Neurite Outgrowth Stimulated by Neural Cell Adhesion Molecules Requires Growth-Associated Protein-43 (GAP-43) Function and Is Associated with GAP-43 Phosphorylation in Growth Cones

Karina F. Meiri,[1] Jane L. Saffell,[2] Frank S. Walsh,[2] and Patrick Doherty[2]

[1]*Department of Pharmacology, State University of New York Health Science Center, Syracuse, New York 13210, and* [2]*Department of Experimental Pathology, Guy's Hospital Medical School, London SE1 9RT, United Kingdom*

The mechanisms whereby cell adhesion molecules (CAMs) promote axonal growth and synaptic plasticity are poorly understood. Here we show that the neurite outgrowth stimulated by NCAM-mediated fibroblast growth factor (FGF) receptor activation in cerebellar granule cells is associated with increased GAP-43 phosphorylation on serine-41. In contrast, neither NCAM nor FGF was able to stimulate neurite outgrowth in similar neurons from mice in which the GAP-43 gene had been deleted by homologous recombination. Integrin-mediated neurite outgrowth was unaffected by GAP-43 deletion. Both neurite outgrowth and rapid phosphorylation of GAP-43 in isolated growth cones required the first three Ig domains of a NCAM-Fc chimera and were stimulated maximally at 5 μg/ml (~50 nM). Likewise, GAP-43 phosphorylation in isolated growth cones also was stimulated by an L1-Fc chimera. Both neurite outgrowth and NCAM-stimulated GAP-43 phosphorylation were inhibited by antibodies to the FGF receptor and a diacylglycerol lipase inhibitor (RHC80267) that blocks the production of arachidonic acid in response to activation of the FGF receptor. Direct activation of the FGF receptor and the arachidonic acid cascade with either basic FGF or melittin also resulted in increased GAP-43 phosphorylation. These data suggest that the stimulation of neurite outgrowth by NCAM requires GAP-43 function and that GAP-43 phosphorylation in isolated growth cones occurs via an FGF receptor-dependent increase in arachidonic acid.

Key words: GAP-43; NCAM; FGF receptor; growth cone; neurite outgrowth; knock-out mouse

Growth cones navigate through embryos by responding to specific guidance cues, many of which belong to highly conserved families (for review, see Tessier-Lavigne and Goodman, 1996; Walsh and Doherty, 1997). Recent experiments have demonstrated a function for the immunoglobulin (Ig) superfamily of cell adhesion molecules (CAMs) in axonal pathfinding (Cohen et al., 1997; Cremer et al., 1997; Dahme et al., 1997; Fazeli et al., 1997), which shows that they function as growth cone receptors for growth and/or guidance cues (Lagenauer and Lemmon, 1987; Doherty et al., 1990; Keino-Masu et al., 1996; de la Torre et al., 1997). CAMs also function in synaptic plasticity associated with learning and memory in the adult (see Muller et al., 1996; Schuster et al., 1996a,b) (for review, see Martin and Kandel, 1996; Walsh and Doherty, 1997).

Recent evidence shows that axonal growth responses stimulated by a number of CAMs involve the stimulation of signaling cascades (Doherty et al., 1995b; Saffell et al., 1997). Thus in some circumstances CAMs activate neuronal fibroblast growth factor (FGF) receptors (Williams et al., 1994d; Saffell et al., 1997), resulting in the sequential stimulation of phospholipase C-γ (PLCγ) and diacylglycerol (DAG) lipase to generate arachidonic acid (for review, see Doherty and Walsh, 1994). The mechanisms whereby CAM-induced activation of a signaling cascade modulates neurite outgrowth and synaptic plasticity are not known. GAP-43 has been implicated in axon growth and growth cone guidance (Skene, 1989) and in the synaptic plasticity that is associated with learning and memory (Fagnou and Tuchek, 1995; Benowitz and Routtenberg, 1997). GAP-43 knock-out mice display errors in axonal pathfinding (Strittmatter et al., 1995), whereas overexpressing GAP-43 causes ectopic axon sprouting in transgenic mice (Aigner et al., 1995). GAP-43 is enriched at the interface between receptors and cytoskeleton, and its phosphorylation state influences cytoskeletal dynamics, including actin polymerization (Meiri and Gordon-Weeks, 1990; Moss et al., 1990; Dent and Meiri, 1992a,b, 1998; Aigner and Caroni, 1993; He et al., 1997). GAP-43 phosphorylation can be stimulated by arachidonic acid in neurites (Dent and Meiri, 1992b), synaptosomal membranes (Schaechter and Benowitz, 1993), and hippocampal slices (Luo and Vallano, 1995). Thus it is a candidate to link CAM-activated signal transduction and the cytoskeleton; however, its regulation by arachidonic acid in growth cones has not been investigated.

Here we show that cerebellar granule cells from GAP-43 knock-out mice are unable to respond to either NCAM or FGF in a neurite outgrowth assay. Furthermore, stimulation of neurite outgrowth, either on cells expressing transfected NCAM or by soluble NCAM chimeras, is accompanied by increased phosphorylation of GAP-43 in neurites and in isolated growth cones. Both maximal neurite outgrowth and maximal GAP-43 phosphorylation required the first three Ig domains of NCAM. The phosphorylation response to NCAM is mimicked by basic FGF and L1 as well as melittin (which stimulates arachidonic acid production). Finally, NCAM-stimulated GAP-43 phosphorylation is inhibited by FGF receptor antibodies and the DAG lipase inhibitor

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Correspondence should be addressed to Dr. Karina F. Meiri at the above address.

(RHC80267) that inhibits the FGF receptor-dependent production of arachidonic acid (Williams et al., 1995b; Hou et al., 1997). Our results show that GAP-43 function is essential for NCAM-stimulated neurite outgrowth and that much of this appears because of the phosphorylation of GAP-43 via the FGF receptor-dependent stimulation of arachidonic acid.

MATERIALS AND METHODS

Cell culture. Cocultures of cerebellar neurons on 3T3 monolayers were established as previously described (Doherty et al., 1990, 1995a). In brief, 80,000 3T3 cells in DMEM/10% FCS were plated into individual chambers of an eight-chamber tissue culture slide coated with poly-L-lysine and fibronectin. After 24 hr to allow for monolayer formation, the medium was removed and 3000 cerebellar neurons taken from postnatal day 2–6 (P2–P6) mice were plated into each well in SATO media (Doherty et al., 1990) supplemented with 2% FCS. Test reagents were added as indicated in the text, and the cocultures were maintained for 26 hr, whereafter they were fixed and stained for GAP-43 immunoreactivity. The mean length of the longest neurite per cell was measured for 150–200 neurons in each population, as previously described (see Doherty et al., 1995a).

