PI3K activation by IGF-1 is essential for the regulation of membrane expansion at the nerve growth cone

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Summary

Exocytotic incorporation of plasmalemmal precursor vesicles (PPVs) into the cell surface is necessary for axonal outgrowth and is known to occur mainly at the nerve growth cone. We have demonstrated recently that plasmalemmal expansion is regulated at the growth cone by IGF-1, but not by BDNF, in a manner that is quasi independent of the neuron's perikaryon. To begin elucidating the signaling pathway by which exocytosis of the plasmalemmal precursor is regulated, we studied activation of the IRS/PI3K/Akt pathway in isolated growth cones and hippocampal neurons in culture stimulated with IGF-1 or BDNF. Our results show that IGF-1, but not BDNF, significantly and rapidly stimulates IRS/PI3K/Akt and membrane expansion. Inhibition of PI3K with Wortmannin or LY294002 blocked IGF-1-stimulated plasmalemmal expansion at the growth cones of cultured neurons. Finally, our results show that, upon stimulation with IGF-1, most active PI3K becomes associated with distal microtubules in the proximal or central domain of the growth cone. Taken together, our results suggest a critical role for IGF-1 and the IRS/PI3K/Akt pathway in the process of membrane assembly at the axonal growth cone.

Key words: IGF-1, PI3K/Akt, Nerve growth cone, Axonal outgrowth, Membrane expansion

Introduction

Neurons are equipped with receptors for multiple trophic factors, such as the 'classical' neurotrophins (NGF, BDNF, and so on) and, e.g. insulin-like growth factor-1 (IGF-1). Neurotrophins are often secreted by an innervation target and promote survival and differentiation of the innervating neuron via a mechanism involving retrograde transport from the nerve terminal to the perikaryon (Reynolds et al., 2000). Among the targets of trophic factors is the regulation of gene expression, and neurotrophin-induced changes in gene expression have been demonstrated (Bonni and Greenberg, 1997). IGF-1 is involved in the regulation of growth and tissue differentiation (Froesch et al., 1985) in various organs, including the brain. Expression of IGF-1 is high in developing brain but decreases in the adult (Rotwein et al., 1988; LeRoith et al., 1992). In culture, IGF-1 stimulates the growth and differentiation of fetal neurons (DiCiccio-Bloom and Black, 1988) and increases neurite sprouting and outgrowth (Aizenman and De Vellis, 1987; Caroni and Grandes, 1990; Beck et al., 1993). The expression of IGF-1 receptors in the central nervous system is high at late embryonic and early post-natal stages and declines significantly thereafter (Ullrich et al., 1986; Werner et al., 1991), again suggesting an important role for this ligand-receptor system in brain development. It is not surprising, therefore, that IGF-1 and its receptor are expressed permanently in the olfactory bulb, where neuronal remodeling and synaptogenesis continue throughout adult life (Bondy, 1991), and that knockout mice lacking IGF-1 receptors exhibit serious defects in CNS development, including a significant reduction in the number of axons (Liu et al., 1993).

We have demonstrated recently that plasmalemmal expansion, a key parameter of axonal growth, is regulated at the growth cone by IGF-1, but not by BDNF, in a manner that is quasi independent of the neuron's perikaryon (Pfenninger et al., 2003). Axons grow primarily by distal assembly of structural elements from components shipped to the growth cone by axoplasmic transport. This assembly process includes microtubule polymerization (Mitchison and Kirschner, 1988) and membrane expansion (Bray, 1970; Feldman et al., 1981; Pfenninger and Maylié-Pfenninger, 1981; Pfenninger and Johnson, 1983; Pfenninger and Friedman, 1993; Craig et al., 1995). However, how IGF-1 regulates membrane expansion at the growth cone is not known. It is important, therefore, to dissect the signaling steps that lead to membrane expansion if we wish to understand how assembly of the growing axon is regulated at the growth cone.

In various cell systems, but not in growth cones, it is known that signaling steps downstream of the IGF-1 receptor include (a) phosphorylation of the insulin receptor substrate (IRS) with (b) concomitant binding of the SH2-containing phosphatase (SHP-2) and (c) binding and phosphorylation of the regulatory subunit, p85, of phosphatidylinositol 3-kinase (PI3K). This

causes PI3K activation and, via PI(3,4,5)P_3, leads to (d) stimulation of PI-dependent kinase (PDK), and (e) to increase in the phosphorylation and, thus, activity of Akt (also known as protein kinase B) (Butler et al., 1998; Cantley, 2002; LeRoith and Roberts, 2003). The studies presented here begin to elucidate the mechanism by which exocytosis of the plasmalemmal precursor is regulated and indicate that PI3K, and its association with microtubules, play a critical role in the process of axonal assembly at the growth cone.

Materials and Methods

Primary antibodies

The following primary antibodies were used: monoclonal antibody (mAb) against tyrosinated α-tubulin (clone TUB-IA2, mouse IgG; Sigma Chemical Co.) diluted 1:2,000; antibody to the phospho-(tyr) p85 PI3K binding motif (cell signaling) diluted 1:500; anti PI3K p85 rabbit antiserum (Upstate) diluted 1:250 (immunofluorescence) and 1:500 (western blots); PY20 monoclonal antibody (Santa Cruz Biotechnology) diluted 1:500 and anti PI3K p110 rabbit antiserum (Upstate) diluted 1:500.

Cell culture

Dissociated hippocampal pyramidal cells were prepared from embryonic rat brain and cultured as described previously (Mascotti et al., 1997; Cáceres et al., 1986). Cells were plated onto polylysine-coated glass coverslips and maintained in DMEM plus 10% horse serum for 1 hour. The coverslips with the attached cells were transferred subsequently to 60 mm Petri dishes containing serum-free medium plus the N2 mixture (Bottenstein and Sato, 1979). To allow for neuronal survival and growth, this medium contains a high level of insulin sufficient to stimulate the receptors for insulin as well as IGF-1. For the experiments involving IGF-1 stimulation (Figs 6 and 7), cells were deprived of insulin for 4 hours before the challenge. Cultures were maintained in a humidified 37°C incubator with 5% CO_2.

