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 neurite extension and is known to occur mainly at the growth cone. This report examines whether this is a regulated event controlled by growth factors. The Golgi complex and nascent PPVs of hippocampal neurons in culture were pulse-labeled with fluorescent ceramide. We studied the dynamics of labeled PPVs upon arrival at the axonal growth cone. In controls and cultures stimulated with brain-derived neurotrophic factor (BDNF), PPV clusters persisted in growth cones with a half-life ($t_{1/2}$) of >14 minutes. Upon challenge with IGF-1, however, fluorescent elements cleared from the growth cones with a $t_{1/2}$ of only 6 minutes. Plasmalemmal expansion was measured directly as externalization of membrane glycoconjugates in resealed growth cone particles (GCPs) isolated from fetal forebrain. These assays demonstrated that membrane expansion could be stimulated by IGF-1 in a dose-dependent manner but not by BDNF, even though intact, functional BDNF receptor was present on GCPs. Because both BDNF and IGF-1 are known to enhance neurite growth, but BDNF did not stimulate membrane expansion at the growth cone, we studied the effect of BDNF on the IGF-1 receptor. BDNF was found to cause the translocation of the growth-cone-specific IGF-1 receptor subunit β_{gc} to the distal axon, in a KIF2-dependent manner. We conclude that IGF-1 stimulates axonal assembly at the growth cone, and that this occurs via regulated exocytosis of PPVs. This mechanism is affected by BDNF only indirectly, by regulation of the β_{gc} level at the growth cone.

Key words: Axonal growth, Growth cone, Membrane expansion, Regulated exocytosis, IGF-1, IGF-1 receptor, BDNF, KIF2

Introduction

Neurites grow primarily by distal assembly of structural elements from components shipped to the growth cone by axoplasmic transport. Such assembly processes include 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). Neurons are equipped with receptors for multiple trophic factors, such as the ‘classical’ neurotrophins [e.g. nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF)] and insulin-like growth factor 1 (IGF-1). The receptors for IGF-1 and BDNF are present in axonal growth cones of many central nervous system neurons [as confirmed here and by others (Quiroga et al., 1995; Tuttle and O’Leary, 1998)]. We have shown earlier that the IGF-1 receptor of growth cones contains an immunochemically distinct β subunit termed β_{gc} (Quiroga et al., 1995). The β_{gc} subunit is highly enriched in axonal growth cones and is therefore of particular interest here. Among the targets of trophic factors is the regulation of gene expression, and neurotrophin-induced changes in gene expression have been demonstrated (e.g. Bonni and Greenberg, 1997). However, the problem of whether growth factors regulate neurite assembly locally at the growth cone has not been addressed. This includes the question of whether the exocytotic insertion of plasmalemmal precursor vesicles (PPVs) for membrane expansion is a constitutive phenomenon or regulated by growth factor(s).

Our laboratories have developed two assays to study plasmalemmal expansion, an important parameter of neurite assembly. One of these, a cell-free assay using isolated growth cones, had demonstrated earlier that membrane insertion at the growth cone can be triggered by depolarization and Ca^{2+} influx (Lockerbie et al., 1991; Wood et al., 1992). The second assay involves the incorporation of fluorescent lipid into PPVs and the study of their dynamics in growth cones of live neurons in culture.

The present report examines the ability of the receptors for IGF-1 and BDNF to regulate plasmalemmal expansion at the growth cone and addresses the question of whether the two growth factors promote neurite outgrowth by similar or different mechanisms.

Materials and Methods

Labeling reagents

The following primary antibodies were used: monoclonal antibody (mAb) against tyrosinated α-tubulin (clone TUB-IA2, mouse IgG; Sigma Chemical) diluted 1:2000; affinity-purified rabbit polyclonal antibody against β_{gc} (Quiroga et al., 1995) diluted 1:100; and a polyclonal antibody against a KIF2 peptide (Morfini et al., 1997). TrkB antibodies were the generous gift of David Kaplan (NCI-Frederick Cancer Research and Development Center). Additional TrkB antibody as well as anti-phosphotyrosine (P-tyr) antibody were purchased from Upstate Biotechnology. Cy3- and Cy5-labeled fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories; Texas-Red-conjugated phalloidin was from Molecular Probes. Beads coated with proteins A and G were from Calbiochem.

Cell culture

Cultures of dissociated hippocampal pyramidal cells from embryonic rat brain were prepared 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 then transferred to 60-mm Petri dishes containing serum-free medium plus the N2 mixture (Bottenstein and Sato, 1979). To allow neuronal survival and growth, this medium contains a high level of insulin sufficient to stimulate the insulin as well as the IGF-1 receptors. 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% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) containing 4% (w/v) sucrose. Cultures were washed with PBS, permeabilized with 0.1% (v/v) 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 stained with labeled 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. F-actin was labeled with Texas-Red-conjugated phalloidin. In most cases, the cells were observed with a Zeiss Axiovert microscope equipped with epifluorescence optics. Fluorescence images were digitized directly into a Metamorph/Metafluor Image Processor (Universal Imaging Corporation, West Chester, PA) and printed using Adobe PhotoShop. For confocal imaging, we used a Zeiss Axiovert 200M epifluorescence microscope equipped for digital deconvolution (Marianas System, Intelligent Imaging Innovations).

Preparation of GCPs

Growth cones particles (GCPs) were prepared 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 M 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 GCPs. Remaining particulate elements of the LSS were collected at the 0.83-2.66 M interface (fraction BC).