Preparation of CAM-Fc chimeras. Production of both NCAM and L1 chimeras has been described previously (Doherty et al., 1995a). To create the panel of NCAM-Fc deletion chimeras, we inserted extracellular domain portions of human NCAM cDNA (Gower et al., 1988) into the PIg vector (Fawcett et al., 1992), using a cloning strategy similar to that used in the production of the L1-Fc chimera (Doherty et al., 1995a). As well as the whole extracellular domain of the five immunoglobulin domains (Ig) and the two fibronectin type III repeats (FNIII), portions of the extracellular domain progressively deleted from the C terminus by PCR were inserted into PIg to produce truncated NCAM-Fc chimeras. Truncation at amino acid (aa) 107 gave Ig1 alone, truncation at aa 212 gave Ig1–2, truncation at aa 304 gave Ig1–3, truncation at aa 405 gave Ig1–4, truncation at aa 534 gave Ig1–5, truncation at aa 598 gave Ig1–5 plus FNIII 1, and finally, truncation at aa 692 gave Ig1–5 plus FNIII 1 and 2. Exon boundaries for human NCAM were derived from Owens et al. (1987). Soluble NCAM-Fc chimeras were harvested from the culture medium of COS-7 cells transiently transfected with plasmid DNA and were purified by binding to protein A-Sepharose beads.

Preparation of isolated growth cones. Isolated growth cones were prepared from P1–P2.5 (day of birth is P0) mouse forebrains as described previously (Gordon-Weeks, 1987), with the following modifications: centrifugation of the discontinuous Ficoll gradient was at a speed of 30,400 × *g* for 20 min with the use of a SW 40.1 rotor. Material banding at the interface between the sample and the 7% Ficoll solution was diluted 1:5 by adding Krebs's solution dropwise while stirring over ice [Krebs's solution contains (in mM) 145 NaCl, 5 KCl, 1.2 $CaCl_2$, 1.3 $MgCl_2$, 1.2 NaH_2PO_4, 10 dextrose, and 20 HEPES, pH 7.4]. The resultant suspension was centrifuged at 15,000 × *g* for 50 min at 4°C, and the resultant pellet was resuspended in Krebs's solution at a concentration of ≈2 mg/ml.

Stimulation of phosphorylation of IGC proteins. Freshly prepared IGCs in Krebs's buffer were incubated for 15 min at 37°C with specific solutions of either NCAMs, L1, or FGF. Then the reaction was stopped by dropping the microfuge tubes into liquid nitrogen, and samples were stored at −20°C until used further. In other experiments in which the inhibition of GAP-43 phosphorylation was studied, freshly prepared IGCs were incubated first for 30 min at 37°C with specific inhibitors of the FGF–receptor–PLCγ pathway before stimulation of phosphorylation with NCAM, L1, or FGF for 30 min at 37°C, as before.

Electrophoresis, Western blotting, and dot blotting. One-dimensional analysis of proteins used 10% SDS gels. Western blotting was performed exactly as described previously (Meiri and Burdick, 1991). Proteins were transferred onto polyvinyl difluoride Immobilon-P paper, and immunoreactivity was detected with 2G12 antibody, followed by peroxidase-labeled anti-mouse IgG. Immunoreactivity was visualized with chemiluminescence, using Pierce (Rockford, IL). To quantify phosphorylation of GAP-43 on dot blots, we solubilized aliquots of IGCs with 0.01 M NaOH and sonicated and dotted the aliquots onto Immobilon membrane. Duplicated samples were probed with the monoclonal antibodies 2G12 and 7B10, as described previously (Meiri and Burdick, 1991). In this case specific immunoreactivity was visualized by the binding of ^{125}I-conjugated sheep anti-mouse IgG (specific activity, 18 μCi/μg), followed by a phosphorimager analysis of the dot blot images, using a Molecular Dynamics Storm 840 PhosphorImager (Sunnyvale, CA). Quantitation of antibody binding was done with Imagequant analysis software, and specific phosphorylation on ser41 was calculated as a percentage of the 2G12 immunoreactivity as compared with total GAP-43 phosphorylation detected with 7B10.

Targeting vector and generation of GAP-43 mutant mice. GAP-43 knock-out mice were created by using conventional gene targeting with a replacement-type targeting vector consisting of a 9.0 kb 129/sv mouse genomic fragment, in which 476 bp of the GAP-43 gene (from intron 1 and nucleotides 133–171 from the cDNA) were replaced with the pPGK-neobpA cassette used as a positive selection marker. The pGK-thymidine kinase cassette was introduced as a negative selection marker. Electroporation and selection were performed with the CJ7 embryonic stem (ES) cell line (129/sv). Genomic DNA derived from 313 G418/FIAU-resistant ES cell clones was screened by using Southern blot analysis after diagnostic restriction enzyme digestion. Recombinant clones containing the expected gene replacements were obtained at a frequency of 1:156. The heterozygote GAP-43 recombinant ES clones injected into C57Bl/6 blastocysts generated chimeras that transmitted the mutated allele to the progeny when mated to C57Bl/6 or 129/sv females. The breeding of two GAP-43 +/− mice gave rise to homozygous mutant mice at a frequency of 25%. Mutant mice produced neither GAP-43 RNA nor protein. Homozygous progeny are born normally, but ~50% die within the first 2 d of postnatal life (PN). Most of the remainder fail to develop past weaning and die between 14 and 21 d PN. A very small percentage (5%) survives past weaning. Two strains of mice were used for these experiments: the A129/sv strain in which the knock-out first was generated and the progeny of a seventh generation back-crossed into C57Bl/6. There were no differences in the phenotype between the two strains.

Other reagents. The polyclonal anti-NCAM antibody (A5) raised against NCAM purified from rat brain and the anti-FGF receptor antiserum raised against the 31-mer CAM homology domain peptide were produced in the Doherty lab and used at 1:200 (see Williams et al., 1994c). The monoclonal anti GAP-43 antibodies 2G12 and 7B10 have been described previously (Meiri et al., 1991) and were used as tissue culture supernatant at 1:5. Iodinated anti-mouse IgG secondary antibody (specific activity, 750–3000 Ci/mmol) was obtained from Amersham. The polyclonal anti-L1 antiserum raised against a fusion protein containing L1 fibronectin domains was a generous gift of Elizabeth Bock (Copenhagen, Denmark) and was used at 1:200. RHC80267 was obtained from Biomol Research Labs (Plymouth Meeting, PA) and was used at 10 μg/ml. Melittin (0.01 M) was purchased from Calbiochem (La Jolla, CA).

