Distribution of Phosphorylated GAP-43 (Neuromodulin) in Growth Cones Directly Reflects Growth Cone Behavior

Erik W. Dent, Karina F. Meiri

Departments of Pharmacology and Anatomy and Cell Biology, SUNY Health Science Center, 750 East Adams Street, Syracuse, New York 13210

Received 16 May 1997; accepted 8 January 1998

ABSTRACT: Phosphorylation of GAP-43 (neuromodulin) by protein kinase C (PKC) occurs at a single site, serine 41. In vivo, phosphorylation is induced after initiation of axonogenesis and is confined to distal axons and growth cones. Within individual growth cones, phosphorylation is nonuniformly distributed. Here, we have used high-resolution video-enhanced microscopy of cultured dorsal root ganglia neurons together with immunocytochemistry with a monoclonal antibody that recognizes PKC-phosphorylated GAP-43 to correlate the distribution of phosphorylated GAP-43 with growth cone behavior. In “quiescent,” nontranslocating growth cones, phosphorylated GAP-43 was confined to the proximal neurite and the central organelle-rich region, and was low in organelle-poor lamellae. However, levels in lamellae were elevated when they became motile. Conversely, levels of phosphorylated GAP-43 were low in either lamellae that were actively retracting or in the central organelle-rich region and proximal neurite of growth cones that had totally collapsed. The results suggest a mechanism whereby phosphorylation of GAP-43 by PKC, potentially in response to extracellular signals, could direct the functional behavior of the growth cone.

Keywords: GAP-43; neuromodulin; growth cones; PKC phosphorylation

INTRODUCTION

Translocation of growth cones in response to extracellular guidance cues requires the elaboration, retraction, and stabilization of specific filopodia and lamellae (Forscher et al., 1992; reviewed by Morris and Tiveron, 1994; Mitchison and Cramer, 1996). Little is known about how extracellular guidance cues cause such fundamental alterations to growth cone structure, but the regulation of interactions between the plasma membrane and the actin cytoskeleton is likely to be important (reviewed by Gumbiner, 1993; Morris and Tiveron, 1994; Goodman, 1996; Tanaka and Sabry, 1995; Yamada and Geiger, 1997). One major growth cone component that may be involved is the nervous-system specific protein GAP-43 (neuromodulin). High levels of GAP-43 (between 50 and 100 μM) (Apel and Storm, 1992) are associated with the growth cone membrane skeleton (Moss et al., 1990; Meiri and Gordon-Weeks, 1990), and are regulated by several Ca²⁺-dependent enzymes, notably protein kinase C (PKC). In fact GAP-43 is the major substrate of PKC in growth cones (Benowitz et al., 1987). Phosphorylation of GAP-43 by PKC thus suggests a route whereby extracellular signals such as guidance cues could directly affect the growth cone cytoskeleton (Baege et al., 1992).

Previously, we used a monoclonal antibody that specifically recognizes the single PKC phosphorylation site on GAP-43 at serine 41 (Coggins and Zwiers, 1989; Apel et al., 1991) to show that in sensory neurons in vivo, PKC phosphorylation of GAP-43 is restricted to the distal axon and the growth cone and is only initiated at the time growing axons arborize upon contacting their targets (Meiri et al., 1991). In sensory neurons in culture, phosphoryla-
tion within individual growth cones is nonuniformly distributed and dynamic (Meiri et al., 1991; Dent and Meiri, 1992) and can be stimulated by a number of extracellular agents including target-derived chemoattractant factors such as nerve growth factor (NGF) (Meiri et al., 1991; Meiri and Burdick, 1992; Liu et al., 1996), membrane-associated molecules, and guidance cues such as NCAM (Dent and Meiri, 1992; Walsh et al., 1996). This highly regulated phosphorylation may serve as a binary switch that regulates GAP-43 interactions with the actin cytoskeleton. Thus, serine\(^{41}\), the PKC site, is situated within a calmodulin-binding IQ motif (Cheney and Mooseker, 1992), such that GAP-43 is able to bind calmodulin (CaM) only when serine\(^{41}\) is unphosphorylated (Alexander et al., 1988; Chapman et al., 1991). In a cell-free assay, both phosphorylated and CaM-bound GAP-43 interacted with F-actin with high affinity but with strikingly different effects on the behavior of actin filaments. PKC-phosphorylated GAP-43 stabilized the formation of long actin filaments, whereas the unphosphorylated, CaM-bound form inhibited filament polymerization (He et al., 1997). Preliminary results suggest that these \textit{in vitro} interactions have functional effects on growth cone behavior. For instance, both phosphorylated GAP-43 and F-actin colocalize at the leading edge of certain growth cones in cultured sensory neurons (He et al., 1997), and likewise, growth cones in which GAP-43 levels had been depleted by oligonucleotides had reduced F-actin levels at the periphery (Aigner and Caroni, 1994).