Immunofluorescence

Cells were fixed for 1 hour at room temperature with 4% (wt/vol) paraformaldehyde in phosphate-buffered saline (PBS) containing 4% (wt/vol) sucrose. Cultures were washed with PBS, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 30 minutes, and again washed in PBS. After labeling with a first primary antibody (1-3 hours at room temperature) and washing with PBS, cultures were incubated with fluorescent secondary antibody (fluorescein- or rhodamine-conjugated; 1 hour at 37°C) and washed with PBS. The same procedure was repeated for the second primary and secondary antibodies. The cells were observed with a Zeiss Axiovert microscope equipped with epifluorescence optics. Fluorescence images were captured with a CCD camera (Hamamatsu), digitized directly into a Metamorph/Metafluor Image Processor (Universal Imaging Corporation, West Chester, PA), and printed using Adobe PhotoShop.

Isolation of growth cones

Axonal growth cones were isolated from developing brain as described (Pfenninger et al., 1983; Lohse et al., 1996). In brief, brains of 18-day gestation fetal rats were homogenized. A low-speed supernatant (LSS) was prepared, loaded onto a discontinuous sucrose density gradient with steps of 0.83 and 2.66 M sucrose, and spun to equilibrium at 242,000 $\boldsymbol{g}_{max}$. The fraction at the load/0.83 M interface (designated 'A') contained the isolated growth cones or growth cone particles (GCPs). Remaining particulate elements of the LSS were collected at the 0.83/2.66 M interface (fraction BC).

Membrane expansion assay

Neurons were cultured as described above, incubated for 12 hours in N2 medium without insulin but containing 50 ng ml^{-1} BDNF, and labeled for 30 minutes with BODIPY-ceramide at room temperature (Pagano et al., 1991; Paglini et al., 2001). After 2.5 hours incubation in the absence of label, at 37°C, fluorescent spots in the growth cones observed in the red channel were examined with the microscope (equipped with a heated stage) in control conditions or after the addition of 10 nM IGF-1.

Gel electrophoresis and western blot

Proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The concentration of acrylamide of the resolving gel varied from 7.5 to 11%. The resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes in Tris-glycine buffer containing 20% methanol. The membranes were either first dried, washed with Tris-buffered saline (TBS; 10 mM Tris pH 7.5, 150 mM NaCl) and then blocked, or directly blocked for 1 hour in TBS containing 5% BSA. The blots were incubated with the primary antibodies in PBS containing 0.05% Tween 20, for 2 hours at room temperature. After washing with TBS containing 0.05% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Promega Corp., Madison WI) for 1 hour at room temperature. After washing the blots were developed using a chemiluminescence detection kit (ECL, Amersham Life Sciences Inc., Arlington Heights, IL). Alternatively, we used secondary antibodies conjugated with Alexa Fluor dye and scanned the labeled blots with a Typhoon 9400 variable mode imager.

Phosphorylation assays

Assays for IRS-2 and Akt activation

The GCP-containing fraction A was mixed gently with an equal volume of 'intracellular buffer' (20 mM Hepes pH 7.3, 50 mM KCl, 5 mM NaCl, 3 mM $MgCl_2$) and permeabilized with 0.01% β-escin. These GCPs were incubated for 5 minutes on ice in the presence or absence of IGF-1 (1 nM) and then, upon the addition of 1 mM ATP, warmed up to 37°C for 30 seconds to 5 minutes. The reaction was terminated by chilling samples and adding 1% Triton X-100 plus 3 mM vanadate, 2 mM NaF, 10 mM EDTA, 100 μM genistein, and a protease-inhibitor cocktail. After 10 minutes on ice, samples were centrifuged at 30,000 $\boldsymbol{g}$ for 1 hour to pellet Triton-insoluble cytoskeletal elements. For immunoprecipitation of IRS-2, the supernatant was incubated with anti-IRS-2 antibody (5 μg ml^{-1}) for 2 hours at 4°C before adding protein-A/G plus-coated beads. Fresh vanadate was added to every solution change. The precipitates were resolved by SDS-PAGE and blotted onto PVDF membrane. After quenching, blots were probed with anti-pTyr monoclonal antibody to reveal IRS-2 phosphorylation, and with anti-SHP2 polyclonal antibody to examine co-precipitation of SHP-2 with IRS-2. Blots were stripped with SDS and re-probed with anti-IRS-2 to generate loading controls. Alternatively, the reaction was terminated by a methanol/chloroform precipitation. The total pellet was analyzed by western blot to detect activated Akt by phospho-specific antibody (anti-Akt-pS473).

PI3K assay

Fraction A was mixed gently with an equal volume of 'intracellular buffer' (20 mM Hepes pH 7.3, 50 mM KCl, 5 mM NaCl, 3 mM $MgCl_2$) and permeabilized with 0.01% β-escin. These GCPs were incubated for 5 minutes on ice either in the presence or the absence of PI3K inhibitor (20 μM LY294002 or 100 nM Wortmannin), followed by another 5 minutes with or without IGF-1 (4 nM). Subsequently, upon the addition of 1 mM ATP, samples were warmed up to 37°C for 1 minute or 5 minutes. The reaction was terminated

by chilling samples and adding 1% Triton X-100 plus 3 mM vanadate, 2 mM NaF, 10 mM EDTA, 100 μM genistein, and a protease-inhibitor cocktail. After 10 minutes on ice, samples were centrifuged at 30,000 ***g*** for 1 hour to pellet Triton-insoluble cytoskeletal elements. The supernatant was incubated with PI3K antibody (anti-p85; Upstate, NY, USA). Immune complexes were then adsorbed using protein-A/G plus Sepharose and subjected to PI3K assay. To this end, precipitates were incubated with 200 μM phosphatidylinositol and 50 μM ^{32}P-ATP (2.5 μCi per assay) at 37°C for 20 minutes. The reaction was terminated by adding ice-cold chloroform:methanol (1:2) containing 2% acetic acid (3.75× assay volume). Following lipid extraction, the radiophosphorylated product was isolated by thin-layer chromatography (TLC) using a mobile phase composed of chloroform:methanol:acetone:glacial acetic acid:water in ratios of 70:50:20:20:20 (Traynor-Kaplan et al., 1989). The TLC plates were analyzed by storage phosphor imaging on a Typhoon 9400 variable mode imager.

