to disallow refilling of the stores. These agents did not block CSPG-induced elevations of $[Ca^{2+}]_i$ (Table 1).

Calcium Channel and Internal Calcium Store Blockers Fail to Prevent Growth Cone Avoidance of CSPG Stripes

Studies show that levels of $[Ca^{2+}]_i$ that increase in the range of those induced by contact with CSPG can result in major changes in the growth cone cytoskeleton, and, therefore, the behavior of growth cones. Since we observed that zero calcium medium (Fig. 7A), or medium to which nickel chloride (Fig. 7B) or lanthanum chloride (not shown) had been added, blocked the elevation of $[Ca^{2+}]_i$ following contact with CSPG, we examined, in particular, the effects of these treatments using the CSPG stripe behavioral assay for CSPG-induced guidance of DRG growth cones. In a series of experiments replicated three times, DRG growth cone encounters with CSPG were analyzed in the presence of either zero calcium media or media containing nickel chloride (10 μ*M*–10 m*M*) or lanthanum chloride (1 m*M*), all conditions or reagents that block elevation of $[Ca^{2+}]_i$ induced by CSPG. Further, in order to compare our results to those of Bandtlow *et al.* (1993), we also included in the study culture dishes treated with dantrolene (40 μ*M*), TG (1 μ*M*), and cyclopiazonic acid (1 μ*M*), which perturb intracellular stores. All of the above reagents were added at the time of culturing DRG neurons. The cultures were qualitatively analyzed using a 20× phase objective. In an additional experiment, the reagents were added just prior to filopodial contact with CSPG and analyzed by time-lapse microscopy using a 63× oil immersion objective. In all cases, growth cones did not elongate onto stripes adsorbed with CSPG, as determined by qualitative examination of DRG cultures in which CSPG stripes were labeled with the fluorescent marker RITC (see Materials and Methods).

Although these initial studies fail to support a role for CSPG-induced elevation of $[Ca^{2+}]_i$ in the CSPG avoidance behavior, more experiments will be necessary before definitive conclusions can be drawn. For example, in order to assure that our treatments were effective in blocking the elevation of $[Ca^{2+}]_i$ throughout the course of the behavior experiments, we must monitor $[Ca^{2+}]_i$ for prolonged periods of outgrowth, as well as document the detailed behavior of growth cones under these conditions.

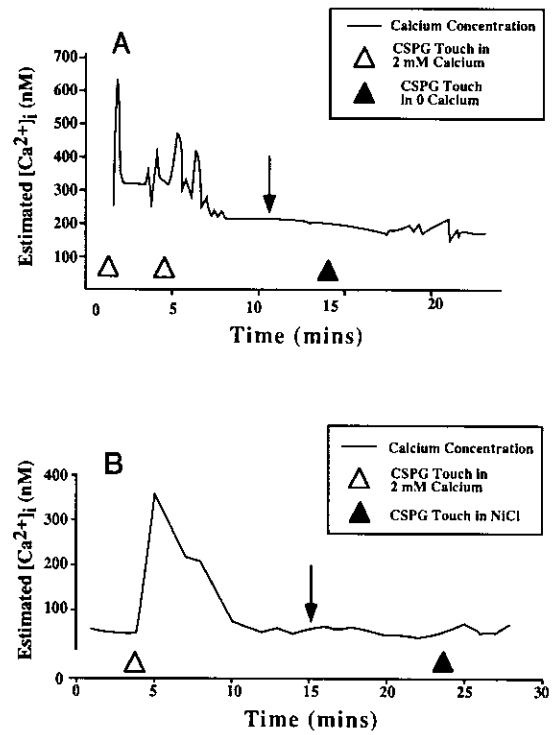

FIG. 7. Influx of Ca^{2+} from the extracellular environment is necessary for the elevation of $[Ca^{2+}]_i$ by CSPG. Contact of DRG neurons with CSPG-coated beads in calcium-containing and calcium-free media (A) and in media containing the general calcium channel blocker, nickel chloride (B). In normal media containing 2 m*M* Ca^{2+}, CSPG contact (white arrowheads) resulted in substantial increases in $[Ca^{2+}]_i$ (mean $\Delta[Ca^{2+}]_i$ = 458 ± 153 n*M*; n = 3; $P > 0.0001$). In calcium-free medium (addition indicated by arrow), however, growth cones did not respond to CSPG with a rise in $[Ca^{2+}]_i$ (black arrowhead; mean $\Delta[Ca^{2+}]_i$ = 22.6 ± 9.8 n*M*; n = 5; $P > 0.5$). (B) In normal media containing 2 m*M* Ca^{2+} without channel blockers, CSPG contact (white arrowhead) resulted in a large increase in $[Ca^{2+}]_i$ (mean $\Delta[Ca^{2+}]_i$ = 498 ± 47.5 n*M*; n = 3; $P < 0.0001$). However, in the presence of 10 m*M* nickel chloride (addition indicated by arrow), growth cones did not elevate $[Ca^{2+}]_i$ (black arrowhead; mean $\Delta[Ca^{2+}]_i$ = 16.1 ± 3.9 n*M*; n = 9; $P > 0.1$).