RESULTS

GAP-43 function is required for NCAM stimulation of neurite outgrowth

Mice in which GAP-43 expression is prevented by homologous recombination are able to extend axons *in vivo* and *in vitro*; however, errors in pathfinding occur (Strittmatter et al., 1995). We tested cerebellar neurons from GAP-43 knock-out mice for their ability to respond to NCAM and FGF by comparing their growth on a monolayer substrate of 3T3 cells with monolayers of 3T3 cells expressing physiological levels of human NCAM, using conditions under which NCAM can stimulate neurite outgrowth from wild-type neurons (Doherty et al., 1990; Williams et al., 1994c). The results are summarized in Figure 1. After 26 hr of culture the mean length of the longest neurite extending from wild-type neurons on NCAM monolayers was 47.4 ± 2.52 μm (n = 274 neurites from four independent experiments). This was significantly greater than the value measured for similar neurons cultured on control 3T3 monolayers (23.6 ± 1.2 μm; n = 290 neurites from four independent experiments). Likewise, after 16 hr of culture in the presence of 10 ng/ml FGF2, the mean length of the longest neurite extending from wild-type neurons on NCAM monolayers was 48.2 ± 3.56 μm (n = 177 neurites from two independent experiments). In contrast, neurons isolated from the GAP-43 knock-out mice extended neurites as normal on 3T3 monolayers (23.66 ± 1.2 μm; n = 394 neurites from four independent experiments) but showed no significant increase in

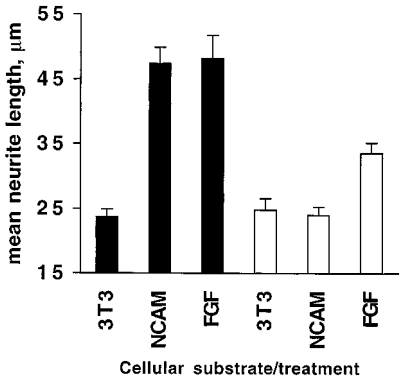

Figure 1. NCAM stimulation of neurite outgrowth is inhibited in GAP-43 knock-out mice. Cerebellar neurons from either wild-type (*black bars 1–3*) or GAP-43 knock-out (*white bars 4–6*) mice were cultured at low density on confluent 3T3 monolayers either alone (*1*, *4*) or in the presence of 10 ng/ml FGF2 (*3*, *6*). Alternatively, cells were grown on NCAM-expressing 3T3 monolayers (*2*, *5*). After 26 hr the cocultures were fixed and stained with a TuJ-1 antibody to visualize neurons for a measurement of the mean length of the longest neurite per cell. The results are combined from four independent experiments and are expressed as mean neurite length ± SEM. Between 200 and 400 neurons were analyzed in each population.

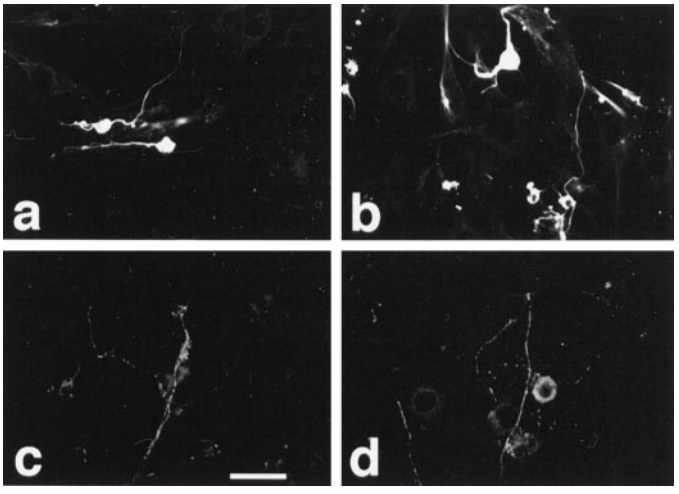

Figure 2. NCAM stimulates increased steady-state levels of phosphorylated GAP-43. Cerebellar neurons were cultured at low density on confluent NCAM-expressing 3T3 cells (*a*, *b*) or on 3T3 cells alone (*c*, *d*). After 16 hr the cocultures were fixed and stained with the monoclonal antibody (mAb) 2G12, which visualizes GAP-43 phosphorylated on serine-41, the PKC site, followed by fluoresceinated secondary antibody. Higher labeling indicates increased levels of phosphorylated GAP-43 in cells cultured on NCAM-expressing monolayers. Scale bar, 50 μm.

length on the NCAM-expressing monolayers (24 ± 1.3 μm; n = 379 neurites from four independent experiments). Moreover, when neurons isolated from the GAP-43 knock-out mice were cultured on 3T3 monolayers in the presence of 10 ng/ml FGF2, they too showed no significant increase in length as compared with controls (33.66 ± 1.6 μm; n = 300 neurites from two independent experiments). These data show that GAP-43 function is required for the neurite outgrowth response stimulated by NCAM and FGF, but not for neurite outgrowth over control 3T3 cells, which is integrin-mediated (Williams et al., 1994b). The latter result is in accord with previous studies that have shown that GAP-43 function is not required for integrin-mediated neurite outgrowth over laminin (Strittmatter et al., 1995).

NCAM stimulates increased steady-state levels of GAP-43 phosphorylation in cerebellar granule cell neurites and growth cones

The phosphorylation status of GAP-43 on serine-41 [the protein kinase C (PKC) site] directly correlates with growth cone function: increased phosphorylation of serine-41 occurs in actively translocating growth cones, whereas collapsing growth cones contain the dephosphorylated form (Dent and Meiri, 1998). GAP-43 phosphorylation can be induced by contact between growth cones and other cells as well as by growth and guidance cues such as nerve growth factor (NGF; Meiri and Burdick, 1991; Dent and Meiri, 1992a,b, 1998). To determine whether NCAM signaling also might influence GAP-43 phosphorylation, we cultured P3–P6 mouse cerebellar granule cells at low density on confluent monolayers of either the NCAM-expressing 3T3 cells or control 3T3 cells. After 26 hr the cultures were fixed and incubated with the monoclonal antibody 2G12 that specifically binds to GAP-43 only when serine-41 is phosphorylated, together with a polyclonal anti-NCAM antibody (Meiri et al., 1991) (see Materials and Methods). Neurons growing on NCAM-expressing 3T3 cells expressed the phosphorylated epitope of GAP-43 throughout the whole neuron; however, this epitope was barely detectable on neurons growing on control 3T3 cells at this stage of culture (Fig. 2). The results suggest that NCAM is able to induce stable increases in the phosphorylation of GAP-43 in both neurites and growth cones.