Results

Initial activation steps of IGF-1 in the growth cone

We have reported recently that IGF-1 regulates plasmalemmal expansion at the axonal growth cone (Pfenninger et al., 2003). To investigate this process further, we have begun to study secondary signaling steps involved in this regulation. We confirmed earlier that the unusual IGF-1 receptor of the growth cone (containing the βgc subunit) indeed become autophosphorylated upon IGF-1 binding (Mascotti et al., 1997). Consistent with this result, the insulin receptor substrate prevalent in growth cones, IRS-2, is also rapidly phosphorylated and binds the SH2-containing protein tyrosine phosphatase, SHP-2, when activated by IGF-1. This is shown by immunoprecipitation in Fig. 1. IGF-1 stimulation increases both IRS-2 phosphorylation and SHP-2 binding 3.5- to 4-fold within the first minute of activation.

Another SH2-containing element downstream of IRS-2 is PI3K. Its regulatory subunit, p85, is known to be phosphorylated upon growth factor stimulation in several cellular systems. Indeed, when we analyzed the pattern of protein phosphorylation in GCPs incubated in control conditions or challenged with 1 nM IGF-1, we found a significant increment in the phosphorylation of an 80-90 kDa protein. This band was subsequently identified by immunoprecipitation as the regulatory subunit, p85, of PI3K (data not shown). To analyze PI3K activation by IGF-1 in GCPs in further detail we stimulated intact GCPs for 5 minutes with 1 nM IGF-1, solubilized them, and immunoprecipitated phosphotyrosine-containing polypeptides using an anti-P-tyr antibody (clone PY20). The resulting immunoprecipitates and remaining soluble polypeptides were analyzed by western blot probed with an anti-p85 antibody. As shown in Fig. 2, the IP of the GCPs stimulated with IGF-1, but not that of control GCPs, contained active p85 (phosphorylated on tyrosine). The increase in p85 phosphorylation was three-fold ($P \leqslant 0.01$). IGF-1 receptor activation also increased PI3K activity shown in enzyme assays. This is described in further detail below.

The product of PI3K, PIP_3, is known to stimulate PDK1 and, thus, to increase phosphorylation of the kinase Akt (or PKB). Therefore, we probed blots of control and IGF-1-stimulated GCPs with an antibody specific for phosphorylated (and activated) Akt. The blots were also probed for p85, which served as a loading control in these experiments. The results are shown in Fig. 3. Within 30 seconds upon IGF-1 stimulation of GCPs, Akt phosphorylation increased to over 150% of control levels and remained there for at least 5 minutes. Overall these results indicate that IGF-1 stimulation of isolated growth cones causes the rapid and sustained activation of signaling steps of the PI3K-Akt pathway.

Subcellular localization of PI3K in the growing axon

To study the subcellular distribution of p85, GCPs were subfractionated into soluble cytosolic proteins, membranes, and a cytoskeletal fraction (Helmke and Pfenninger 1995). Western blots of these fractions were probed with an anti-

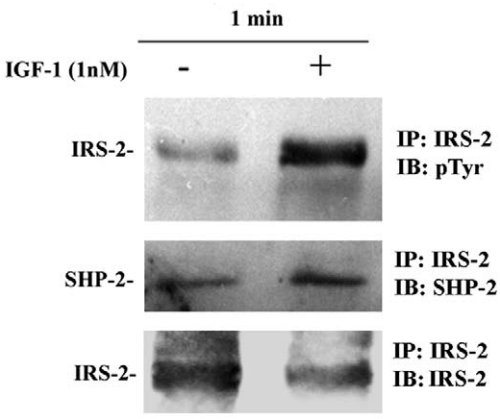


Fig. 1. IGF-1 stimulates the phosphorylation and SHP-2 binding of IRS-2 in growth cones. GCPs were stimulated with IGF-1 or incubated in control conditions for 1 or 5 minutes. IRS-2 was immunoprecipitated, and blots of the precipitates were probed with antibodies to pTyr, SHP-2 and IRS-2 as a loading control.

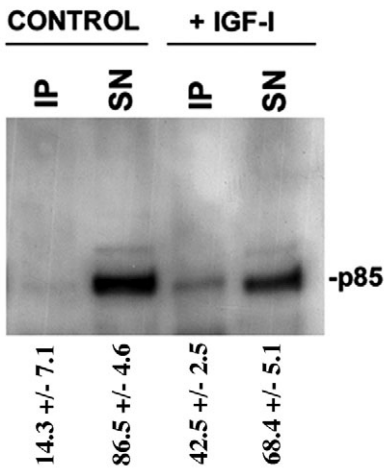


Fig. 2. IGF-1 increases phosphorylation of the PI3K subunit p85 in growth cones. Phosphotyrosine (PY20) immunoprecipitates were prepared from GCPs incubated in control medium or with 1 nM IGF-1 for 5 minutes. Blots were probed with anti-p85 antibody. IP, immunoprecipitate; SN, supernatant polypeptides after immunoprecipitation. A substantial increment of p85 is evident in the immunoprecipitate of GCPs stimulated with IGF-1. Relative optical densities of p85 are shown at the bottom of the figure (arbitrary units; means±s.e.m. of three independent experiments).

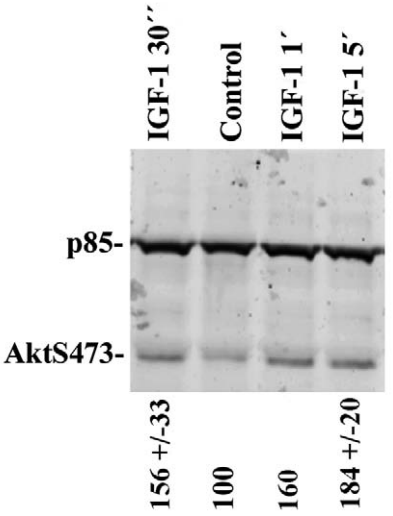


Fig. 3. IGF-1 enhances Akt phosphorylation in growth cones. GCPs were incubated in control conditions or stimulated with IGF-1 for 30 seconds, 1 minute or 5 minutes. Western blots of polypeptides were probed with anti-p85 and with a phosphorylation-specific antibody to Akt (AktS473). p85 served as a loading control. The relative O.D. (A.U.) of AktS473 normalized over p85 signal are shown at the bottom of the figure. Those results are the means±s.e.m. of three (30 seconds and 5 minutes) or two (1 minute) independent experiments.