DISCUSSION

The primary goal of this study was to test whether growth cone contact with CSPG could have a signaling function and affect, specifically, the intracellular calcium concentration. Our results demonstrate that the answer to this question is yes. Another goal of this study was to determine the role of the elevation of $[Ca^{2+}]_i$ in growth cone avoidance of substratum-bound CSPG. Here, the answer is incomplete.

CSPG Activates a Calcium Second Messenger System

The results of the present study, taken together, demonstrate that DRG neurons are capable of responding to contact with CSPG by elevation of $[Ca^{2+}]_i$. Whether DRG neurons contacted a CSPG-adsorbed surface at the cell body or at the growth cone, the results were identical. Large rises in $[Ca^{2+}]_i$ on the order of five to seven times resting levels were routinely observed. These rises required the carbohydrate portion of CSPG since enzymatic removal of the carbohydrate abolished the elevation of $[Ca^{2+}]_i$. Furthermore, the rise occurred irrespective of whether surface-bound CSPG was brought to the neuron or the neuron was allowed to grow out and encounter CSPG. It is of interest that in previous guidance experiments (Snow *et al.*, 1990a), the degree of inhibition of growth cone migration was related to the amount of CSPG bound to a substratum. In the present experiments, there was also a clear relationship between the amount of contact with CSPG and the calcium response. Contact with one or two CSPG-coated beads rarely evoked a calcium rise. However, three or more CSPG beads routinely evoked the large rises we observed. These results taken together, and coupled with the magnitude and time course of the calcium rises we observed, show that PGs can induce a rise in $[Ca^{2+}]_i$ that could clearly provide a signaling function in DRG neurons.

Possible Roles for the Elevation of $[Ca^{2+}]_i$ in DRG Neurons

There are many cellular functions which could be directly affected by a rise in growth cone $[Ca^{2+}]_i$. Our particular interests have led us to focus on the relationship between elevated $[Ca^{2+}]_i$ and growth cone migration. We can rely on the fact that CSPG-induced elevation of $[Ca^{2+}]_i$ in growth cones is within the range of calcium rises shown by previous work to significantly alter the structural components necessary for growth cone behavior (Goldberg, 1988; Lankford and Letourneau, 1989, 1991; Letourneau and Cypher, 1991; Davenport and Kater, 1992; Kater and Mills, 1991; Rehder and Kater, 1992). Based on these data, we propose that calcium might fulfill sufficient conditions to act as an intermediate in PG-induced inhibition of growth cone migration by promoting reorganization of the cytoskeleton for stopping and turning at a CSPG border.

Elevation of $[Ca^{2+}]_i$ in response to contact with CSPG may play a number of roles in cytoskeletal reorganization. First, contact with CSPG may have direct effects on the cytoskeleton that increase the dynamic motility of the growth cone. In the leading margin of the growth cone, actin filaments are broken down in response to an elevation of $[Ca^{2+}]_i$ (Lankford and Letourneau, 1989, 1991; Lankford *et al.*, 1990). This action on actin filaments may regulate the behavior of filopodia that continuously sample the CSPG-adsorbed substratum. Actin filaments in growth cones may be cross-linked by the actin binding proteins α-actinin and filamin (Letourneau and Shattuck, 1989). When $[Ca^{2+}]_i$ is elevated, α-actinin dissociates from actin filaments, which could break down the filament network and destabilize filopodia and lamellipodia (Burridge and Feramisco, 1982; Blanchard *et al.*, 1989; Hartwig and Kwiatkowski, 1991; Weeds *et al.*, 1991). Gelsolin, another actin binding protein, is activated by elevated $[Ca^{2+}]_i$ to sever actin filaments and cap the barbed, or growing, end of actin filaments (Yin, 1988). Immunocytochemical evidence indicates that gelsolin is present in the leading margin of DRG growth cones (Letourneau, unpublished). Thus, the effects of elevated $[Ca^{2+}]_i$ on both α-actinin and gelsolin could lead to a rapid turnover of filopodia and lamellipodia and thereby facilitate redirection of the growth cone.

In addition to these direct effects that stimulate cytoskeletal reorganization, elevation of $[Ca^{2+}]_i$ in growth cones that contact CSPG may affect the motile apparatus of the growth cone by triggering other signaling cascades. Prolonged responses within growth cones may be activated in this manner. Neurons contain other major cytosolic targets for Ca^{2+}: protein kinase C (PKC) (Hyman and Pfenninger, 1987; Bixby, 1989), calpain (Siman and Noszek, 1988), and calmodulin (Kennedy, 1989). When activated, PKC can phosphorylate and thereby regulate membrane proteins. Similarly, the Ca^{2+}-dependent protease calpain, when activated, can proteolyze membrane proteins and has been shown to regulate cytoskeletal dynamics in neurons (Siman and Noszek, 1988). Calmodulin is a Ca^{2+}-binding regulatory protein that is progressively activated as intracellular calcium is raised above resting levels, with maximum binding of calcium at the micromolar range (Kennedy, 1989). An important function of activated calmodulin is to bind to Ca^{2+}/calmodulin-dependent protein kinases, which in turn phosphorylate a variety of proteins.