An NCAM-Fc chimera stimulates neurite outgrowth and phosphorylation of GAP-43 in isolated growth cones

We have shown previously that Fc chimeras containing the extracellular domains of NCAM and L1 are as effective as cell-expressed CAMs at stimulating neurite outgrowth (Doherty et al., 1995a; Saffell et al., 1997). Here we quantified the neurite outgrowth response to show that a concentration of 1 μg/ml (~10 nM) of NCAM-Fc significantly increased neurite length, whereas a maximal response was obtained at 5 μg/ml (Fig. 3*a*). Neurite outgrowth stimulated by the NCAM-Fc chimera could be blocked fully by an antibody that binds to mouse NCAM, whereas it was unaffected by an antibody against L1 (Table 1). Significantly, neurite outgrowth stimulated by the NCAM chimera also was inhibited by an antibody that blocks neuronal FGF receptor function (Table 1). This result is in accord with our previous finding that NCAM stimulation of neurite outgrowth also is prevented by the expression of a dominant-negative FGF receptor in neurons (Saffell et al., 1997) and supports the hypothesis that homophilic CAM binding triggers neurite outgrowth by activation of the FGF receptor signal transduction cascade. We used a deletion panel of NCAM-Fc constructs to demonstrate that the first three Ig domains of NCAM are as effective as the whole molecule at stimulating neurite outgrowth (Fig. 3*b*). This too is in accord with the observation that the third Ig domain contains an important homophilic adhesion site (Rao et al., 1994; Ranheim et al., 1996).

To quantify the ability of NCAM-Fc chimeras to stimulate increases in GAP-43 phosphorylation, we used a subcellular fraction of intact growth cones isolated from neonatal mouse brain (IGCs; Meiri and Burdick, 1991). As demonstrated previously, the monoclonal antibody 2G12 specifically binds to GAP-43 only when serine-41, the PKC site, is phosphorylated, whereas monoclonal antibody 7B10 shows no such constraints. Because these

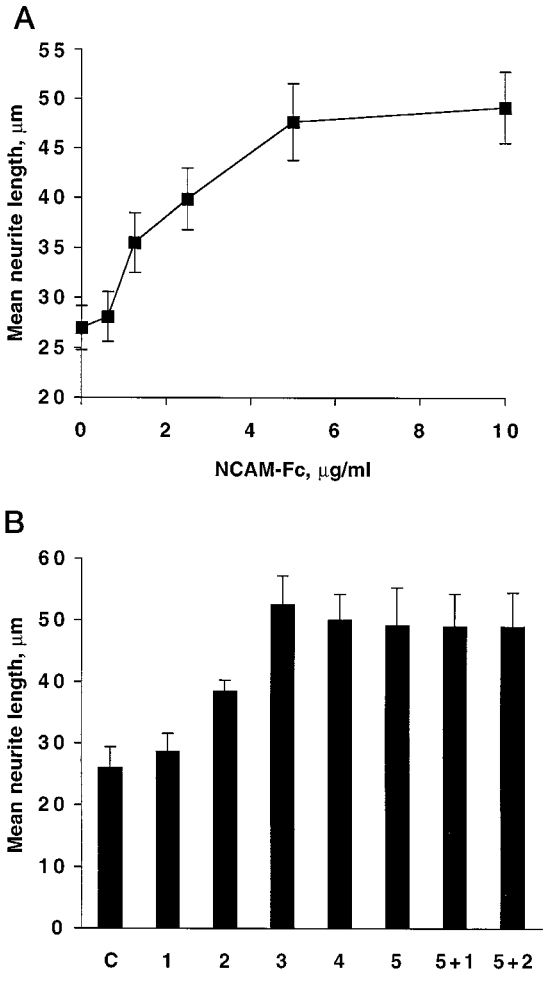

Figure 3. *A*, Soluble NCAM-Fc stimulates neurite outgrowth. Cerebellar neurons were cultured at low density on confluent 3T3 monolayers in the presence of 0, 0.625, 1.25, 2.5, 5, and 10 μg/ml NCAM-Fc. After 16 hr the cocultures were fixed and stained with a GAP-43 antibody to visualize neurons for a measurement of the mean length of the longest neurite per cell. The results of a single representative experiment are shown and expressed as the mean neurite length (in micrometers) ± SEM for 150–200 neurons analyzed in each population. *B*, Maximal outgrowth is elicited by the first three immunoglobulin-like (Ig) domains of NCAM. Cerebellar neurons were cultured on 3T3 monolayers in the absence (*C*) or the presence of Fc chimeras containing NCAM Ig domain 1 only (*1*), Ig domains 1–2 (*2*), Ig domains 1–3 (*3*), Ig domains 1–4 (*4*), Ig domains 1–5 (*5*), Ig domains 1–5 plus the fibronectin type III-like domain 1 (*5+1*), or full-length NCAM (*5+2*). All NCAM-Fc chimeras were used at 5 μg/ml. After 16 hr the cocultures were fixed and stained for a determination of mean neurite length (see Fig. 1 legend). The results show the mean neurite length (in micrometers) ± SEM pooled from three independent experiments with the whole panel of domain-deleted NCAM-Fc chimeras.

two antibodies interact with similar affinities to independent epitopes, the relative ratio of their binding can be used to determine the "specific activity" of the phosphorylation status of GAP-43 on serine-41 (Meiri and Burdick, 1991; He et al., 1997) (see Materials and Methods for details). In results that closely paralleled those obtained for axon outgrowth, the soluble NCAM-Fc chimera caused a significant increase in GAP-43 phosphorylation (Fig. 4*a*). Phosphorylation occurred at a concentration of 1 μg/ml, with the maximal response occurring at 5 μg/ml (Fig. 4*b*). Thus, the steady-state level of phosphorylated GAP-43 increased from a basal value of 43.5 ± 2.2% to 95.3 ± 3.9% of total GAP-43 phosphorylated (mean ± SEM; $n = 10$; $p < 0.0001$). Likewise, to establish whether the three Ig domain chimera also is required to induce GAP-43 phosphorylation, we incubated IGCs with 5 μg/ml of the same deletion chimeras that were used in the neurite outgrowth studies (see Fig. 3*b*). The results in Figure 4*b* show that a single Ig domain had little effect on GAP-43 phosphorylation (48.75 ± 3.8% vs 41.08 ± 2.2% of the total was phosphorylated). However, a significant increase in phosphorylation to 55.94 ± 1.36% was seen when the growth cones were incubated with the Fc chimera containing the first two Ig domains of NCAM ($p < 0.001$; $n = 6$). The three Ig domain NCAM chimera increased phosphorylation to 88.37 ± 1.49%, a value that was not significantly different from the levels obtained when the growth cones were incubated with the full-length NCAM-Fc chimera (five Ig plus two fibronectin domains). Thus there is a clear correlation between the axonal growth-promoting ability of the NCAM deletion mutants and their ability to phosphorylate GAP-43 (compare Figs. 3*b*, 4*b*).