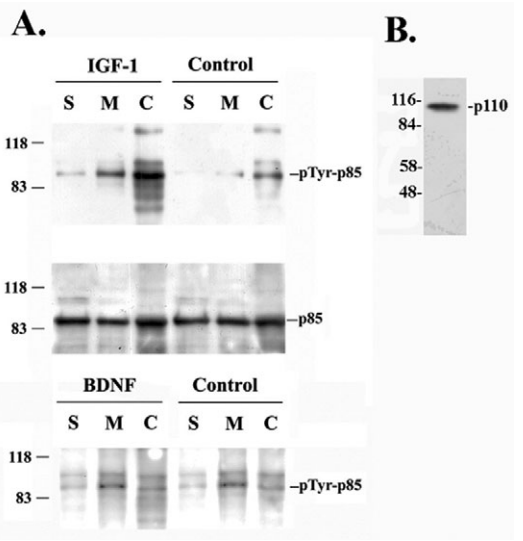


Fig. 4. (A) The phosphorylated regulatory subunit of PI3K, pTyr-p85, is associated primarily with the growth cone cytoskeleton. Western blots of GCP subfractions (S, cytosol; M, membranes; and C, cytoskeletal fraction). GCPs were preincubated in control medium, with IGF-1 (10 nM, 5 minutes) or with BDNF (0.2 nM, 5 minutes). Blots were probed with anti p85 (middle) or anti-pTyr-p85 (top and bottom). Equal amounts of protein were loaded in each lane. Note the significant activation of PI3K with IGF-1 (top), but not with BDNF (bottom). pTyr-p85 was highly enriched in the cytoskeletal fraction and, to a lesser extent, in the membrane fraction. (B) Western blot of GCP polypeptides probed with anti-p110 antibody. Note mono-specificity of the antibody.

p85 antibody. Fig. 4A (middle) shows roughly equal immunoreactivity at ~85 kDa in all GCP subfractions. This assay also serves as a loading control indicating that, for each fraction, the amounts of p85 present in control and IGF-1 stimulation conditions are the same. Parallel blots were prepared from control GCPs or from GCPs stimulated with IGF-1 or BDNF for 5 minutes and then subfractionated. These blots were probed with an antibody specific for active PI3K, anti-Ptyr-p85. The results show significant activation of PI3K by IGF-1 (Fig. 4A, top), but not by BDNF (Fig. 4A, bottom). Interestingly, most active PI3K (anti-Ptyr-p85 label in the IGF-1-stimulated samples) is recovered in the cytoskeletal fraction (Fig. 4A, top). These results indicate that IGF-1, but not BDNF, significantly stimulates PI3K in GCPs, and that most active PI3K is associated with the cytoskeleton.

To study PI3K activation in intact neurons, we cultured hippocampal pyramidal cells in the presence of 50 ng ml^{-1} BDNF. After control incubation or challenge with 10 nM IGF-1 for 5 minutes, the cultures were fixed, detergent-treated under microtubule-stabilizing conditions (Black et al., 1994), and then immunostained with antibodies to the catalytic subunit of PI3K (p110) and to tyrosinated α-tubulin. As shown in Fig. 4B, the anti-p110 antibody was specific. Immunofluorescence results are shown in Fig. 5A and indicate that IGF-1 stimulation triggers the association of p110 with microtubules, primarily in the distal third of the axon and in the axonal growth cone. Neurons not detergent-extracted before fixation exhibited strong p110 immunoreactivity in the perikaryon, minor processes, and axons in all conditions assayed (data not shown). To exclude the possibility that the label in the p110 channel was not artifactual 'bleed-through' of the tubulin signal, we also immunostained hippocampal pyramidal neurons with anti-p110 alone (Fig. 5B). Results indicate a significant IGF-1-induced increase in p110 immunostaining in the distal axon and growth cone compared with controls. In these conditions, growth cone labeling with anti-p110 only (lower left panel of Fig. 5B) mimicks the tubulin pattern seen in Fig. 5A. Fig. 5C shows the growth cone of a hippocampal pyramidal neuron double-immunostained with p110 and phalloidin. Most of the p110 labeling does not co-localize with the actin cytoskeleton

We also double-immunostained hippocampal neurons challenged with IGF-1 or insulin with antibodies to Ptyr-p85 and to tyrosinated tubulin. As shown in Fig. 6A, IGF-1, but not the very closely related factor, insulin, significantly activated PI3K in the distal third and growth cone of the axon. In this example some immunostaining can also be seen associated with the proximal axon, near what appears to be a branch point. Thus, staining most probably is due to an emerging growth cone attached to the long axon. Fig. 6B shows immunostaining with pTyr-p85 alone and confirms significant activation of PI3K at the distal third axon and the growth cone. These observations indicate that IGF-1 triggers PI3K activation and microtubule association in the distal third and growth cone of the axon, the area where βgc is located (Mascotti et al., 1997) The morphological observations are thus consistent with the biochemical results described above.

PI3K and membrane expansion

The observed rapid activation of PI3K and Akt by IGF-1 in the growth cone raises the question of whether PI3K is involved

