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Wortmannin Blocks Goldfish Retinal Phosphatidylinositol 3-Kinase and Neurite Outgrowth*

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The goldfish retina has been used extensively for the study of nerve regeneration. A role for phosphatidylinositol 3-kinase (PI3K) in neurite outgrowth from goldfish retinal explants has been examined by means of wortmannin (WT), a selective inhibitor of the enzyme. The presence of PI3K in retinal extracts was determined by means of immunoprecipitation as well as by an in vitro assay system for catalytic activity. The relative amount of the p85 subunit of PI3K detected by western blot in the retina following optic nerve crush was unchanged. WT inhibited goldfish brain PI3K activity at concentrations as low as 10^{-9} M, approximating that reported for inhibition of mammalian PI3K's. Daily addition of 10^{-8} M WT to retinal explants, activated by prior crush of the optic nerve, significantly inhibited neurite outgrowth during a 7 day in vitro culture period, while a single addition of WT to freshly explanted retina had no effect on neurite outgrowth. These results suggest that a PI3K-mediated process may be critical for nerve regrowth.

KEY WORDS: Goldfish retina; neurite outgrowth; neuronal regeneration; phosphatidylinositol 3-kinase; Wortmannin.

INTRODUCTION

The enzyme phosphatidylinositol 3-kinase (PI3K) catalyzes phosphorylation of the 3'-hydroxyl group of the inositol moieties of phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5 bisphosphate to produce the corresponding 3'-phosphorylated derivatives. PI3K is emerging as the initial step in a signal transduction pathway employed by a wide range of growth-related and neurotrophic factors (1,2). The 3'-phosphoinositides have been proposed to play a role in NGF-stimulated neurite outgrowth from PC12 cells (3), growth factor-induced mitogenesis (4), insulin-stimulated glucose uptake and glycogen synthesis (5), and PDGF-stimulated membrane ruffling (6,7). PI3K is also involved in the action of certain oncoproteins, such as $pp60^{c\text{-}src}$ with which PI3K interacts (8). The enzyme is a heterodimer composed of a 85 kDa regulatory (p85) and a 110 kDa (p110) catalytic subunit (9,10). Upon binding of the regulatory subunit to tyrosine phosphorylated proteins, the catalytic subunit is activated. The fungal metabolite wortmannin (WT) is a potent inhibitor of PI3K activity in vitro and in vivo, and has been shown to bind covalently to the 110-kDa subunit of PI3K (11). It has not previously been employed to study nerve regeneration.

The goldfish visual system has been explored extensively as a model of nerve regeneration in the ver-

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Abbreviations used: anti-p85, antibody for the 85-kDa subunit of PI 3-kinase; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; NGF, nerve growth factor; NGI, neurite growth index; CNGI, computerized nerve growth index; NP-40; Nonidet P-40; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PI3K, phosphatidylinositol 3-kinase; TLC, thin-layer chromatography; WT, wortmannin.

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tebrate central nervous system, since injured optic nerve axons regrow and reestablish their highly selective pattern of synaptic connections with postsynaptic neuronal sites in the optic tectum (12). During regeneration, there is increased RNA and protein synthesis in the retinal ganglion cells (RGC's; 13) and enhanced routing of newly synthesized RGC protein to their regrowing axons (14). A number of potential growth factors appear to play a role in goldfish optic nerve regeneration (15–17), and could exert their effects via PI3K-mediated signal transduction. In addition, tubulins, which increase dramatically during regeneration (18), have been shown to associate with PI3K (19). In this study, we used WT to investigate the possible role of PI3K on the process of neurite outgrowth from goldfish retinal explants, and by implication, on nerve regeneration.

EXPERIMENTAL PROCEDURE

Materials. ATP, aprotinin, bovine serum albumin (BSA), leupeptin, phosphatidylinositol (PI; from bovine liver), polylysine and wortmannin (WT) were purchased from Sigma (St. Louis, MO) [γ^{32}P]ATP (3000 Ci/mmol) was from DuPont NEN (Wilmington, DE). Silica gel 60 thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L15 culture medium and protein-A agarose were purchased from GIBCO BRL (Grand Island, NY). Anti-Rat PI3K (anti-p85) antibody was from Upstate Biotechnology Incorporated (Lake Placid, NY). Panasorbin cells were from Calbiochem (La Jolla, CA).