Table 1. Function-blocking antibodies to neuronal NCAM and FGFRs inhibit neurite outgrowth stimulated by soluble NCAM-Fc

	Mean neurite length ± SEM (*n*)
Control	25.9 ± 2.3 (197)
NCAM-Fc	42.8 ± 3.1 (167)
NCAM-Fc + anti rat NCAM ab	27.6 ± 2.2 (187)
NCAM-Fc + anti FGFR ab	25.0 ± 2.0 (177)
NCAM-Fc + anti-L1 ab	45.0 ± 3.1 (154)

PND2 mouse cerebellar neurons were plated on confluent 3T3 monolayers at a density of 3000 neurons per chamber in medium supplemented with NCAM-Fc (3 Ig version), 5 μg/ml, in the absence or presence of anti-rat NCAM, −FGFR, or -L1 antisera, all diluted 1:200 (see Materials and Methods for details of antibodies). After 16 hr the cocultures were fixed and stained for GAP-43 immunoreactivity to determine the mean length of the longest neurite per cell (mean neurite length) for 150–200 neurons in each population as shown.

FGF stimulates the phosphorylation of GAP-43 in isolated growth cones

NCAM-stimulated neurite outgrowth can be accounted for by an activation of the FGF receptor signal transduction cascade (see above and introductory remarks). To determine whether activation of the FGF receptor also could mimic NCAM-stimulated phosphorylation of GAP-43, we incubated intact IGCs with FGF2. FGF2 also stimulated the phosphorylation of GAP-43, with a maximal response (an increase from 41 ± 4% to 84.5 ± 1.9% of the total GAP-43 being phosphorylated) ($p < 0.0001$; $n = 6$) seen after 15 min of treatment with 25 ng/ml FGF2 (Fig. 5*a*). Phosphorylation of GAP-43 was diminished at higher concentrations of FGF, resembling the similar "biphasic" curves obtained when axonal growth responses were stimulated by FGF (Williams et al., 1994a). The ability of both NCAM and FGF to stimulate GAP-43 phosphorylation in isolated growth cones raises the question of whether other CAMS also can stimulate GAP-43 phosphorylation in the same way. To determine whether L1 also could mimic NCAM-stimulated phosphorylation of GAP-43, we incubated intact IGCs with L1-Fc. L1-Fc also stimulated the phosphorylation of GAP-43, with a maximal response (an increase from 40.5 ± 5.5% to 93.1 ± 5% of total GAP-43 being phosphorylated) ($p < 0.001$; $n = 3$) seen after 15 min of treatment with 1 ng/ml L1-Fc (Fig. 5*b*). The activated FGF receptor stimulates arachidonic acid production in both neurons and nonneuronal cells, and this requires the sequential activity of PLCγ

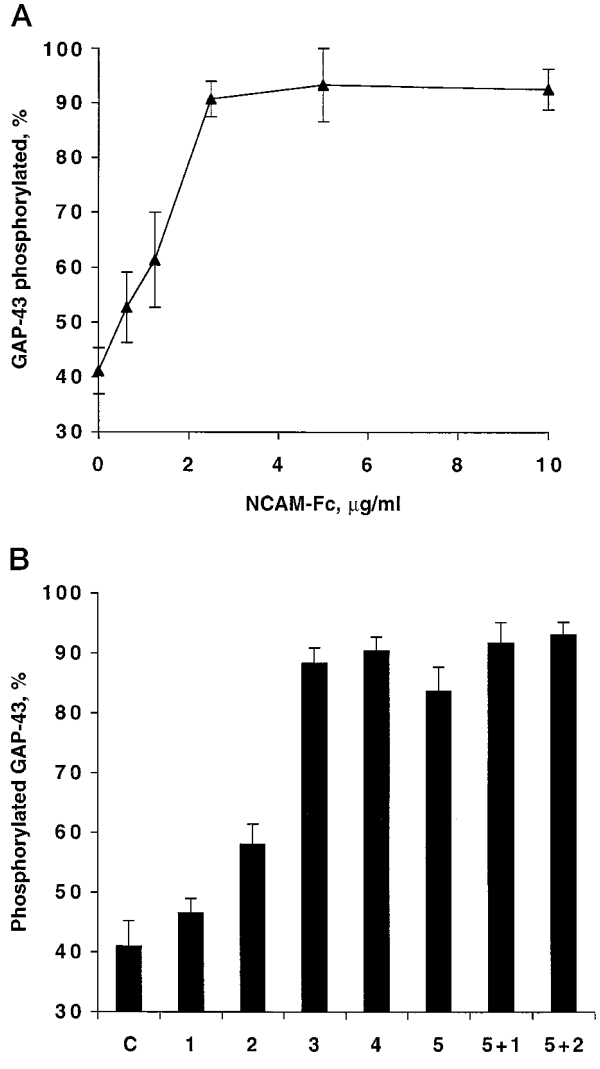

Figure 4. *A*, Soluble NCAM-Fc stimulates increases in phosphorylated GAP-43. IGCs prepared from P2 mice forebrains were treated with 0, 0.625, 1.25, 2.5, 5, and 10 μg/ml NCAM-Fc. After 15 min the samples were frozen quickly in liquid nitrogen. Equal amounts of total protein (5 μg) were slot-blotted in triplicate, and parallel blots were reacted with the mAb 2G12 to detect phosphorylated GAP-43 or with 7B10 to detect total GAP-43. Immunoreactivity was detected with an iodinated secondary antibody and quantified by a phosphorimager. The results show the specific activity of phosphorylated GAP-43 and are the mean of three independent experiments ± SEM. *B*, Maximal phosphorylation of GAP-43 is elicited by the first three immunoglobulin-like (Ig) domains of NCAM. IGCs prepared from P2 mice forebrains were treated in the absence (*C*) or presence of Fc chimeras containing NCAM Ig domain 1 only (*1*), Ig domains 1–2 (*2*), Ig domains 1–3 (*3*), Ig domains 1–4 (*4*), Ig domains 1–5 (*5*), Ig domains 1–5 plus fibronectin type III-like domain 1 (*5+1*), or full-length NCAM (*5+2*). All NCAM-Fc chimeras were used at 5 μg/ml. After 15 min the samples were frozen quickly in liquid nitrogen. Equal amounts of total protein (5 μg) were slot-blotted in triplicate, and parallel blots were reacted with the mAb 2G12 to detect phosphorylated GAP-43 or with 7B10 to detect total GAP-43. Immunoreactivity was detected with an iodinated secondary antibody and quantified by a phosphorimager. The results show the specific activity of phosphorylated GAP-43 and are the mean of three independent experiments ± SEM with the whole panel of domain-deleted NCAM-Fc chimeras.