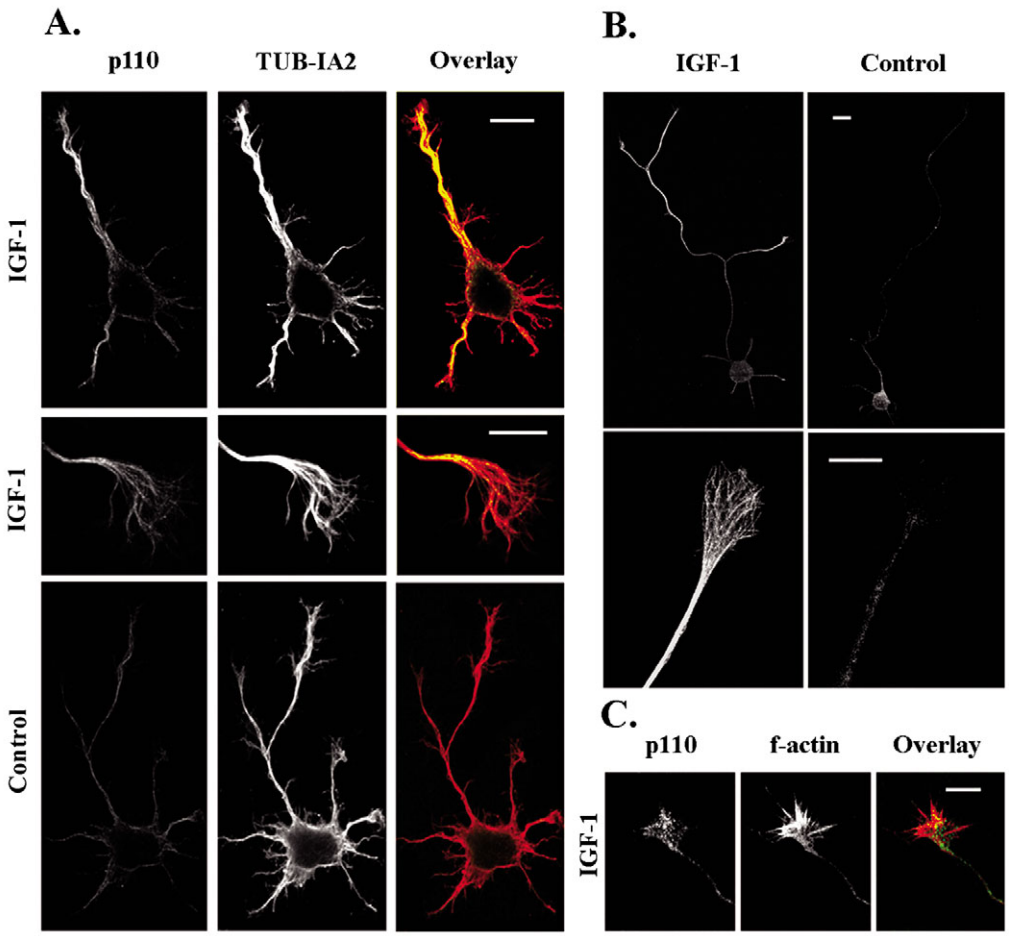


Fig. 5. (A) Double immunofluorescence micrographs showing the distributions of tyrosinated α-tubulin (TUB-IA2) and of the p110 subunit of PI3K in hippocampal pyramidal neurons after 18 hours in culture (in the presence of 50 ng ml^{-1} BDNF). Cells were incubated for 5 minutes in control medium (bottom) or with 10 nM IGF-1 (top and middle), permeabilized in microtubule-stabilizing conditions, and then fixed for antibody labeling. Note that stimulation with IGF-1 triggers the association of p110 with microtubules primarily in the distal axon (top) and the axonal growth cone (middle). Bar, 20 µm (top and bottom) and 5 µm (middle). (B) Double immunofluorescence micrographs showing the distribution of the p110 subunit of PI3K in hippocampal pyramidal neurons after 36 hours in culture (in the presence of 50 ng ml^{-1} BDNF). Cells were incubated for 5 minutes in control medium (right) or with 10 nM IGF-1 (left), permeabilized in microtubule-stabilizing conditions, and then fixed for antibody labeling. Bar, 20 µm (top) and 5 µm (bottom). (C) Double immunofluorescence micrographs showing the distribution of p110 and F-actin at the growth cone of an hippocampal pyramidal cell culture for 18 hours in the presence of 50 ng ml^{-1} BDNF and incubated for 5 minutes with 10 nM IGF-1. Note that most of p110 immunostaining does not co-localize with F-actin. Bar, 5 µm.

in the regulation of membrane expansion. To test this, we performed experiments with the PI3K inhibitors, LY294002 (LY; 20 µM) and Wortmannin (Wm; 100 nM). First we measured PI3K inhibition by these reagents. GCPs were pre-incubated either in control conditions or in the presence of IGF-1, with or without LY or Wm. Then we assayed PI3K activity using PI as a substrate. The results are shown in Fig. 7. In the absence of the inhibitors, IGF-1 stimulates PI3K activity nearly twofold. In the presence of LY or Wm, however, IGF-1-stimulated activity drops to below control or to 10% of the stimulated activity, respectively.

The preceding experiments indicate that LY and Wm effectively reduce IGF-1-stimulated PI3K activity in growth cones and can be used to test whether PI3K activity is necessary for IGF-1-stimulated plasmalemmal expansion. Hippocampal neurons cultured in the presence of 50 ng ml^{-1} BDNF were pulse-labeled at room temperature with BODIPY-ceramide, a fluorescent sphingomyelin and glucosylceramide precursor (Pagano et al., 1991). After further incubation for 2.5-3 hours at 37°C, this resulted in intense fluorescence of the Golgi complex and in the presence of variously-sized, fluorescent compartments along the axon and in the axonal growth cone (Pfenninger et al., 2003). Because of the high concentration of BODIPY label in these compartments and excimer formation, fluorescence is red; as membrane vesicles become inserted into the plasmalemma and the labeled ceramide is diluted, excimer formation ceases and fluorescence shifts to green. Thus, membrane insertion causes the dissipation of red fluorescence in the growth cone in these experiments (Pfenninger et al., 2003). As reported earlier, the half-life of fluorescent puncta (vesicles or clusters recorded in the red channel) in unstimulated growth cones exceeded 14 minutes. In the presence of IGF-1, however, vesicle dissipation was accelerated, and the half-life of puncta dropped to 5 to 6 minutes (Figs 8 and 9) (Pfenninger et al., 2003). Preincubation of cultures with LY prior to IGF-1 challenge had no obvious effect on overall growth cone configuration, while Wm caused growth cones to be more filopodial than lamellipodial (Fig. 8). In both cases, labeled compartments were evident as in growth cones treated with only IGF-1. However, the fluorescent puncta persisted for extended periods of time in the presence of the PI3K inhibitors (Fig. 8). These observations were quantified by counting fluorescent spots in each growth cone as a function of time starting at the onset of the challenge. Numbers were normalized to 1 at the onset of the challenge. As shown in Fig. 9, the PI3K inhibitors Wm and LY, in the presence of IGF-1, increased the half-life of fluorescent puncta from 5-6 minutes to >14 minutes (similar or greater than that of control

conditions without IGF-1) (Pfenninger et al., 2003). These results indicate that LY and Wm block or greatly reduce IGF-1-stimulated membrane addition at the growth cone.