We therefore set out to determine how the distribution of phosphorylated and unphosphorylated GAP-43 correlates with specific growth cone behaviors. Using high-resolution video-enhanced microscopy together with a monoclonal antibody to GAP-43 that identifies the PKC-phosphorylated form, we show here that phosphorylated GAP-43 is always found in areas in which organelles are clustered, usually in the central region of the growth cone and the adjacent neurite.Transient phosphorylation could also be induced in growth cone lamellae that are expanding rapidly. In contrast, phosphorylated GAP-43 was low in the lamellae of growth cones that were retracting and in both lamellae and central regions of growth cones that were collapsing. Our results suggest that the regulation of GAP-43 by PKC phosphorylation may allow the growth cone to generate a cytoskeletal response to extracellular guidance cues, presumably mediated by interactions between GAP-43 and F-actin.

**MATERIALS AND METHODS**

**Tissue Culture**

Dissociated cultures of dorsal root ganglion (DRG) cells were prepared as follows: Briefly, dorsal root ganglia collected from embryonic day E15–18 fetal rats were dissociated by incubation in 0.25% trypsin in Hank’s balanced salt solution (Sigma, St. Louis, MO) for 45–60 min at 37°C. After addition of an equal volume of media [Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum; Gibco, Grand Island, NY], the trypsinized ganglia were pelleted, resuspended in 1 mL of media, triturated, and finally plated onto either 22-mm\(^2\) etched coverslips (Belloco, Vineland, NJ) or eight-compartment Labtek wells (Nunc, Naperville, IL) that had previously been coated with laminin (16.6 \(\mu\)g/mL in carbonate buffer, pH 9.5) at a concentration of about 2 \(\mu\)g/cm\(^2\). Cells were plated at a density of 6000 cells/cm\(^2\) and were grown in DMEM containing 10% fetal calf serum and 5 U/mL 7S NGF (DMEM/FCS/NGF; Sigma, St. Louis, MO) at 37°C with 5% CO\(_2\). For time-lapse imaging, dissociated DRG cultures were plated on etched 22-mm\(^2\) coverslips with 0.8-mm spacers attached. A similar sized coverslip coated with silicon grease was lowered onto this one, and the “sandwich” was transferred to the imaging chamber (Forscher and Smith, 1988). Using this method, cells are not exposed to an air–liquid interface, which often causes severe retraction of processes and growth cone collapse. Fresh, warmed CO\(_2\)-equilibrated media were perfused into the chamber at a rate of 50–100 \(\mu\)L/min (total chamber media change within 4–8 min), and the cell chamber was kept at 37°C using a thermostatically coupled forced air heater within a custom Plexiglas chamber that surrounded the stage of the microscope. Cells were viewed with a Zeiss Axiolab IM-35 microscope (Carl Zeiss, Thornwood, NY) equipped with a 63X/1.4 NA Plan-Apochromat objective and matching 1.4 NA oil condenser along with a \(\times\)1.6 optivar magnifying lens. A 100-W halogen light source was used for illumination and shuttered using a Uniblitz V62 shutter. For IRM imaging a Plan-Neofluar \(\times\)63/1.25 NA oil Ph antiflex objective with the quarter wave plate adjusted to maximum brightness was used in combination with a 50-W mercury epillumination light source, 546 ± 10-nm precision filter, Zeiss double reflector HD, and pre- and postobjective polarizers adjusted to extinction, similar to that used by Gomez and Letourneau (1994). Images were collected with a Hamamatsu C2400 camera and saved directly to optical discs using a Panasonic TQ-2028F optical magnetic disk recorder. All images were background-subtracted, shading-corrected, and sharpened before saving using Image 1 software (Universal Imaging, Brandywine, PA).
Fixing and Staining Cultured Cells for Immunofluorescence