Membrane expansion assays

GCP binding of ^{125}I-labeled wheat germ agglutinin (WGA) was used as a measure of membrane area. WGA was radioiodinated using chloramine T and carrier-free Na^{125}I (Hunter and Greenwood, 1962) in the presence of 0.2 M hapten sugar, *N*-acetylglucosamine (GlcNAc). Labeled lectin was purified by size-exclusion chromatography on Sephadex G-25 and affinity chromatography on a GlcNAc column (Pierce Chemical, Rockford, IL). The resulting ^{125}I-WGA had a specific activity of ~0.8 μCi μg^{-1} protein. This was diluted with unlabeled WGA so that each assay tube contained ~300,000 cpm at a final concentration of 5 μM WGA. Under these conditions, WGA binding was linear; non-specific binding, determined in the presence of 0.2 M GlcNAc, was ~5%. For expansion assays, 1.3 ml of ice-cold GCP-containing band A was mixed first with 0.5 ml cold 2× dilution buffer (100 mM sucrose, 20 mM glucose, 200 mM NaCl, 10 mM KCl, 2.4 mM NaH_2PO_4, 44 mM HEPES, 2.4 mM $MgCl_2$, pH 7.3) and, after 20 minutes, with a further 0.8 ml of the same buffer. After an additional 20 minutes on ice, 100 μl aliquots of the suspension were added to assay tubes containing 100 μl L-15 culture medium plus factor, saponin or vehicle. Samples were equilibrated on ice for 30 minutes and then warmed up in a water bath to 36°C (controls were kept on ice), typically for 6 minutes, and subsequently chilled in ice slurry for 5 minutes. Ice-cold ^{125}I-WGA, diluted in L-15 as described above, was added to each tube and incubation continued for 15 minutes on ice. Samples were diluted with 200 μl cold L-15 and 300 μl aliquots loaded onto 0.5 ml cushions of 0.4 M sucrose in PBS, in siliconized conical tubes. These were spun at 37,500 $\boldsymbol{g}_{max}$ for 1 hour and then frozen. The tips containing the pellets were cut off and counted in a γ-radiation counter. Every experimental set included in triplicate (i) 0°C controls and (ii) samples containing 0.01% (w/v) saponin that were warmed up to 36°C for 6 minutes. This allowed us to determine external labeling in control conditions and total labeling after permeabilization, respectively. The difference between the two measurements was the size of the internal pool, which was used (separately for each set) as the bias for expressing membrane externalization.

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 a 2.5 hour 'chase' at 37°C (in the absence of label), the dissipation of the fluorescent spots (red channel) at the growth cones was examined with the microscope (equipped with a heated stage) in control conditions or after the addition of 20 nM IGF-1. Other cultures were kept in the absence of exogenous growth factors and then challenged with 50 ng ml^{-1} BDNF.

Gel electrophoresis and western blot

Proteins were separated by SDS/polyacrylamide-gel electrophoresis (SDS-PAGE) (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 (Millipore) in Tris-glycine buffer containing 20% methanol. The filters were dried, washed with Tris-buffered saline (TBS; 10 mM Tris pH 7.5, 150 mM NaCl) and blocked for 1 hour in TBS containing 5% bovine serum albumin (BSA). For probing blots with a single antibody, the filters were incubated with the primary antibody in PBS containing 0.05% Tween 20 for 2 hours at room temperature. After washing with TBS containing 0.05% Tween 20, the filters were incubated with horseradish-peroxidase-conjugated secondary antibody (Promega, Madison, WI) for 1 hour at room temperature. After washing, the blots were developed using a chemiluminescence detection kit (ECL, Amersham Life Sciences, Arlington Heights, IL).

TrkB activation in GCPs

The GCP suspension retrieved from the gradient was mixed gently with an equal volume of 'intracellular buffer' (20 mM Hepes pH 7.3, 50 mM KCl, 5 mM NaCl, 6 mM $MgCl_2$) and permeabilized with 0.02% β-escin. These GCPs were incubated for 5 minutes in the presence or absence of BDNF (0.2 nM) on ice and then, upon the addition of 1 mM ATP, 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 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. For immunoprecipitation of the receptor, the supernatant was incubated with anti-TrkB antibody (5 μg ml^{-1}) for 2 hours at 4°C before adding protein-A/G-coated beads. The precipitates were resolved by SDS-PAGE and blotted onto PVDF membrane. After quenching, blots were double-probed with mouse anti-P-tyr mAb and rabbit anti-TrkB primary antibodies. Blots were washed and then incubated with both Cy5-conjugated anti-mouse and Cy3-labeled anti-rabbit secondary antibodies. Finally, blots were imaged with a Typhoon 9200 scanner and data collected in the red and green channels to allow for simultaneous quantification of TrkB and Ptyr.

Results

Effects of IGF-1 and BDNF on membrane addition at the growth cone

To observe and measure membrane addition at the growth cone we (i) studied intact hippocampal neurons in culture by fluorescence microscopy after labeling plasmalemmal precursor compartments, and (ii) quantified externalization of membrane glycoproteins in isolated growth cones.

Live neurons in culture

These experiments depend on the expression of the appropriate receptors on the growth cones under investigation. The presence of β_{gc}-containing IGF-1 receptor in hippocampal neurons in culture has been reported (Mascotti et al., 1997). The distribution of TrkB and F-actin in growth cones of such neurons is illustrated in Fig. 1A. This confocal image shows almost complete overlap of the two labels, indicating the presence of TrkB in the growth cone periphery, including the finest filopodia.