Discussion

Earlier data from these laboratories have shown that IGF-1, unlike the classic neurotrophins, has a direct local effect on the growth cone by stimulating exocytotic insertion of plasmalemmal precursor vesicles (PPVs) for axonal membrane expansion (Pfenninger at al., 2003). This occurs via activation of a receptor that contains the unusual βgc subunit (Quiroga et al., 1995; Mascotti et al., 1997). The present report addresses the question of how regulation of exocytosis is linked to the IGF-1 receptor, and how this may relate to other mechanisms that control assembly of the axon at the growth cone.

PI3K involvement in plasmalemmal expansion of the axon

Intracellular signaling by the IGF-1 receptor is relatively well understood. The activated receptor typically stimulates one or both of two cascades, (1) the ras/MAP kinase pathway, via the adaptor protein SHC, or (2) the PI3K/Akt cascade, via IRS (Butler et al., 1998; Cantley, 2002; LeRoith and Roberts, 2003). Our results show that, in isolated growth cones, IGF-1 stimulates (1) IRS-2 phosphorylation and SHP-2 binding, (2) PI3K (p85) phosphorylation and activity, and (3) Akt phosphorylation. At least at the level of Akt the effect occurs rapidly (30 seconds) and is long-lasting (5 minutes). BDNF, however, does not increase p85 phosphorylation even though functional TrkB is present in the isolated growth cones (Pfenninger et al., 2003). Zheng and Quirion recently studied differential effects of IGF-1 and BDNF on survival of hippocampal neurons and found that IGF-1 activation of the PI3K pathway was rapid and sustained, whereas that of the MAP kinase pathway was transient (Zheng and Quirion, 2004; Zheng et al., 2000a). For BDNF they reported the inverse. The relevant assays were carried out with whole neurons in culture.

The results presented here were obtained with GCPs and, thus, reveal the events occurring specifically in axonal growth cones, where βgc-containing IGF-1 receptors are located and most of the new membrane is inserted into the plasmalemma. It seemed logical, therefore, to assess whether inhibition of

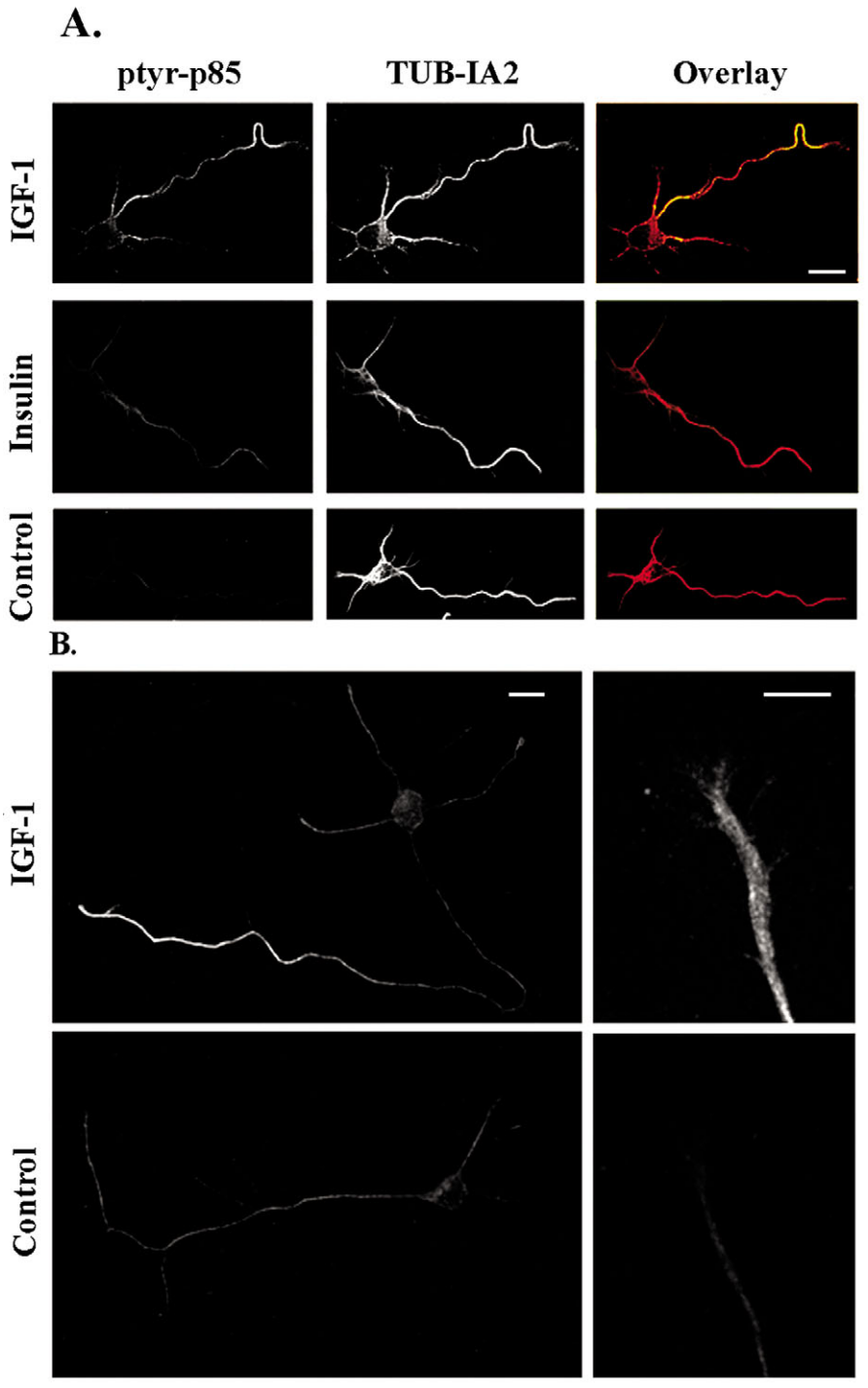


Fig. 6. (A) Double immunofluorescence micrographs showing the distributions of pTyr-p85 and tyrosinated α-tubulin in hippocampal pyramidal neurons after 24 hours in culture. Cells were incubated in control medium or in the presence of 10 nM IGF-1 or insulin for 5 minutes. pTyr-p85, indicating activation of PI3K, is present only in the cells stimulated with IGF-1 and is evident especially in the distal axon and growth cone. Bar, 20 μm. (B) Inmunofluorescence micrographs showing the distribution of pTyr-p85 in hippocampal pyramidal neurons after 36 hours in culture and incubated for 5 minutes with 10 nM IGF-1 (top) or in control medium (bottom). Note the intense staining of the distal third and the growth cone of the axon. Bar, 20 μm (left), 5 μm (right).