Labile lamellae and filopodia are commonly resorbed during fixation (Dent and Meiri, 1992). We found optimum preservation of growth cone morphology and optimal immunoreactivity with anti-GAP-43 antibodies following fixation for 15 min in 50% Bouin’s fixative in growth cone Krebs’ with 0.4 M sucrose (50% BKS). Growth cone Krebs’ is 145 mM NaCl, 5 mM KCl, 1.2 mM CaCl₂, 1.3 mM MgCl₂, 1.2 mM Na₂HPO₄, 10 mM glucose, and 20 mM Hepes, pH 7.4. Fixed cells were permeabilized for 20 min in 0.02% digitonin in goat block [10% normal goat serum/4% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)] and washed in 0.5% BSA in PBS. When the 2G12/C7 monoclonal antibody (Meiri et al., 1991) was used to double label cells together with the polyclonal anti-GAP-43 antiserum (Curtis et al., 1991), the cultures were incubated first in 2G12/C7 for 1 h, washed, and then incubated with high-fluorescein goat anti-mouse immunoglobulin G (IgG) (Antibodies Inc., Davis, CA), in goat block for 30 min. The 2G12-labeled cells were then incubated overnight at 4°C in the polyclonal anti-GAP-43 antiserum, followed by Texas red donkey anti-rabbit, secondary antibody (Jackson Immunocells) in horse block for 30 min. Overnight incubation with the polyclonal antiserum does not cause dissociation of 2G12/C7 from phosphorylated GAP-43 (Dent and Meiri, 1992). Cells were then coverslipped with Citifluor antifade mountant (City University, London, UK) and stored at 4°C.

Photographing Fluorescently Labeled Cells

Ektachrome 800/1600 slide film was used for immunofluorescence photography. All cultures were photographed on a Nikon Microphot microscope fitted with B2E and G1A filter sets. Black-and-white images were photographed at 400 ASA, and color at 800 ASA. For double-labeled cells, the exposure for each fluorochrome was independently metered, as described previously (Dent and Meiri, 1992). DIC images were directly imported from .tif files into Photoshop, whereas fluorescent images were digitized using a Polaroid Sprintscan 35 and color corrected using Adobe Photoshop 4.0. Figures were printed using a Kodak XP8600 printer.

Experimental Protocols

When dissociated cultures of DRG cells are grown on laminin substrates, GAP-43 is not phosphorylated during the initial extension of neurites, but can be detected in distal neurites and growth cones by 12 h after plating. Every growth cone contains phosphorylated GAP-43 by 36 h (Dent and Meiri, 1992). To determine how phosphorylated GAP-43 is distributed in individual growth cones, we therefore elected to use cultures between 8 and 24 h after plating. For video analysis, cells were plated onto coverslips having etched letters and numbers (Belco, Vineland, NJ), which allowed us to locate the growth cones which we had observed under video. Individual growth cones were identified under high power by their behavior, followed over time, and imaged at regular intervals, usually once per minute. Only one growth cone was imaged per coverslip to minimize the time the cultures spent in the perfusion chamber. Cells were illuminated only during image acquisition, and were rapidly fixed within 15 s of the last acquired image by perfusion with a warm fixative that was specifically developed to preserve labile lamellae and filopodia that are normally resorbed during conventional fixation procedures. A total of 122 growth cones from 16 independent experiments were used for these analyses. An independent experiment refers to different dissections and cultures. Several coverslips were obtained from each independent experiment. Some growth cones were used in more than one analysis—for instance, single growth cones often contained a rapidly expanding lamella as well as a retracting area. However, growth cones that were touching other cells were not also used for analyses of motility.