To study membrane addition, hippocampal neurons cultured in the presence of 50 ng ml^{-1} BDNF or in the absence of exogenous growth factors (see Materials and Methods) were pulse-labeled at room temperature with BODIPY-ceramide, a fluorescent sphingomyelin and glucosylceramide precursor, and then chased for 2.5-3 hours at 37°C (Pagano et al., 1991). This resulted in intense fluorescence of the Golgi complex and in the presence of variously sized fluorescent compartments in the perikaryon, along the neurites and, eventually, in the axonal growth cone (Fig. 2A,B). Fig. 3 shows the labeled compartments in the growth cone in a confocal image at higher resolution. BODIPY-ceramide has a concentration-dependent emission spectrum, with a maximum at 515 nm (green) at low concentration. In the growth cone in Fig. 3A, green label is evident in the plasma membrane, extending into the filopodia and lamellipodia. At high concentration, BODIPY-ceramide forms excimers with an emission maximum at ~620 nm (red). This enables selective visualization of Golgi-derived vesicles. Hence, all membrane addition experiments were recorded in the red channel (Figs 2, 4). We attempted to correlate the Golgi-derived compartments in growth cones with structures detectable in phase-contrast images (Fig. 3B). However, there was no obvious correlation between the elements labeled red and specific structures discernible under phase contrast.

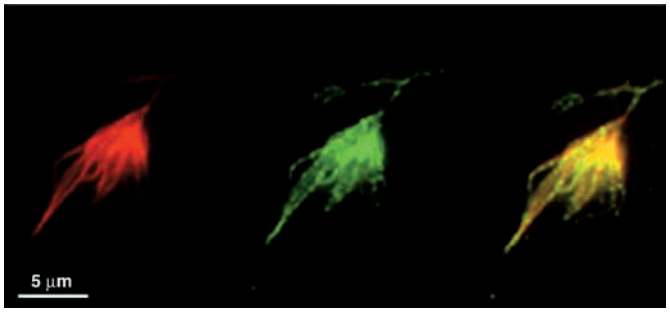

Fig. 1. Confocal fluorescence micrographs of a growth cone double-labeled for F-actin (red) and TrkB (green). The merged image shows almost complete overlap of the two labels (yellow), indicating predominant localization of the receptor in the growth cone periphery. The growth cone is from a culture of hippocampal pyramidal neurons grown for 24 hours in vitro.

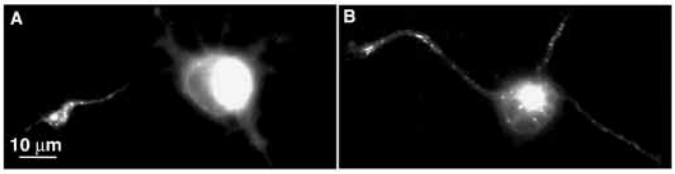

Fig. 2. Fluorescence micrographs of hippocampal neurons, labeled with BODIPY-ceramide for 30 minutes at room temperature and chased for 2.5-3 hours at 37°C. The images were recorded in the red channel, which reveals only highly concentrated label. Intense labeling of the Golgi complex and of vesicle clusters in neurites and growth cones is evident. The growth cones of these neurons are shown in the time series in Fig. 4.

In pulse-chase experiments, in control medium or upon challenge with 50 ng ml^{-1} BDNF, the fluorescent spots in the distal axon persisted for extended periods of time (>14 minutes). Upon challenge with IGF-1 (20 nM), however, fluorescent puncta in growth cones dissipated relatively rapidly (Fig. 4), whereas those in the perikaryon and axon shaft seemed unaffected by the challenge. This observation was quantified by counting fluorescent spots in each growth cone as a function of time after onset of the challenge and by expressing the numbers normalized to 1 at the onset of challenge (Fig. 5). Fluorescent puncta under control and BDNF conditions exhibited a half life ($t_{1/2}$) of >14 minutes, whereas the $t_{1/2}$ for IGF-1 was only ~6 minutes following challenge. In other words, IGF-1, but not BDNF, accelerated the dissipation of ceramide label from plasmalemmal precursor compartments at the growth cone.

Isolated growth cones

To determine directly and quantitatively whether growth factors regulated membrane addition in growth cones, we used fetal forebrain GCPs in our cell-free membrane expansion assay (Lockerbie et al., 1991). This membrane expansion assay measures membrane externalization from the internal vesicular pool as an increase in exposed glycoconjugates. First, we determined the internal pool of WGA binding sites in GCPs by comparing intact, unstimulated GCPs to permeabilized fractions. We observed that the internal pool varied from ~20% to ~50% of total binding sites from experiment to experiment (mean±s.d.=34.7±13.5%; n=7). Therefore, this pool was measured for each experiment, and the changes in externally exposed WGA binding sites were expressed as a percentage of

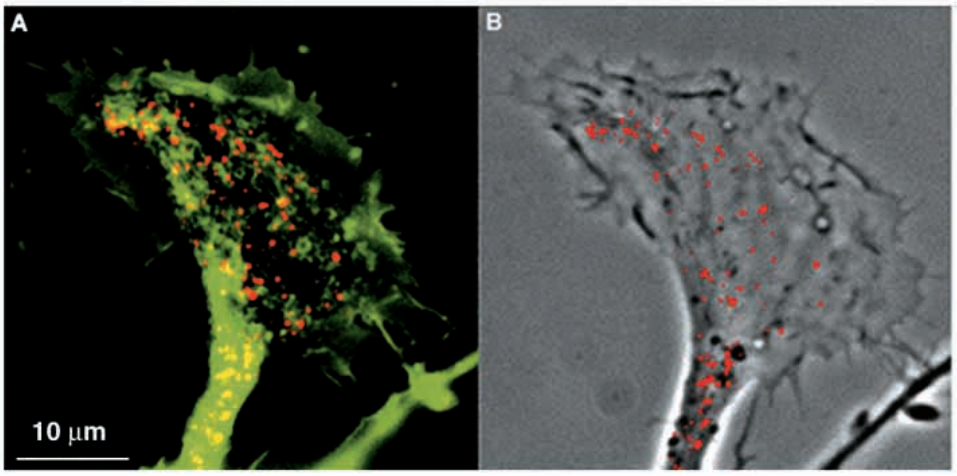

Fig. 3. Confocal fluorescence image of the unusually large, live axonal growth cone of a BODIPY/ceramide-labeled hippocampal neuron (24 hours in culture). The labeling protocol was as described for Fig. 2. (A) A merged image showing the BODIPY label recorded separately in the red (high concentration) and green (low concentration) channels. The many red puncta show Golgi-derived vesicles or vesicle clusters, whereas the green fluorescence outlines the growth cone's plasmalemma, labeled with small amounts of BODIPY. As soon as possible (~30 seconds) after recording BODIPY fluorescence, a phase-contrast image was taken of the same growth cone and then superimposed on the red BODIPY image. The merged image shows the relationship of Golgi-derived vesicles to structures seen under phase contrast.