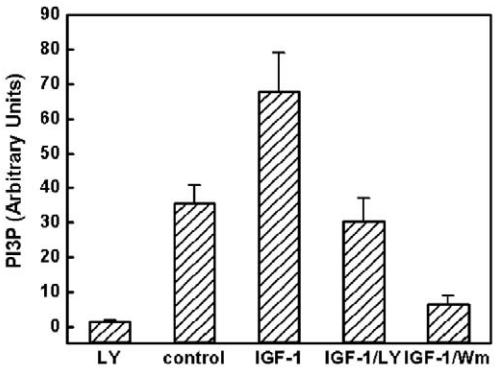
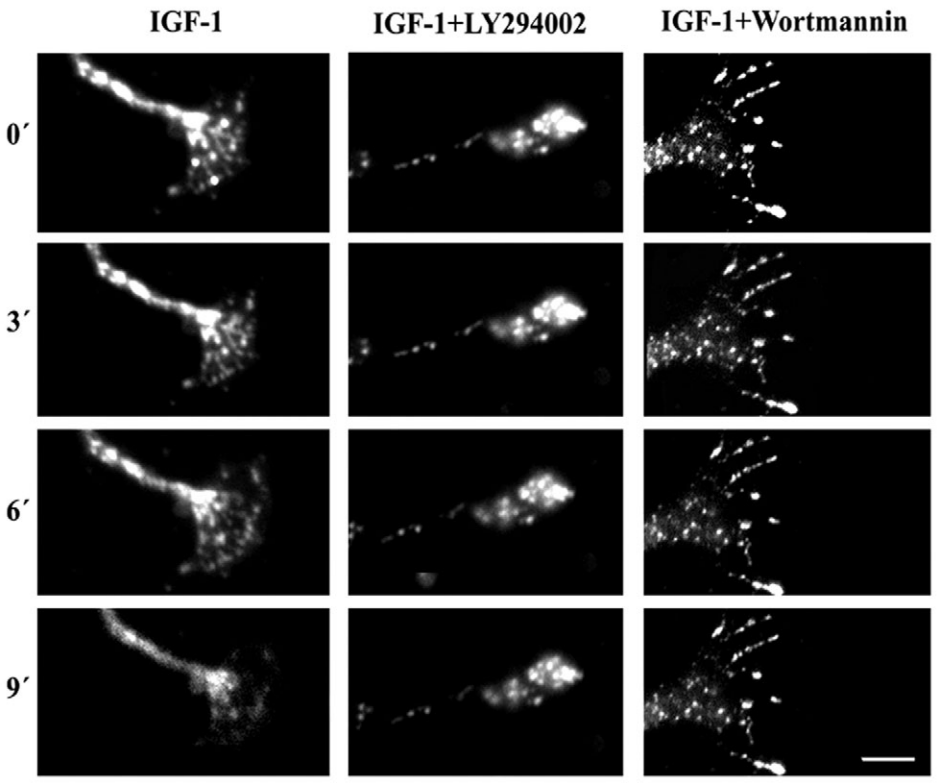


Fig. 7. Stimulation and inhibition of growth cone PI3K activity by IGF-1 and by LY/Wm, respectively. GCPs, in the presence or absence of 20 μM LY or 100 nM Wm, were pre-incubated for 5 minutes at 37°C with or without IGF-1 (4 nM). PI3K was immunoprecipitated and subjected to kinase assay, using PI as a substrate. PI3P was isolated by TLC and phosphorylation assessed by storage phosphor imaging. LY reduced control levels and inhibited IGF-1-stimulation of PI3K activity. Wm reduced IGF-1-stimulated kinase activity to below control levels.

PI3K with Wm or LY blocked plasmalemmal expansion. Wm at 1 μM, a concentration similar or lower than those commonly used for such experiments (Zheng et al., 2000a; Zheng et al., 2000b; Zhu et al., 2003), did indeed block the insertion of BODIPY-ceramide into the plasmalemma, but it also altered overall growth cone configuration, suggesting that it affected more than just vesicle exocytosis. However, at the concentration (20 μM) that inhibited PI3K activity in isolated growth cones, the more specific LY also inhibited exocytosis of the plasmalemmal precursor. We can conclude that PI3K activity is necessary for IGF-1-stimulated vesicle insertion and plasmalemmal expansion at the axonal growth cone.

Inositol lipids have been linked to membrane traffic (Cockroft and De Matteis, 2001), and several reports have established the requirement of PI3K for exocytosis in non-neuronal cells (Gatof et al., 2004; Chasserot-Golaz et al., 1998). Of particular interest in the present context are studies on the growth-factor-regulated exocytotic insertion of membrane proteins into the plasmalemma. These include insulin regulation of the recruitment of the GLUT4 transporter (Cheatham et al., 1994; Clarke et al., 1994), EGF/NGF/IGF-1 regulation of insertion of TRP (transient receptor potential) family ion channels (Kanzaki et al., 1999; Bezzerides et al., 2004), and angiotensin II regulation of exocytosis of the Na^+/H^+ exchanger 3 (du Cheyron et al., 2003). In all cases, PI3K activity has been shown to be necessary for insertion into the plasmalemma. The variety of cell systems and membrane proteins involved in these examples suggest a common theme of regulation: growth factor activation of receptor tyrosine kinases and of the PI3K pathway for exocytotic recruitment of membrane components to the plasmalemma. This apparent rule also applies to axonal membrane growth at the growth cone as shown herein (Bezzerides et al., 2004).

One of the implications is that activation of the PI3K/Akt pathway would regulate neurite or axonal growth. This has indeed been reported (Kimura et al., 1994; Kita et al., 1998; Kobayashi et al., 1997; Markus et al., 2002). In addition, PI3K activity seems to be involved in the establishment of neuronal polarity (Shi et al., 2003; Menager et al., 2004). While all these observations support the concept of PI3K-dependent vesicle exocytosis for axonal elongation, PI3K's functional role may be broader, as suggested by its interaction with microtubules and its involvement in the delivery of synaptic vesicles to the readily releasable pool (Cousin et al., 2003).