Creating Ratio Images of Phosphorylated and Total GAP-43

The monoclonal antibody 2G12/C7 recognizes an epitope on GAP-43 that includes phosphoserine⁴¹ (Meiri et al., 1991), whereas the polyclonal antibody does not discriminate between phosphorylated and unphosphorylated forms (Curtis et al., 1992). To obtain information about levels of phosphorylated GAP-43 relative to levels of the total protein, ratio images were constructed from the phospho- (2G12) and total (poly GAP-43) signals as follows: 24-bit color images in .tif format were changed to gray scale in Adobe Photoshop. Gray-scale images were then imported into Metamorph version 2.5 and the maximum gray value was determined using the ‘region statistics’ command. The maximum gray value for each image was brought to 255, the highest possible value for an 8-bit image, by multiplying each original image by a constant that varied between images. Thus, most of the dynamic range was used for each normalized image. Next, the ratio of the normalized 2G12/poly GAP-43 images was produced in Metamorph using the following formula: Normalized 2G12 image/Normalized poly GAP-43 image × 200 = Ratio image. The multiplication factor of 200 was used to keep each image within the dynamic range of the 8-bit image format. Resulting images were pseudocolored and a color scale was included with each one. Using this method, colors to the right of green signify higher ratios of phosphorylated GAP-43, and red designates high ratios of the phospho form compared with total. On the other hand, colors to the left show areas in which GAP-43 is significantly dephosphorylated compared with total protein. Purple areas show GAP-43 almost totally dephosphorylated.
Figure 1 Nonuniform distribution of phosphorylated GAP-43 in growth cones of DRG growth cones in culture. (a–l) Fluorescent photomicrographs of dissociated E15-18 DRGs that have been double labeled with polyclonal anti-GAP-43 followed by Texas red anti-rabbit 2nd antibody and 2G12/C7 followed by fluoresceinated anti-mouse 2nd antibody. Growth cones could be separated on the basis of morphology into symmetrical (a–g), asymmetrical (h–k), and filopodial (l), and further divided based on location of phosphorylated GAP-43. (a) Phosphorylated GAP-43 restricted to the proximal neurite at the neck of the growth cones (arrows). (b,c) High levels of phosphorylated GAP-43 in both the neurite and the central region of the growth cone with lower levels in the lamellae. (d–f) High levels in the neurite and central region, but areas of highly phosphorylated GAP-43 in discrete zones at the leading edge. (g) High levels of phosphorylated GAP-43 throughout the neurite and growth cone. (h,i) Asymmetric growth cones with high levels of phosphorylated GAP-43 in the neurite and also localized within discrete regions of the lamella. (j,k) Asymmetric growth cone where lamella extends outward from a curving neurite. High levels of phosphorylated GAP-43 in the neurite, the extension of the neurite into the growth cone and in discrete areas of lamellae (asterisks). (l) Filopodial growth cone with high levels of phosphorylated GAP-43 in some filopodia (arrows), but not others (asterisk). Scale: 1 cm = 10 μm.
RESULTS
Nonuniform Distribution of Phosphorylated GAP-43 in Growth Cones

By examining the fluorescent photomicrographs, we could designate lamellar growth cones as either largely symmetrical or obviously asymmetrical, and then categorize the distribution of 2G12/C7 immunoreactivity within them. In symmetrical growth cones, four distinct localizations of phosphorylated GAP-43 were obvious: In the first, it was confined to the neurite proximal to the neck of the growth cone [Fig. 1(a)]. In the second, it was still high in the neurite, but was also present in the central region of the lamellar expansion [Fig. 1(b,c)]. In the third type of symmetrical growth cone, phosphorylated GAP-43 was still found in the central region but was also enriched in discrete areas of the lamella [Fig. 1(d−f)]. It is in this type of growth cone that phosphorylated GAP-43 colocalizes with actin filaments (He et al., 1997). Finally, high levels of phosphorylated GAP-43 could also be found throughout the central region and lamellar expansion [Fig. 1(g)]. We also identified two types of asymmetric growth cones; in the first, only the lamellar expansion itself appeared asymmetric, and phosphorylated GAP-43 was commonly restricted to one specific area of the lamella [Fig. 1(h,i)]. In the second type, the whole growth cone appeared curved as though it were turning. In these growth cones, high levels of phosphorylated GAP-43 extended from the neurite along the curved inner edge of the growth cone, and levels were also often high within discrete areas of the lamellae [Fig. 1(j,k)]. Finally, in growth cones that were largely filopodial, some filopodia but not others were usually enriched in phosphorylated GAP-43 [Fig. 1(l)]. This study did not focus on GAP-43 distribution in filopodia.

Although these results present a picture confirming our previous observation that GAP-43 phosphorylation is dynamically regulated within growth cones (Dent and Meiri, 1992), they provide no specific information as to relative increases in phosphorylated GAP-43 in any of the areas, nor do they provide information about the functional states of DRG growth cones that might underlie any changes in phosphorylation. To address this issue directly, we coupled examination of live cells with double-labeling immunocytochemistry using the GAP-43 antibodies, and then produced ratio images in which changes in the proportion of phosphorylated GAP-43 relative to total protein were more apparent.