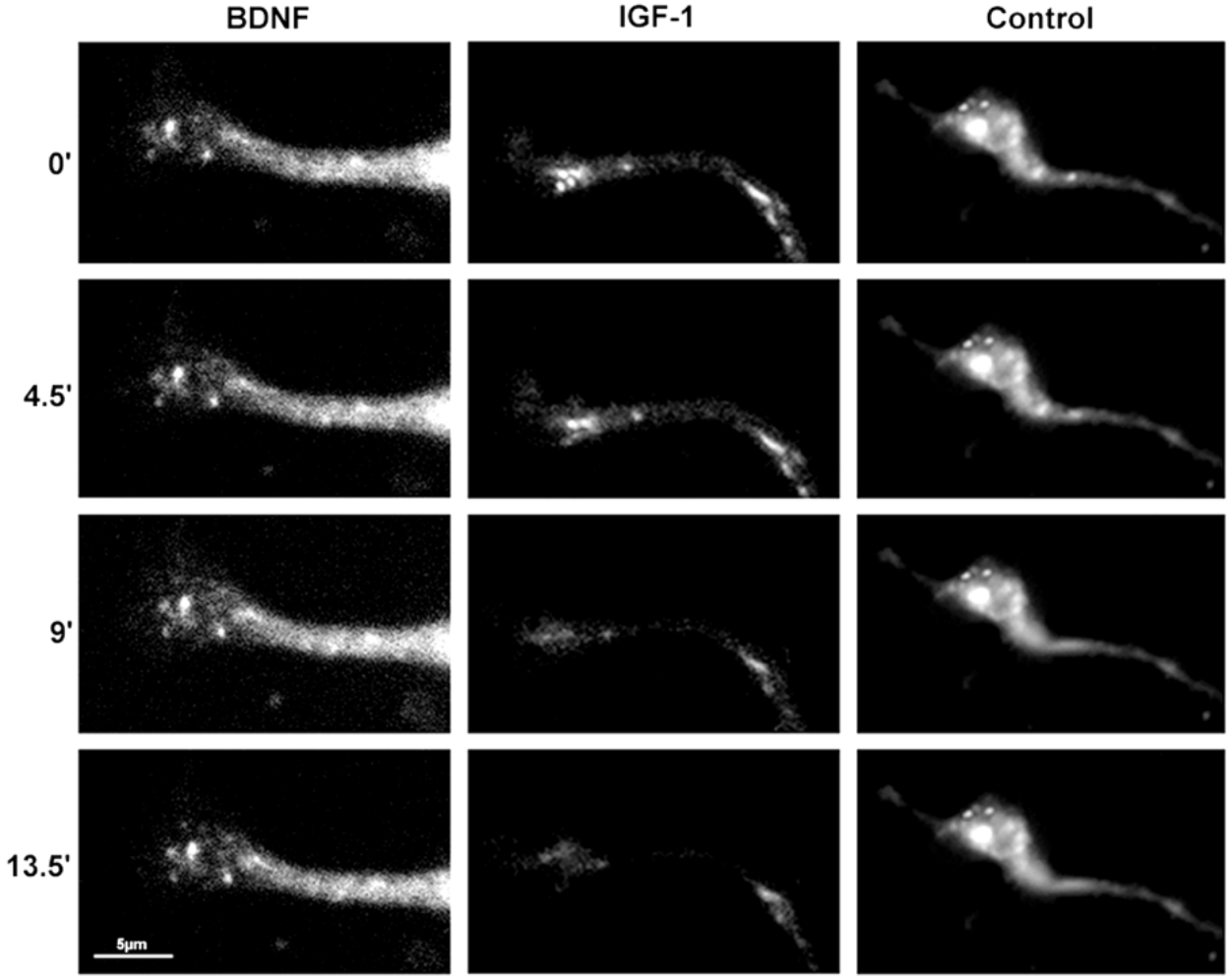

Fig. 4. Fluorescence micrographs (recorded in the red channel) of the axonal growth cones of hippocampal neurons from membrane addition experiments. Neurons were labeled with BODIPY-ceramide for 30 minutes at room temperature and then chased for 2.5-3 hours at 37°C. Growth cones were challenged with control medium, IGF-1 or BDNF, after factor deprivation, for the time indicated in minutes. Whereas fluorescent vesicle clusters persist in controls (vehicle only; growth cone of the neuron shown in Fig. 2A) and in the presence of BDNF, red fluorescence rapidly dissipates upon challenge with 20 nM IGF-1 (growth cone of the neuron shown in Fig. 2B).