Microtubule association of activated PI3K in the growth cone

Our results demonstrate that the PI3K regulatory subunit, p85, is fairly uniformly distributed among the three

Fig. 8. LY and Wm inhibit IGF-1 stimulation of membrane expansion at the growth cone. Representative fluorescence micrographs (recorded in the red channel) of the axonal growth cones of hippocampal neurons in culture are shown. Neurons were labeled with BODIPY-ceramide for 30 minutes at room temperature and then chased for 2.5-3 hours at 37°C. Growth cones were challenged with 10 nM IGF-1 in the absence (control) or the presence of the PI3K inhibitors Wm (1 μM) or LY (20 μM). Note the rapid dissipation of fluorescence upon challenge with IGF-1 versus the persistence of fluorescent puncta in the presence of the PI3K inhibitors. Bar, 3 μm.

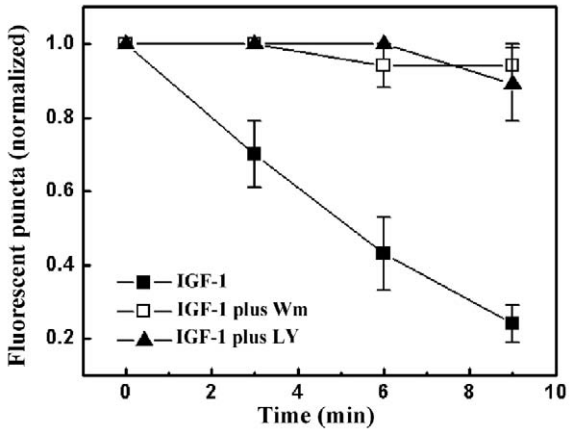


Fig. 9. Relative number of fluorescent puncta (vesicles or clusters recorded in the red channel) in growth cones challenged with 10 nM IGF-1 in control conditions or in the presence of 1 μM Wm or 20 μM LY. Values were normalized to 1 at the onset of challenge. Data points are means + s.e.m. from more than three independent growth cone assays.

subfractions prepared from GCPs (soluble, membrane and cytoskeleton fractions). However, IGF-1-stimulated increases in pTyr-p85 are observed in the membrane fraction and, most dramatically, in the cytoskeleton fraction. This observation is confirmed by the immunofluorescence results, which show a substantial increase in microtubule-bound pTyr-p85 upon IGF-1 stimulation. p85 Phosphorylation is confined to the most distal segment of the growing axon (including the growth cone). The catalytic subunit of PI3K, p110, is recruited to the same microtubule region upon IGF-1 stimulation, indicating the association of active PI3K with these distal axonal microtubules.

The association of PI3K regulatory subunits with microtubules (α, β tubulin) has been reported before (Kapeller et al., 1993; Kapeller et al., 1995; Inukai et al., 2000). However, our data reveal that, in the growing axon, it is the microtubule-associated p85 that exhibits the largest increase in phosphorylation upon IGF-1 stimulation, and the activated p85 recruits p110 to the microtubules. These effects are selective and cannot be replicated, e.g. by BDNF (in biochemical assays) or by insulin (in culture, as shown by immunofluorescence). Interestingly, the growth cone's IGF-1 receptors (containing βgc) are located in the distal third of the axon and proximally in the growth cone, in its 'central region'. Apparently, they are associated somehow with microtubules (Mascotti et al., 1997). This contrasts the distribution of many receptors, such as TrkB (Pfenninger et al., 2003) or neuropilin (Mikule et al., 2002), which are predominantly found in the growth cone periphery, together with the bulk of actin microfilaments. The proximal distribution of βgc and PI3K is reflected in the localization of PPV clusters and insertion sites, which are also found primarily in the central region of the growth cone (Pfenninger and Friedman, 1993; Pfenninger et al., 2003). The localization of the PPV clusters may explain why there seems to be no significant membrane insertion along the axonal shafts, even in the presence of active PI3K.

The precise mechanisms by which PI3K regulates plasmalemmal expansion at the growth cone remains to be investigated. Several possibilities are open since PI3K regulates a number of signal transduction pathways [for review see Cantley (Cantley, 2002)], including those involving the protein serine/threonine kinases Akt and PDK1, protein tyrosine kinases (Tec family), exchange factors for GTP-binding proteins (Grp1 and Rac exchange factors), and adaptor proteins (GAB-1). These proteins initiate complex sets of events that control a wide spectrum of phenomena, such as protein synthesis, actin and tubulin polymerization, cell survival and cell-cycle entry. In the context of regulation of axonal growth, two targets of Akt are of special interest: (1) glycogen synthase kinase 3 (GSK3). GSK3 phosphorylation by Akt turns off its catalytic activity, which is involved in microtubule polymerization (Hong and Lee, 1997; Zhou et al., 2004; Drechsel et al., 1992; Zumbrunn et al., 2001) and in kinesin-based membrane transport towards the distal end of growing axons (Sanchez et al., 2001; Morfini et al., 2002). (2) The Rho-family GTPase Cdc42, which is involved in regulation of the actin cytoskeleton and micrutubule capture and stabilization (Fukata et al., 2003). Thus, PI3K may regulate several mechanisms that jointly result in axon assembly.

In conclusion, our results show that PI3K activation by IGF-1 is necessary for plasmalemmal expansion at the growth cone, and that most active PI3K is associated with distal microtubules reaching into the proximal or central domain of the growth cone. Published data link PI3K activation to microtubule stabilization, polymerization and organelle transport (via GSK3) (Hong and Lee, 1997; Zhou et al., 2004; Morfini et al., 2004), and to the regulation of the actin and tubulin cytoskeleton (via Cdc42) (Okkenhaug and Vanhaesebroeck, 2001; Fukata et al., 2003). However, activation of GSK3β also leads to MAP-1B phosphorylation. Phosphorylated MAP-1B is required to maintain a population of unstable microtubules in the growth cone, a prerequisite for axonal growth (Goold and Gordon-Weeks, 2004; Gonzalez-Billault et al., 2004). Together with those data our results suggest that the IGF-1-activated IRS/PI3K/Akt pathway could play a critical and comprehensive role in the growth of the axon by controlling plasmalemmal expansion as well as the dynamics of the cytoskeleton.

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