Distribution of Phosphorylated GAP-43 in Organelle-Rich Central Regions of the Growth Cone

When dissociated DRGs are plated on laminin at this density, individual growth cones spend a significant time either stationary or making no net advances (Dent and Meiri, 1992). We examined 35 growth cones that did not translocate over the time viewed (n = 6 independent experiments). These live “quiescent” growth cones showed a clearly defined central region in which organelles were concentrated, and double-labeling immunocytochemistry with 2G12/C7 and the polyclonal anti-GAP-43 antiserum followed by ratio imaging revealed that 83% (29 of 35) had high levels of total GAP-43.
Figure 3  High ratios of phosphorylated GAP-43 in motile lamellae. (a,b,f,g,k,l) DIC images of growth cones whose lamellae were motile between time 0 (a,f,k) and time 9 min (b), 7 min (g), and 12 min (l). Cells were then fixed within 30 s and double labeled with polyclonal anti-GAP-43 followed by Texas red anti-rabbit 2° antibody (c,h,m) and 2G12/C7 followed by fluoresceinated anti-mouse 2° antibody (d,i,n). (e,j,o) Ratio images of the same growth cones. White arrows indicate that expanding lamella have high ratios of fluorescent GAP-43 at their leading edge as indicated by the red color. Scale bar = 25 μm.
within the organelle-rich central region and high ratios of phosphorylated GAP-43 in discrete areas of this region compared with the organelle-poor lamellae [Fig. 2(c,d)]. Quiescent lamellae overall had low ratios of phosphorylated GAP-43, but occasional, discrete spots of the phosphorylated form were apparent [Fig. 2(c,g)]. The ratio of phosphorylated GAP-43 was also increased in the organelle-rich region of five asymmetric growth cones (\( n = 4 \) experiments) which were turning to one side [Fig. 2(g,h); see also Fig. 1(e,f)].

Correlation of Phosphorylated GAP-43 Distribution with Growth Cone Motility

Phosphorylated GAP-43 Is Enriched in Certain Highly Motile Lamellae. Phosphorylated GAP-43 was not always confined to the organelle-rich central region. In 17% (6 of 35) of “quiescent” growth cones described above, ratios of phosphorylated GAP-43 were very high throughout the growth cone, as shown in Figure 1(g). However, even though these growth cones did not actually translocate, they all had highly active lamellae and filopodia that were motile at the time of fixation, suggesting a relationship between motility of lamellae and distribution of phosphorylated GAP-43. The behavior of 20 growth cones with highly motile lamellae was followed (\( n = 5 \) independent experiments). Subsequently, four were rejected because either the lamella or a filopodium touched another cell. Of the remainder, 94% (15 of 16) had high ratios of phosphorylated GAP-43 in either the whole lamella or in the part of the lamella that was particularly active [Fig. 3(d,e,i,j,n,o)]. The single growth cone with low ratios of phosphorylated GAP-43 in an active lamella had only just begun to extend a neurite [Fig. 3(k–o)]. Initial axon extension in sensory neurons in culture as well as in vivo occurs without phosphorylated GAP-43 (Meiri et al., 1991; Dent and Meiri, 1992).

Phosphorylated GAP-43 Is Depleted in Retracting Lamellae. We next examined growth cones undergoing lamellar retraction for 2G12/C7 immunoreactivity. Fourteen growth cones were examined (\( n = 4 \) experiments). In 78% (11 of 14), levels of phosphorylated GAP-43 were lower in the retracting areas than in the central region or in the growth cone neck. Two examples are illustrated in Figure 4: In the first two, lamellae arose from a single neurite [Fig. 4(a–e)]. There was clearly less phosphorylated GAP-43 in the retracting lamella compared with the second lamella that expanded over the time viewed [Fig. 4(d,e)]. One growth cone.