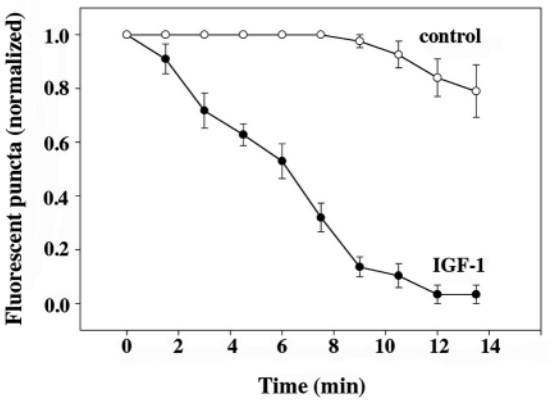

Fig. 5. Relative numbers of fluorescent puncta (vesicles or clusters recorded in the red channel) in growth cones for controls and IGF-1 (normalized to 1 at the onset of challenge). The values for $t_{1/2}$ are >14 minutes and 6 minutes, respectively. Data points are means ± s.e.m. from ten growth cones.

that pool. Fig. 6 shows dose-response curves of these changes for IGF-1 and BDNF. It is evident that BDNF did not affect WGA labeling over a wide concentration range. By contrast, IGF-1 caused a dose-dependent increase in WGA labeling that reached ~40% of the internal pool at ≤1 nM. The effect was blocked on ice and was time dependent, with an optimal response between 5 minutes and 10 minutes at 36°C (data not shown). It follows that IGF-1, but not BDNF, stimulates externalization of WGA binding sites in forebrain GCPs.

The apparent lack of a growth cone response to BDNF raises the question of whether its receptor, TrkB, is present and/or functionally intact. In order to address this question, we prepared subcellular fractions from 18-day fetal rat brains and probed western blots with antibodies to TrkA, TrkB and TrkC (generous gift of D. R. Kaplan). All Trk proteins were detectable (data not shown) (Knüsel et al., 1994) but TrkB immunoreactivity was the most abundant. Fig. 4 shows TrkB

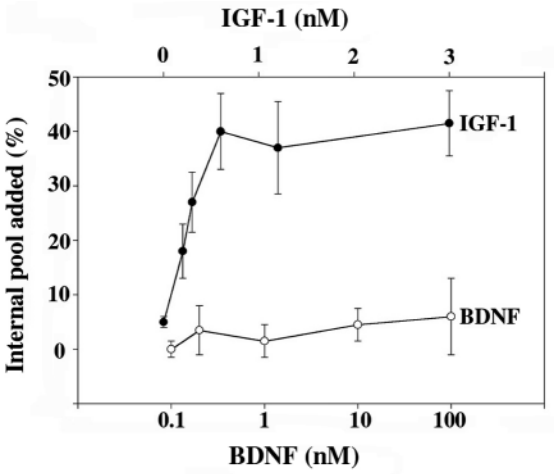

Fig. 6. Effects of trophic factors on membrane expansion in GCPs. The ordinate of the plot shows the percentage of the internal membrane pool added to the surface (determined by ^{125}I-WGA binding). The incubation was for 6 minutes at 36°C. Data points are means ± s.e.m. from five to ten determinations.

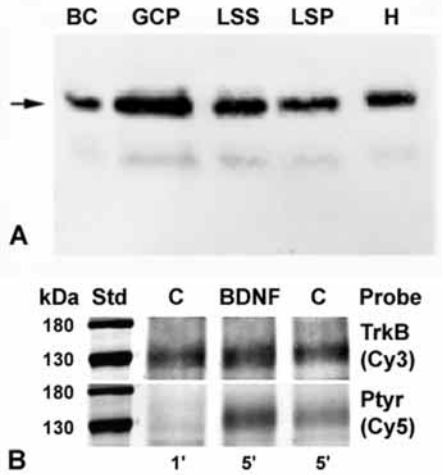

Fig. 7. TrkB in isolated growth cones. (A) Western blot of fetal rat brain (F18) subfractions probed with antibody to the external domain of TrkB. Equal amounts of protein were loaded in each lane. Arrowheads point at intact TrkB (~140 kDa). (B) TrkB immunoprecipitated from GCPs incubated in control medium or with BDNF (0.2 nM) for 1 minute or 5 minutes. Blots were probed with anti-TrkB (external domain) antibody to show the amount of receptor in each lane (top; Cy3 label) as well as with anti-P-tyr to demonstrate phosphorylation (bottom; Cy5). Although receptor levels are equal in all lanes, there is a substantial increase in P-tyr with BDNF relative to control at 5 minutes' incubation.

immunoreactivity at ~140 kDa, the size of the full-length receptor (Chao, 1992). TrkB was enriched increasingly as one moved from homogenate to LSS and then to its derivative fraction, the GCPs. Densitometric analysis of a representative experiment indicated an increase of >80% in GCPs compared with the homogenate. The low-speed pellet (LSP), containing large cell fragments, exhibited a similar amount of immunoreactivity to the homogenate. By contrast, the LSS subfraction BC was depleted to about one-half of the immunoreactivity seen in LSS. Because of the primarily axonal origin of GCPs (Saito et al., 1992; Lohse et al., 1996), these blots indicate enrichment of intact TrkB in axonal growth cones isolated from rat forebrain. To ascertain that TrkB was indeed activatable in GCPs, we stimulated the receptor with BDNF, immunoprecipitated it using an anti-TrkB antibody and probed blots with both anti-TrkB and anti-P-tyr antibodies, using two different chromophores as reporter groups (Fig. 7B). TrkB labeling (Cy3 chromophore; top) reveals that equal amounts of the receptor were loaded in each lane of this gel. Tyrosine phosphorylation of the receptor (Cy5 chromophore; bottom), however, is quite different in the three lanes: Comparison of controls at 1 minute and 5 minutes indicates some kinase activity without the addition of exogenous BDNF. In the presence of BDNF (5 minutes), however, there is a 2.5-times increase in P-tyr relative to the corresponding control. Thus, TrkB in GCPs can indeed be activated by BDNF.