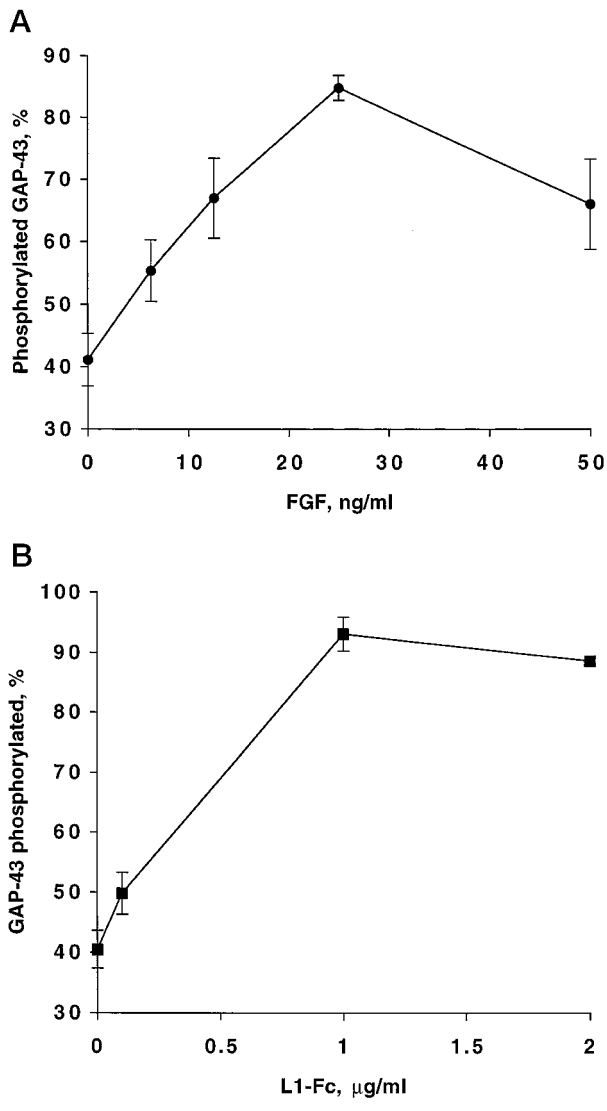

Figure 5. *A*, FGF2 stimulates increases in phosphorylated GAP-43. IGCs prepared from postnatal day 2 (P2) mice forebrains were treated with 0, 6.25, 12.5, 25, and 50 ng/ml FGF2. After 15 min the samples were frozen quickly in liquid nitrogen. Equal amounts of total protein (5 μg) were slot-blotted in triplicate, and parallel blots were reacted with the mAb 2G12 to detect phosphorylated GAP-43 or with 7B10 to detect total GAP-43. Immunoreactivity was detected with an iodinated secondary antibody and quantified by a phosphorimager. The results show the specific activity of phosphorylated GAP-43 and are the mean of three independent experiments ± SEM. *B*, Soluble L1-Fc stimulates increases in phosphorylated GAP-43. IGCs prepared from P2 mice forebrains were treated with 0, 0.5, 1, and 2 μg/ml L1-Fc. After 15 min the samples were frozen quickly in liquid nitrogen. Equal amounts of total protein (5 μg) were slot-blotted in triplicate, and parallel blots were reacted with the mAb 2G12 to detect phosphorylated GAP-43 or with 7B10 to detect total GAP-43. Immunoreactivity was detected with an iodinated secondary antibody and quantified by a phosphorimager. The results show the specific activity of phosphorylated GAP-43 and are the mean of two independent experiments ± SEM.

and DAG lipase. Likewise, GAP-43 phosphorylation can be stimulated by arachidonic acid in neurites in culture, in synaptosomal membranes, and in hippocampal slices (see introductory remarks). Here we treated IGCs with melittin to stimulate arachidonic acid production via the activation of PLA2. Melittin could mimic fully the NCAM/FGF stimulation of GAP-43 phosphorylation in the isolated growth cones so that 92.5 ± 3.5% of total GAP-43 was phosphorylated in the presence of 0.01 μM melittin (Fig. 6). Higher concentrations of melittin (0.1 μM) failed to increase phosphorylated GAP-43 above control levels (Fig. 6).

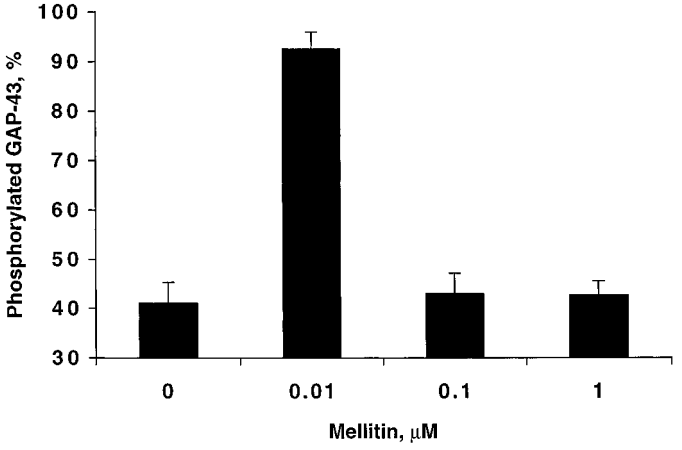

Figure 6. Melittin stimulation of increases in phosphorylated GAP-43 is biphasic. IGCs prepared from P2 mice forebrains were treated with 0, 0.01, 0.1, and 1 μM melittin. After 15 min the samples were frozen quickly in liquid nitrogen. Equal amounts of total protein (5 μg) were slot-blotted in triplicate, and parallel blots were reacted with the mAb 2G12 to detect phosphorylated GAP-43 or with 7B10 to detect total GAP-43. Immunoreactivity was detected with an iodinated secondary antibody and quantified by a phosphorimager. The results show the specific activity of phosphorylated GAP-43 and are the mean of three independent experiments ± SEM.