---

**Figure 4** Decreased ratios of phosphorylated GAP-43 in retracting areas of growth cones. (a,b) DIC images of a split growth cone, with one expanding lamella and one which retracted from time 0 (a) to (b) 17 min later. Cells were then fixed within 30 s and double labeled with polyclonal anti-GAP-43 followed by Texas red anti-rabbit 2° antibody (c), and 2G12/C7 followed by fluoresceinated anti-mouse 2° antibody (d). (e) Ratio image of the same growth cone. The ratio of phosphorylated GAP-43 is higher in the expanding lamella (asterisk) and lower in the retracting lamella and the region between the two lamellae (arrow). This does not reflect a differences between the overall amount of GAP-43 in the two lamellae, since labeling with the polyclonal antibody (c) was similar in both. (f,g) DIC images of a single growth cone in which the central lamellae retracted between 0 min (f) and 14 min (g). Cells were then fixed within 30 s and double labeled with polyclonal anti-GAP-43 followed by Texas red anti-rabbit 2° antibody (h), and 2G12/C7 followed by fluoresceinated anti-mouse 2° antibody (i). (j) Ratio image of the same growth cone. The ratio of phosphorylated GAP-43 is higher in the expanding lamella (asterisk) than in the central retracting region where the growth cone split (arrow). The overall amount of GAP-43 at the lamellar edge is similar to the level where they divide. Scale bars: (a,b,f,g) = 15 μm; (c–e,h–j) = 20 μm.
Figure 5  Decreased phosphorylation of GAP-43 in retracting and collapsing growth cones. (a,b) DIC images of a growth cone which was retracting from time 0 (a) for 18 min (b). (c) Cells were then fixed within 30 s and double labeled with polyclonal anti-GAP-43 followed by Texas red anti-rabbit 2° antibody (c) and 2G12/C7 followed by fluoresceinated anti-mouse 2° antibody (d). (e) Ratio image of the same growth cone. The black arrows indicate that retraction of the lamella and proximal neurite is accompanied by low ratios of phosphorylated GAP-43 in the remaining filopodia, the residual central region of the growth cone, and in the retracting neurite. (f,g) DIC images of a single growth cone which retracted at time 0 (f) or
that split into two [Fig. 4(f–j)] had a reduced ratio of phosphorylated GAP-43 at the retracting zone that bisected the dividing lamella [Fig. 4(i,j)]. Reduced ratios of phosphorylated GAP-43 did not simply reflect less total GAP-43 in the retracting areas, since levels of polyclonal antibody immunoreactivity were the same in the retracting areas as in the nonretracting lamellae [Fig. 4(c,h)]. The results suggest that the predominant form of GAP-43 in retracting lamellae is not phosphorylated on serine 41.

Phosphorylated GAP-43 Is Depleted in the Central Region of Growth Cones that Are Collapsing. Growth cone collapse involves retraction of the central region and often the proximal neurite as well as the lamella. However, growth cones that completely collapsed without being in contact with another cell proved difficult to find. In fact, from a total of 10 independent experiments from which nearly 100 growth cones were sampled, we were able to find only three that completely collapsed without touching another cell first. Two are illustrated: In the first [Fig. 5(a–e)], the neurite retracted as well as the lamella, and phosphorylated GAP-43 was low in both areas [Fig. 5(d,e)]. In the second growth cone [Fig. 5(f–j)], both the filopodia and the central region retracted, and again phosphorylated GAP-43 was low in both places [Fig. 5(i, j)]. However, in this case the neurite did not retract significantly, and higher levels of phosphorylated GAP-43 persisted in this region [Fig. 5(j)]. The results suggest that phosphorylation of GAP-43 in the central region and neurite is stable provided they are not retracting [Fig. 5(e, j); compare with Fig. 1].

Phosphorylated GAP-43 Is Also Low in Growth Cones That Are Induced to Collapse Experimentally. Acute retraction of growth cone lamellae can be induced by perfusing DRG cultures in defined media without NGF (Aletta and Greene, 1988; González-Agosti and Solomon, 1996). Likewise, in these cultures, NGF deprivation caused complete collapse of growth cones and extensive retraction of the proximal neurite [arrows, Fig. 5(k–o)]. By 1 h after perfusing with media without NGF, 72% of growth cones (48 of 65) had no lamellae. By comparison, 75% of control cultures (78 of 104) still retained lamellae. Low levels of phosphorylated GAP-43 were found in the residual growth cone and retracted proximal neurite of 81% (39 of 48) of collapsed growth cones [Fig. 5(n,o)]. This is in contrast to the situation during lamellar retraction in which the central region of the growth cone and proximal neurite remained stable. Under those circumstances, phosphorylated GAP-43 remained high in both the central region and the proximal neurite.