Effects of BDNF on neurite growth and distribution of β_{gc}

The lack of BDNF stimulation of plasmalemmal expansion in our assays prompted us to examine the effects of BDNF on neurite growth in our cultured hippocampal neurons. To assess

Table 1. Effects of BDNF on axonal growth and on β_{gc} immunofluorescence in the distal axon

	Control	BDNF*	BDNF+asKIF2†
Average axonal length (μm)	105±28.2	189±24.8	86.7±31.6
β_{gc} staining intensity (A.U.)	65.8±36.2	335±92.8	58.8±21.4

Hippocampal pyramidal cells were cultured for 36 hours in control conditions in the presence of either 50 ng/ml BDNF or 50 ng/ml BDNF plus anti-sense KIF2 (asKIF2). Images were recorded and digitized, and the axonal lengths and β_{gc} staining intensities were measured using the morphometric menu of Metamorph 2.0 software. 50 cells were scored in each condition. Results are the averages±s.d.

*BDNF values were significantly different from controls at $P<0.001$ (Student's *t*-test).

†BDNF+asKIF did not differ significantly from controls.

the degree of differentiation, a neuron was considered to be in stage 3 when the length of one of the processes (the axon) exceeded the average length of the other processes (the minor processes) by at least 20 μm (Cáceres et al., 1986; Dotti et al., 1988); without a discernible axon, neurons were in stage 2. In the cultures grown without BDNF, ~65% of the neurons were in stage 2 after 36 hours of differentiation in vitro (DIV). In the presence of 50 ng ml^{-1} of BDNF, however, almost 80% of the cells were in stage 3 (36 hours of DIV), indicating that BDNF significantly accelerated the rate of axon growth and neuronal polarization in our experimental system. This was borne out in measurements of axon length (Table 1). BDNF nearly doubled axon length over a 36-hour growth period. These differences are evident in Fig. 8A,C vs Fig. 8E,G.

We have reported previously (Quiroga et al., 1995) that β_{gc} is highly enriched in GCPs isolated from fetal brain. In hippocampal neurons in culture, however, growth cone enrichment of β_{gc} was transient and decreased significantly before the cells began dendritic differentiation. This suggested that the lack of a trophic factor in the cultures might have been responsible for the decline in β_{gc}. To test this hypothesis, hippocampal neurons were cultured in the presence or absence (control cultures) of 50 ng ml^{-1} BDNF and then labeled with antiserum to β_{gc}. The control cells developed several minor processes within 24-36 hours DIV and a relatively short axon (Fig. 8A,C) with a weakly β_{gc}-positive growth cone (Fig. 8B,D). Cells cultured for 24 hours in the presence of BDNF (Fig. 8E,F) were similar to controls. After 36 hours in culture with BDNF, however, a significant increment in β_{gc} immunostaining was evident in the distal axons and growth cones (Fig. 8D,H). β_{gc} immunoreactivity in distal axons was measured in 36-hour cultures and the data are shown in Table 1. The results demonstrate that BDNF increased the fluorescence intensity of β_{gc} more than fivefold in the distal third of the axon, including the growth cone.

Effects of BDNF and KIF2 expression

The increase in β_{gc} in the distal axon could be the result of enhanced β_{gc} production and/or redistribution by export from the perikaryon into the axon. Surprisingly, western blots failed to reveal an increase in β_{gc} immunoreactivity in hippocampal neurons cultured with BDNF (Fig. 9). By contrast, BDNF greatly enhanced the expression of the central-domain microtubule motor KIF2 (Fig. 9). Thus, KIF2 might be