The overall result of the elevation of $[Ca^{2+}]_i$ in growth cones is dependent upon the spatial organization of these target proteins and their affinities for Ca^{2+}, as well as the location of downstream components of the signaling cascade. The elevation of $[Ca^{2+}]_i$ elicited by CSPG contact may signal multiple changes via actin-binding

proteins, PKC, calpain, calmodulin, and/or related proteins that facilitate turning at a boundary with CSPG.

Another interpretation of our data is that CSPG may inhibit growth cone advance by a mechanism unrelated to changes in $[Ca^{2+}]_i$, and that the rise in $[Ca^{2+}]_i$ is acting as a signal for a function not yet described. Given that PGs could potentially play a role in signaling events such as synaptogenesis (Gordon *et al.*, 1993), this possibility is plausible.

CSPG Contact Does Not Result in Growth Cone Collapse

Unlike interactions of growth cones with other inhibitors described in the literature (Kapfhammer and Raper, 1987; Walter *et al.*, 1987; Schwab and Caroni, 1988; Cox *et al.*, 1990; Davies *et al.*, 1990; Muller *et al.*, 1990; Raper and Kapfhammer, 1990; Johnston and Gooday, 1991), growth cones that contact PGs rarely collapse; i.e., they do not show a temporary or permanent reduction of the growth cone to a spindle shape with rearward movement of the growth cone and axon. Other studies similarly report that growth cone collapse does not occur when dissociated motor neurons contact cells from posterior sclerotome (Oakley and Tosney, 1993), a tissue rich in PGs (Tosney and Landmesser, 1985), and collapse is not always accompanied by elevated calcium (Ivins *et al.*, 1991). In some instances, filopodia do retract following contact with CSPG (Fig. 2B), but collapse most often does not occur (Fig. 2A), and retraction of filopodia is a normal occurrence and is sometimes seen in response to control beads (Fig. 3A).

Although the mechanisms of inhibition involved when growth cone collapse occurs in response to other agents are unknown, differences between the results of other studies describing growth cone collapse and this study using CSPG suggest that: (1) CSPG may represent a different mechanism of inhibition of growth cone migration than other agents, (2) the apparatus of growth cone motility is affected differently by CSPG than by other agents, and (3) calcium influx may be important, but as one of two or more components required to initiate collapse.

Growth Cone Avoidance of Bound CSPG May Not Be Dependent upon Elevation of Growth Cone $[Ca^{2+}]_i$

A goal of this study was to test whether growth cone avoidance of surface-bound CSPG requires intracellular calcium signaling. Behavioral experiments in this study represent preliminary data to this end, in that we cultured neurons under conditions in which the elevation of $[Ca^{2+}]_i$ was presumably absent and qualitatively analyzed the results. However, we have not verified this assumption by monitoring $[Ca^{2+}]_i$ in these analyses. Experiments are in progress to fully characterize the behavior of growth cones at a CSPG stripe by monitoring changes in $[Ca^{2+}]_i$ over prolonged periods using reagents to block the elevation of $[Ca^{2+}]_i$. Further, we are undertaking a detailed analysis of growth cone behaviors such as filopodial lifetimes, rates of lamellipodial expansion and neurite outgrowth, and degree of adhesion to the substratum, during normal encounters with CSPG and when the elevation of $[Ca^{2+}]_i$ is blocked.

It may turn out that growth cones still avoid a CSPG-containing substratum in the absence of an elevation of $[Ca^{2+}]_i$. If so, this may indicate that an additional affect of the CSPG is important in the growth cone avoidance response. Integrins play an important role in neurite outgrowth and the guidance of growth cones, including those of DRG neurons (Letourneau *et al.*, 1988; Neugebauer *et al.*, 1988; Tomaselli *et al.*, 1988; Lein *et al.*, 1991; Haugen, 1992; Tomaselli *et al.*, 1993). Previous evidence indicates that CSPG can weaken cell adhesion to adhesive components of the ECM (Culp *et al.*, 1986; Rouslahti, 1988; Gallagher, 1989). The negative charge of the polysaccharide chains of surface-bound CSPG may interfere with integrin binding to the substratum-bound adhesive ligands fibronectin and laminin. This would comprise another way to promote growth cone turning at a CSPG boundary by a mechanism that is independant of CSPG-induced elevation of $[Ca^{2+}]_i$.

In addition to these immediate effects of CSPG on integrin function, CSPG may also exert long range effects on expression of integrin genes. Recent data show that $\alpha 3$ integrin mRNA is up-regulated when DRG growth cones migrate on low concentrations of CSPG bound to laminin in a CSPG step gradient (Condic *et al.*, 1993), and that this up-regulation may facilitate adaptation for growth cone migration in a complex extracellular environment. Together, these data indicate that CSPG may affect integrin function and cell behavior.

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