**DISCUSSION**

Video-enhanced microscopy of live cultures of dissociated DRG neurons followed by immunocytochemistry with our monoclonal antibody that detects steady-state phosphorylation of GAP-43 on serine 41, the kinase C phosphorylation site, allowed us to investigate how changes in levels of GAP-43 phosphorylation correlate with functional states of growth cones. The results show how the ratio of both persistently or transiently phosphorylated GAP-43 changes with the functional state of growth cones. Persistent or stable phosphorylation was localized in neurites proximal to the neck of the growth cone and in organelle-rich regions of growth cones provided that they were not completely collapsing. In contrast, transient phosphorylation of GAP-43 was induced in lamellae that became motile. Our results therefore suggest that phosphorylation of GAP-43 by PKC is both spatially and temporally coupled to the functional state of the growth cone, and imply that the regulation of steady-state phosphorylation of GAP-43 is critical for growth cone motility.
Table 1  Results Showing Distribution of Phosphorylated GAP-43 in Growth Cones

<table>
<thead>
<tr>
<th>Functional State</th>
<th>P-GAP-43</th>
<th>% Total</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quiescent</td>
<td>Low in lamellae</td>
<td>83% (29/35)</td>
<td>6</td>
</tr>
<tr>
<td>Loosely apposed lamellae</td>
<td>Low in lamellae</td>
<td>86% (12/14)</td>
<td>6</td>
</tr>
<tr>
<td>Motile lamellae</td>
<td>High in lamellae</td>
<td>94% (15/16)</td>
<td>5</td>
</tr>
<tr>
<td>Retracting lamellae</td>
<td>Low in retracting areas</td>
<td>78% (11/14)</td>
<td>4</td>
</tr>
<tr>
<td>Naturally collapsing</td>
<td>Low in residual growth cone</td>
<td>100% (3/3)</td>
<td>10</td>
</tr>
<tr>
<td>NGF-deprived collapsing</td>
<td>Low in residual growth cone</td>
<td>81% (39/48)</td>
<td>6</td>
</tr>
<tr>
<td>Contact with other cells</td>
<td>High in region of contact</td>
<td>94% (16/17)</td>
<td>6</td>
</tr>
<tr>
<td>Central region tightly attached to substrate</td>
<td>High in central region</td>
<td>84% (16/19)</td>
<td>6</td>
</tr>
</tbody>
</table>

* Number of independent experiments.

levels of phosphorylated GAP-43 plays an important role in growth cone motility and guidance. Table 1 summarizes the quantitative data used in this analysis.

**Unphosphorylated GAP-43 in Lamellar Retraction and Growth Cone Collapse**

Levels of phosphorylated GAP-43 were consistently low in the quiescent (nonmotile) lamellae of growth cones that did not translocate, except for small discrete spots which we have previously shown may represent areas of tight attachment to the substrate (Meiri and Gordon-Weeks, 1990). Cessation of translocation is a normal part of the motility cycle (Dent and Meiri, 1992) and stationary growth cones frequently resumed translocating when viewed for extended periods. Areas of lamellae that had been motile but which were quiescent at the time of fixation were also low in phosphorylated GAP-43. In contrast, quiescent lamellae that became motile and remained so at the time of fixation also acquired high levels of phosphorylated GAP-43. The transition from lamellar quiescence to motility, and vice versa, is therefore accompanied by dynamic phosphorylation and dephosphorylation of GAP-43 in the lamellae. Low levels of phosphorylated GAP-43 were also found in both lamellae that were retracting and whole growth cones that were collapsing. Whole growth cone collapse differs from lamellar retraction in that it involves retraction of the central region, and often the proximal neurite as well. Retraction of lamellae and collapse of whole growth cones seem to be correlated with dephosphorylation of GAP-43 in different areas of the growth cone. For example, the transient dephosphorylation of GAP-43 was not sufficient to induce retraction of lamellae, suggesting that dephosphorylation in this area is not involved in retraction. Likewise, GAP-43 in the central region of the growth cone was not dephosphorylated when lamellae themselves retracted. The key correlate to whole growth cone collapse therefore seems to be dephosphorylation of GAP-43 in the central region, which remained high during lamellar retraction, but which was always low in growth cones that had collapsed completely upon NGF withdrawal.