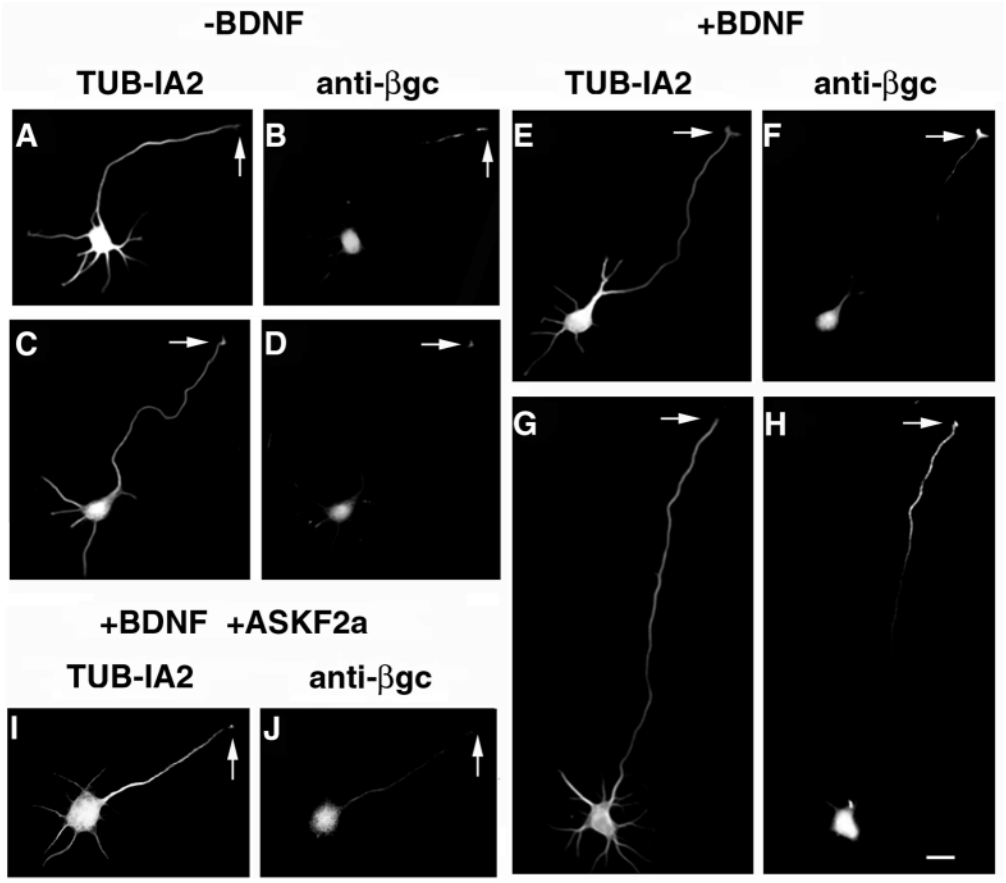

Fig. 8. Double immunofluorescence micrographs showing the distribution of tyrosinated α-tubulin (A,C,E,G,I) and β_{gc} (B,D,F,H,J) in hippocampal pyramidal neurons after 24 hours (A,B,E,F) or 36 hours (C,D,G-J) in culture, in the absence (A-D) or the presence (E-J) of 5 ng ml^{-1} BDNF. In the absence of BDNF, neurons exhibit faint β_{gc} immunofluorescence in the growth cones (B and D, arrows). In the presence of BDNF, β_{gc} immunofluorescence is strong in the growth cones and distal third of the axons (F and H, arrows). The addition of anti-sense *KIF2* (*asKIF2*), even in the presence of BDNF (I and J, 36 hours in culture), greatly reduces axonal growth and abolishes distal β_{gc}. Bar, 10 μm.

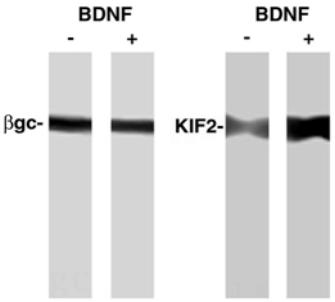

Fig. 9. β_{gc} and KIF2 protein levels as determined by western blot of homogenates of hippocampal neurons cultured in the absence (–) or the presence (+) of 50 ng ml^{-1} BDNF for 36 hours. Whereas β_{gc} levels are unchanged, there is a striking increase in KIF2 production in the cells treated with BDNF.

responsible for β_{gc} redistribution during axonal differentiation. To test this hypothesis, we cultured hippocampal pyramidal cells in the presence of both BDNF and an antisense oligonucleotide to *KIF2* (*asKIF2*). Western blots of total extracts of such cultures demonstrated significantly diminished KIF2 production compared with controls. However, *asKIF2* did not affect cell survival and/or the expression of other proteins such as tyrosinated α-tubulin, β_{gc}, kinesin heavy chain and synaptophysin (data not shown) (see Morfini et al., 1997). Immunofluorescence shows that *asKIF2* substantially reduced axon outgrowth (Fig. 8G,I) and caused the absence of β_{gc} in the distal axon (Fig. 8J,H). The effects are shown quantitatively in Table 1. In the presence of BDNF and *asKIF2*, both axonal length and β_{gc} fluorescence intensity in the distal third of the axon were attenuated significantly compared with those in cultures treated with BDNF alone. Thus, β_{gc} distribution to the axonal growth cone is KIF2-dependent.

Discussion

Many observations indicate, directly or indirectly, that plasmalemmal expansion of the growing neurite or axon occurs by exocytotic incorporation of PPVs into the cell surface, primarily at the growth cone: the nearly quantitative, rapid axoplasmic transport of newly synthesized lipid to the growth cone (Pfenninger and Johnson, 1983); the accumulation in growth cones of clear vesicles that contain newly synthesized lipid (Pfenninger and Friedman, 1993); the externalization of new glycoconjugates on the plasmalemma covering the PPV clusters (Pfenninger and Maylié-Pfenninger, 1981) (see also Feldman et al., 1981; Craig et al., 1995); the dependence of neurite growth on proteins known to be involved in exocytosis (Osen-Sand et al., 1993; Kabayama et al., 1998); and the dependence of membrane expansion on Ca^{2+} influx in isolated growth cones (Lockerbie et al., 1991). Exocytotic membrane expansion is consistent with well-established cell-biological concepts (e.g. Burgess and Kelly, 1987), but the question of its regulation has remained open. Thus, it seemed possible that axon growth was controlled simply by growth-factor-regulated synthesis of neurite components (e.g. Bonni and Greenberg, 1997) in the perikaryon, followed by constitutive assembly of the plasma membrane (and cytoskeleton) at the growth cone (De Camilli, 1993). Increasing recent evidence indicates, however, that the export pathways to the cell surface of many membrane proteins are regulated [see, for example, the glucose transporter (Lund et al., 1995; Martin et al., 2000)]. The stimulation of membrane insertion at the growth cone by depolarization (Lockerbie et al., 1991) had already suggested regulation but the problem of whether growth-promoting factors controlled this process remained to be investigated. The results presented here address this problem.

Regulation of plasmalemmal expansion by trophic factors

The present results extend our earlier observations on the role of depolarization and Ca^{2+} influx in plasmalemmal expansion to the action of growth factors in both intact neurons and isolated growth cones. As expected, BODIPY-ceramide incorporation into sphingomyelin and its glycosylation to glucosylceramide (Pagano et al., 1991) result in strong, stable labeling of the Golgi complex. Intense red fluorescence indicates a high concentration of the label in this organelle. Labeled smaller compartments, presumably vesicles and their clusters, emerge from the Golgi and begin to appear at the growth cones within ~2 hours. Excimer formation by the BODIPY label and recording in the red channel enable the selective imaging of these Golgi-derived membranes. Although overlap of these compartments with specific phase-dense or phase-bright structures was not evident, their overall distribution is consistent with that of PPV clusters labeled with another lipid precursor, glycerol (Pfenninger and Friedman, 1993), as expected. The time-lapse observations shown here reveal that IGF-1, but not BDNF, accelerates the disappearance of Golgi-derived, BODIPY-labeled structures from the growth cones. This is consistent with the coalescence into the plasmalemma of vesicles or vesicle clusters visible initially as bright red spots owing to their high BODIPY content. Upon plasmalemmal insertion, the BODIPY label becomes diluted into a much larger lipid pool and so the fluorescent signal in the red channel disappears and shifts to green.