Table 2. Inhibitors of the FGFr-diacylglycerol pathway inhibit NCAM and FGF-induced increased GAP-43 phosphorylation

	Control	NCAM	FGF
Control	47.2 ± 3.5	98.2 ± 4.0*	60.4 ± 7.1**
αFGFR	44.1 ± 4.2	63.9 ± 3.1***	47.7 ± 4.5
766	42.1 ± 3.8	74.4 ± 2.69***	45.9 ± 8
766P	36.4 ± 2.6	39.2 ± 3.4	35.9 ± 4.7
RHC-80267	42.6 ± 6.8	38.4 ± 5.9	37.5 ± 5.0

IGS prepared from P2 mice forebrains were treated at 37°C with either Krebs's buffer alone or containing αFGFR antiserum, used at 1:200; the peptide 766 and its phosphorylated form 766P, used at 5 μg/ml; or the diacylglygerol lipase inhibitor RHC-80267, used at 10 μg/ml. After 30 min the samples were centrifuged and resuspended in either buffer alone or buffer containing NCAM-Fc, used at 5 μg/ml or FGF2, used at 25 ng/ml. After 15 min the samples quickly were frozen in liquid nitrogen. Equal amounts of total protein (5 μg) were slot-blotted in triplicate, and parallel blots were reacted with the mAb 2G12 to detect phosphorylated GAP-43 or with 7B10 to detect total GAP-43. Immunoreactivity was detected with an iodinated secondary antibody and quantitated by a phosphorimager. The results show the specific activity of phosphorylated GAP-43 and are the mean of three independent experiments ± SEM. Specific activities were compared with controls, using two-tailed Student's *t* test.

*$p < 0.001$; **$p > 0.05$; ***$p > 0.01$.

This result is interesting in the context of the fact that axonal growth responses stimulated by melittin are also biphasic (Williams et al., 1994a).

FGF receptor function is required for NCAM-mediated phosphorylation of GAP-43

Finally, we investigated whether the antibodies to the FGF receptor that can inhibit NCAM-stimulated neurite outgrowth (Table 2) (Williams et al., 1994b) also inhibit the NCAM-stimulated phosphorylation of GAP-43 in IGCs. The results presented in Table 2 show that preincubation of the growth cones with the antibody 36.2/37.2, which binds to the CAM homology domain in the FGF receptor, reduced the NCAM-Fc chimera-stimulated GAP-43 phosphorylation by ~70% ($p < 0.0001$; $n = 6$). As expected, this antibody also inhibited the response to FGF (Table 2). Likewise, FGF2-stimulated arachidonic acid synthesis can be blocked by a DAG lipase inhibitor RHC80267 (Williams et al., 1994b; Hou et al., 1997), and this agent also inhibits the neurite outgrowth response stimulated by NCAM (Williams et al., 1994b) and FGF (Williams et al., 1994c; Lom and Holt, 1997). In the present study we found that treatment of the IGCs with this DAG lipase inhibitor completely inhibited both the NCAM- and FGF-stimulated increase in GAP-43 phosphorylation (Table 2). Interestingly, none of these agents had any effect on the basal level of GAP-43 phosphorylation in the isolated growth cones.

DISCUSSION

Ig CAMs play important roles in axonal growth and guidance and in the synaptic plasticity associated with learning and memory (see introductory remarks). Neuronal responsiveness to CAMs is unlikely to be explained by simple adhesion; for example, the neuronal CAM deleted in colorectal cancer (DCC), a netrin receptor, is required for their growth-promoting and guidance functions (Keino-Masu et al., 1996; de la Torre et al., 1997). However, some DCC-positive neurons do not respond to netrins (Keino-Masu et al., 1996) or become unresponsive over time (see Shirasaki et al., 1998). Thus the spatiotemporal regulation of neuronal responsiveness to CAMs is crucial for both neuronal development and adult plasticity.

Some aspects of NCAM function are explained best by its ability to activate signaling cascades. For example, neurite outgrowth stimulated by cell-expressed and soluble NCAM can be accounted for fully by a CAM-dependent activation of neuronal FGF receptors (for review, see Walsh and Doherty, 1997). Likewise, neurite outgrowth stimulated by either cell-expressed or soluble L1 can be blocked by agents that inhibit the activity of the FGF receptor (Williams et al., 1994a, 1995a). The proximal steps in the FGF receptor signaling cascade involve the sequential activities of PLCγ and DAG lipase to generate arachidonic acid; however, we know little about how this modulates the motile behavior of a growing neurite.

The growth cone is responsible for orchestrating axonal growth and guidance (Kater and Rehder, 1995). A major growth cone component implicated in axonal growth and guidance and the synaptic plasticity associated with learning and memory is GAP-43 (see introductory remarks). It therefore becomes important to determine whether GAP-43 functions downstream from the NCAM-activated signal transduction cascade. In this regard, it has been established that arachidonic acid, which plays a central role in the NCAM-stimulated axonal growth response (Doherty and Walsh, 1994) and which has been implicated in signaling associated with learning and memory (Williams et al., 1994a), can stimulate GAP-43 phosphorylation by PKC (see introductory remarks).

Our results clearly demonstrate a requirement for GAP-43 in both NCAM- and FGF2-stimulated neurite outgrowth. In contrast, the lack of GAP-43 did not affect neurite outgrowth over fibroblasts (this study) or laminin (Strittmatter at al., 1995), both of which depend on integrin function. Considerable evidence has shown that FGF receptor function is required for NCAM, but not for integrin-dependent neurite outgrowth (for review, see Walsh and Doherty, 1997). It thus appears that GAP-43 function is essential for specific environmental cues to stimulate growth.

Growth cone behavior directly correlates with the phosphorylation status of GAP-43; for example, GAP-43 phosphorylation can be stimulated by cell–cell contact, and phosphorylated GAP-43 is enriched in lamellae that are actively translocating. In

contrast, retracting growth cones contain unphosphorylated GAP-43 (Dent and Meiri, 1992a,b, 1998). This correlation, together with the requirement for GAP-43 function in NCAM-stimulated neurite outgrowth, raises the question of whether GAP-43 phosphorylation is modulated directly by the signal transduction cascade stimulated by NCAM.

Our results show the phosphorylation of GAP-43 in neurons that are growing on NCAM-transfected cells, but not on untransfected control cells, suggesting that the homophilic binding of NCAM might stimulate the phosphorylation of neuronal GAP-43. Because this system is not amenable to quantitative biochemical studies, we used subcellular fractions of isolated growth cones to investigate how soluble NCAM-Fc chimeras affect GAP-43 phosphorylation. We have used them previously to demonstrate the increased phosphorylation of GAP-43 during depolarization-induced Ca^{2+} influx and in response to NGF (Meiri and Burdick, 1991).