**Transiently and Stably Phosphorylated GAP-43 in Motile Growth Cones**

The high levels of stably phosphorylated GAP-43 in the central region may result from the activation of PKC in response to the presence of NGF in the culture medium. In a subcellular fraction of intact isolated growth cones, NGF stably increased steady-state levels of GAP-43 phosphorylated by PKC. When NGF was removed, phosphorylated GAP-43 declined to baseline levels in a Ca$^{2+}$ dependent manner, because of phosphatase activity (Meiri and Burdick, 1992). Interestingly, the half-time for dephosphorylation of GAP-43 closely paralleled the time taken for maximal growth cone collapse upon NGF deprivation in these experiments (Meiri and Burdick, 1992). Growth cone retraction upon withdrawal of NGF resembles that seen when PKC was rapidly down-regulated by phorbol ester in similar DRG cultures (Dent and Meiri, 1992), and similarly, inhibition of PKC is known to induce growth cone collapse (Campenot et al., 1994; Theodore et al., 1995), further confirming the ability of PKC phosphorylation to confer stability to growth cones. In neither case do we know the temporal sequence of the dephosphorylation events that lead to either lamellar retraction or growth cone collapse; however, neuronal cells that were stably transfected with an unphosphorylatable form of GAP-43 were unable to attach successfully to laminin substrates, implying that dephosphorylation may precede the detachment that gives rise to retraction and collapse.
The results present evidence that the spatial regulation of GAP-43 dephosphorylation throughout the growth cone, like its phosphorylation, is complex.

The results describe a temporal and spatial correlation between the functional state of a growth cone and the phosphorylation status of GAP-43. Our previous experiments showing how phosphorylation of GAP-43 acts as a binary switch that can regulate F-actin behavior suggest a molecular mechanism that might account for these findings: In vitro, unphosphorylated GAP-43 behaves as a barbed-end capping protein preventing actin filament polymerization (Hug et al., 1995; He et al., 1997). Likewise, growth cones that are collapsing have reduced levels of F-actin (Fan et al., 1993). Overexpression of CapZ, an actin-binding protein whose behavior resembles that of unphosphorylated GAP-43, results in decreased membrane stability (Hug et al., 1995); and likewise, overexpression of an unphosphorylatable form of GAP-43 in PC12 cells results in unstable membranes (Meiri et al., 1996). The results therefore suggest a correlation between unphosphorylated GAP-43, depletion of F-actin, and growth cone instability. Phosphorylated GAP-43 has different effects on F-actin behavior: In vitro, phosphorylation of GAP-43 stabilizes the formation of long actin filaments by removing them from the dynamic polymerization/depolymerization cycle (He et al., 1997). We have previously shown that contact with other cells can induce phosphorylation of GAP-43 at the growth cone membrane (Dent and Meiri, 1992), and this, together with the colocalization of phosphorylated GAP-43 with F-actin at the leading edge of lamellae (He et al., 1997), strongly suggests that the spatial regulation of phosphorylated GAP-43 at the growth cone membrane may be important in determining F-actin behavior within growth cones. Several proteins that regulate actin interaction with membranes are acylated (Aderem, 1992), and the dynamic palmitoylation of GAP-43 on cys$^4$ and cys$^5$ in growth cones may contribute to the spatial regulation of the phosphorylated form of GAP-43 (Liu et al., 1991; Skene and Virag, 1989). Likewise, given the importance of GTP-binding proteins in both growth cone signaling and regulation of the actin cytoskeleton (Tapon and Hall, 1997), the ability of GAP-43 to modulate GTP binding to G proteins (Strittmatter et al., 1993) may also play a role in the regulation of actin interactions. During axonogenesis in vivo, sensory neurons, such as DRGs, begin to phosphorylate GAP-43 as they approach their peripheral targets, and the increased phosphorylation coincides with both the arborization of DRG axons and acquisition of electrical excitability (Fields and Itoh, 1996). Thus phosphorylation of GAP-43, potentially regulatable by calcium changes in the periphery (Fitzgerald and Fulton, 1992), is both spatially and temporally positioned for involvement in the direction of synaptogenesis in DRG neurons (Gottman et al., 1988). Furthermore, these experiments imply that phosphorylated GAP-43 in different parts of the growth cone may play different roles in this process. "The mechanics of the growth cone cytoskeleton is at the heart of understanding movement and growth, and their relationship to axon guidance" (Morris and Tiveron, 1994).

The high levels of GAP-43 in growth cones and its position in the signal transduction cascade that links extracellular signals with the cytoskeletal response underscore its importance to growth cone function.

The authors thank Dr. Shyamala Mani, Anjili Mathur, and Yiping Shen for critical comments on the manuscript, and Amanda Bass for assistance. This work was supported by NS 26091 to KFM.

REFERENCES