These fluorescence microscopic observations document the regulated ‘disappearance’ of BODIPY-labeled structures at the growth cone but do not provide direct evidence about membrane insertion. Therefore, we complemented these experiments with an assay of membrane externalization using GCPs. We have characterized this cell-free assay in detail (Lockerbie et al., 1991) and shown that changes in WGA binding (e.g. induced by Ca^{2+} influx) reflect changes in B_{max} (maximum lipid bound) not in K_D. We have shown, furthermore, that depolarization-induced externalization of WGA binding sites is paralleled by an ultrastructurally measurable and comparable decrease in the membrane area of internal precursor vesicles (operationally defined as vesicles <180 nm in diameter). This earlier characterization of the assay readily applies to the experiments presented here. BDNF was used over a wide concentration range (0.01-100 nM) in order to test whether it triggered externalization of WGA binding sites. However, WGA binding was not affected in these assays, even though western blots had demonstrated the presence and enrichment of intact TrkB, as well as its activation, in the same GCP preparation. By contrast, IGF-1 stimulated the externalization of WGA binding sites in a dose-, time- and temperature-dependent manner. A maximal effect was observed at ~1 nM (the K_D of the IGF-1 receptor was 0.75 nM) and amounted to ~40% externalization of the internal pool. This value was comparable to, or higher than, the numbers obtained for K^+ depolarization and Ca^{2+} ionophore treatment (Lockerbie et al., 1991). It follows from both sets of experiments that insertion into growth-cone plasmalemma of PPVs is a regulated phenomenon and stimulated by the growth factor IGF-1, but not by BDNF.

BDNF and redistribution of β_{gc}

BDNF was discovered as a neuronal survival, differentiation and axonal-elongation factor (Davies et al., 1986; Hofer and Barde, 1988). Our data confirm in our experimental system that BDNF increases the axonal growth of hippocampal pyramidal neurons in culture almost twofold. However, unlike IGF-1, BDNF does not promote outgrowth by stimulating plasmalemmal assembly at the growth cone. Instead, our data show that it affects IGF-1 receptor distribution. Without exogenous BDNF, β_{gc} appears in our cultures only transiently. BDNF is required for, and greatly stimulates, the sustained distal axonal enrichment of β_{gc}. These results parallel our previous finding that enrichment of β_{gc} in the distal neurites of PC12 cells is tightly regulated by nerve-growth factor (NGF) (Mascotti et al., 1997). Together these observations suggest that neurotrophins of the NGF family regulate IGF-1-receptor distribution in the growing neuron.

Experiments performed in whole cells or with cell extracts indicate that KIF2 is a plus-end-directed microtubule-based motor protein (Noda et al., 1994; Morfini et al., 1997; Santama et al., 1998). BDNF causes a striking increase in KIF2 protein level in hippocampal pyramidal cells, together with the distal axonal enrichment of β_{gc}. Conversely, transfection of neurons with *asKIF2* blocks distal accumulation of β_{gc} and reduces axonal growth. These results suggest that β_{gc} reaches the axonal growth cone by KIF-dependent anterograde transport that is regulated by BDNF.

Conclusions

Our results demonstrate that plasmalemmal expansion, a key parameter of axonal growth, is regulated at the growth cone by IGF-1, in a manner that is quasi-independent of the neuron's perikaryon. That IGF-1 is involved in this regulation is perhaps not surprising in view of the many reports of its positive effects on neuronal differentiation and outgrowth (DiCiccio-Bloom and Black, 1988; Aizenman and DeVellis, 1987; Caroni and Grandes, 1990; Beck et al., 1993; Ishii et al., 1993). However, IGF-1's direct and local effect on plasmalemmal expansion at the growth cone is a novel result.

The growth cone receives BDNF signals, as indicated by the presence of full-length, activatable TrkB. Interestingly, the distribution of TrkB at the growth cone is different to that of the β_{gc}-containing IGF-1 receptor. TrkB is present throughout the growth-cone periphery, together with filamentous actin. By contrast, β_{gc} is located primarily in the proximal growth cone, together with microtubules (Mascotti et al., 1997). The significance of this different receptor distribution is not known, but the distinct patterns are likely to be relevant to the receptors' different biological effects. Neurotrophins have been established as target-derived polypeptides that promote neuron survival via retrograde transport from the nerve terminal to the perikaryon (Reynolds et al., 2000). However, it has been suggested that the temporal and spatial characteristics of many effects of BDNF in the central nervous system are more consistent with local actions than with signaling mediated by long-distance retrograde transport (e.g. Altar et al., 1997). Indeed, neurotrophins might act as chemoattractants and have been implicated in the modulation of growth-cone responses to guidance molecules (Campenot, 1977; Paves and Saarma, 1997; Tuttle and O'Leary, 1998; Ming et al., 1999; Gallo and Letourneau, 2000). Yet, at least in the two assays used here, BDNF does not directly influence membrane addition at the growth cone. Our findings suggest instead an indirect effect. By controlling the distribution of β_{gc}, BDNF probably influences distal growth regulation via retrograde signaling to the perikaryon, the synthesis of KIF2 (and probably other proteins) and increased transport of β_{gc} to the axonal growth cone.

Overall, our studies support the hypothesis that the actions of BDNF and IGF-1 on the growth cone are different but interdependent. They seem to occur in sequence, with BDNF regulating the distribution of β_{gc}-containing IGF-1 receptors. Once the IGF-1 receptor is present in the distal axon, IGF-1 directly regulates membrane assembly and outgrowth at the growth cone.

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