A similar concentration range of an NCAM-Fc chimera stimulated both neurite outgrowth and phosphorylation of GAP-43. Likewise, the three Ig domain chimera stimulated the phosphorylation of essentially all of the GAP-43 (95.3 ± 3.9% of total) within minutes. Recent results suggest that NCAM–NCAM binding involves pairwise antiparallel binding, with the third Ig domains binding to each other with higher activity than the other domains (Rao et al., 1994; Ranheim et al., 1996).

Previous results (Williams et al., 1994a, 1995a) predicted that a soluble L1-Fc chimera, which, like NCAM, is able to activate the FGF receptor giving rise to PLCγ activation and arachidonic acid synthesis, also should cause the stimulation of GAP-43 phosphorylation. As with NCAM, the L1-Fc chimera stimulated the phosphorylation of a significant amount of GAP-43 (93 ± 5% of total) within minutes. The results confirm that different CAMS use similar second messenger systems intracellularly.

The FGF receptor can be activated directly by basic FGF (FGF2; Jaye et al., 1992; Green et al., 1996). FGF2 stimulated phosphorylation of 84.5 ± 1.9% ($n = 6$) of the GAP-43 in the growth cone preparation, not statistically different from NCAM-Fc. Thus we conclude that both agents stimulate phosphorylation of the same pool of GAP-43 and that the majority of growth cones containing GAP-43 must be responsive to both FGF and NCAM. Likewise, melittin, which stimulates arachidonic acid production in neurons, also stimulates the phosphorylation of essentially all of the GAP-43 in the isolated growth cone preparations.

Both NCAM- and L1-stimulated neurite outgrowths depend on the activation of the FGF receptor in neurons. The sequential activities of PLCγ and DAG lipase are key steps that lead to the production of arachidonic acid and subsequent neurite outgrowth (Walsh and Doherty, 1997). We showed here that an FGF receptor-blocking antibody inhibits not only the FGF-stimulated increase in GAP-43 phosphorylation but also the NCAM-stimulated response. Inhibition of the NCAM response was incomplete (~70%), conceivably reflecting the activation of additional pathway(s) by NCAM. Alternatively, the CAM–FGF receptor interaction may be inhibited incompletely by this antibody. Our own lab and others have demonstrated that a specific inhibitor of DAG lipase blocks both FGF receptor-dependent increases in arachidonic acid production (Williams et al., 1994a,c; Hou et al., 1997) and inhibits neurite outgrowth stimulated by FGF from several neuronal types (Williams et al., 1994a,c; Brittis et al., 1996; Lom and Holt, 1997). FGF receptor function also is required for the projection of axons in both the mammalian and *Xenopus* retina (Brittis et al., 1996; McFarlane et al., 1996), and inhibition of DAG lipase with RHC80267 can mimic the effects of direct inhibition of the FGF receptor function (Brittis et al., 1996; Lom and Holt, 1997). Here we have shown that this DAG lipase inhibitor completely prevents GAP-43 phosphorylation induced by NCAM and FGF. Thus we conclude that axonal growth and guidance responses and the phosphorylation of GAP-43 by FGF and NCAM requires the activity of DAG lipase, most probably reflecting its role in arachidonic acid production.

Neurite outgrowth stimulated by NCAM and FGF is not additive (Williams et al., 1994b), and similarly each stimulated phosphorylation of the complete pool of GAP-43 in growth cones. In contrast, neurite outgrowth stimulated by FGF and arachidonic acid is biphasic such that low levels are stimulatory, whereas higher levels can be inhibitory. This has been attributed to "set-point" properties of arachidonic acid (Williams et al., 1994a,c). Likewise, the dose–response curve for both FGF- and melittin-induced phosphorylation of GAP-43 was biphasic. We speculate that NCAM and FGF do not have additive effects on neurite outgrowth because each can induce complete phosphorylation of GAP-43 in growth cones. Likewise, melittin and arachidonic acid might have biphasic effects on neurite outgrowth because they have biphasic effects on GAP-43 phosphorylation.

Evidence *in vitro* and in culture implicates GAP-43 and F-actin interactions as one mechanism whereby GAP-43 phosphorylation affects growth cone behavior; when GAP-43 levels in growth cones are depleted, F-actin is reduced and motility is inhibited (Aigner and Caroni, 1993). *In vitro*, phosphorylated GAP-43 stabilizes long actin filaments, whereas unphosphorylated GAP-43 prevents actin polymerization (He and Meiri, 1997). Alternatively, neurite outgrowth stimulated by the NCAM and L1 also requires activation of the Ca^{2+}/calmodulin-dependent kinase (Williams et al., 1995a). In this context the Ca^{2+}-dependent dissociation of calmodulin from GAP-43 (Chapman et al., 1991) might contribute to kinase activation, thereby enhancing neurite outgrowth (see Kuhn et al., 1998). Our results with the GAP-43 knock-out mouse suggest *either* that it is not functionally redundant with mechanisms directly activating the Ca^{2+}/calmodulin kinase or that both kinase and actin behaviors contribute independently.

How arachidonic acid stimulates GAP-43 phosphorylation remains to be determined. In a number of paradigms PKCβII, which is enriched in the growth cone membrane skeleton along with GAP-43, phosphorylates GAP-43 on serine-41 (Coggins and Zwiers, 1989; Apel et al., 1990). Arachidonic acid might activate PKC directly or might increase calcium influx via N- and L-type channels, thereby stimulating kinase activity. In this regard, Ca^{2+} channel function is required for NCAM-stimulated neurite outgrowth (for review, see Walsh and Doherty, 1997) and can activate PKC to stimulate GAP-43 phosphorylation (see Fukura et al., 1996).

Our results suggest that the NCAM axonal growth response from intact neurons and the phosphorylation of GAP-43 in isolated growth cones are well correlated and that GAP-43 function is required for NCAM-stimulated neurite outgrowth. Likewise, our results with L1 predict that GAP-43 function will be required for L1-induced neurite outgrowth. Both neurite outgrowth and GAP-43 phosphorylation can be accounted for fully by an FGF receptor-dependent signaling cascade that generates arachidonic acid as a consequence of the sequential activities of PLCγ and DAG lipase. Other CAMs, and in particular L1 and N-cadherin, also stimulate neurite outgrowth via an FGF receptor-dependent

mechanism (Walsh and Doherty, 1997), and we found that L1-Fc also will phosphorylate GAP-43 in isolated growth cones. Our results provide a framework for a model whereby CAMs and FGFs influence axonal growth and guidance by modulating the ratio of phosphorylated to unphosphorylated GAP-43 in discrete areas of the growth cone.

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