Animals. Goldfish (*Carassius auratus*), 6–7 cm in body length, obtained from Grassy Forks Fisheries (Martinsville, IN) were anesthetized in tank water containing 0.04% tricaine methanesulfonate. Intraorbital crush of the right optic nerve was performed as previously described (20).

Explant Preparation and Culture. We have demonstrated previously that explanted goldfish retinas do not extend neurites onto a substratum, but will do so if the optic nerve is crushed several days prior to explantation (20,21). Retinas were removed from goldfish 7 days following optic nerve crush, explanted as previously described (21) onto polylysine-coated plastic dishes, and were maintained at 20–22°C in a humid chamber in L15 complete medium (21). After 7 days in culture, the extent of neurite outgrowth was evaluated (22). Briefly, the average length of the neurites, in units of explant widths (500 μm), was multiplied by an estimate of neurite density, on a scale of 1–4, to yield a nerve growth index (NGI). In parallel, a computerized NGI (CNGI) was assayed by an automated program using digitized low power video images of the explants from a Wild dissecting microscope (23). For each experimental group, 5 dishes, containing 9 explants each, were scored.

For the WT experiments, explants were allowed to attach firmly to the substratum for 24 h. The culture medium was replaced every 24 h and contained fresh WT, prepared from a 2 mM WT stock solution in DMSO. This was serially diluted to yield the desired experimental range (10^{-6} to 10^{-10} M) in L15 complete medium. DMSO was added to all incubations, including controls to yield a final concentration of 0.02% (v/v). At this concentration, DMSO had no effect on neurite outgrowth.

Immunopurification of PI3K from Goldfish Retina. Goldfish were dark-adapted, anesthetized and euthanized by severing the spinal cord. Retinas were gently lifted from the underlying pigment layer, separated from the optic nerve, rinsed with phosphate-buffered saline (PBS), pH 7.4, and mechanically homogenized in lysis buffer (1 ml/retina) containing 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 1% Nonidet P-40 (NP-40), 10 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. The homogenates were centrifuged at 6,000 *g* for 10 min at 4°C. The supernatants were incubated on ice for 15 min with Panasorbin cells (50 μl/ml) and centrifuged at 6,000 *g* for 10 min at 4°C. The resulting clear supernatants were incubated overnight at 4°C with anti-p85 antiserum (5 μl/mg protein), followed by addition of protein A-agarose beads (50 μl/ml) for 2 h with mixing. Immune complexes bound to the beads were washed three times with PBS containing 1% NP-40, twice with PBS containing 0.5 M LiCl and 0.1 M Tris-HCl (pH 7.5), and twice with 0.1 M NaCl containing 1 mM EDTA and 10 mM Tris-HCl (pH 7.5).

PI3K Activity Assay. The reaction mixtures for measuring PI3K activity contained 50 μl of the immunoprecipitated enzyme preparation (representing 1 mg of retinal protein) and 50 μl of PI micelles prepared by sonicating 800 μg of PI in 1 ml of 20 mM HEPES buffer (pH 7.6) containing 1 mM EDTA for 45 sec in a Kontes sonicator with a microtip probe. The mixture was preincubated for 15 min at room temperature with or without various concentrations of WT in 50 μl of HEPES buffer. The reaction was initiated by the addition of 50 μl of 40 mM $MgCl_2$ containing 200 μM [γ-^{32}P]ATP (20 μCi per assay). The reaction was terminated after 10 min by the addition of 750 μl of ice-cold $CHCl_3$/MeOH/2 M HCl (20:40:0.5) followed by 250 μl of chloroform and 250 μl of 2 M HCl. The resulting organic phase and the interface were extracted with an equal volume of methanol/0.1 M EDTA (1:0.9). The organic phase was dried, resuspended in 25 μl of chloroform/methanol (1:1), and spotted on a TLC plate which had been precoated with 1% potassium oxalate. The plate was developed in chloroform/methanol/H_2O/7.7N ammonium hydroxide (60:47:11.3:2) (22). ^{32}P-labeled PI 3-phosphate (P13P) was visualized by autoradiography and compared with an iodine-stained PIP standard (PI4P was used, since it is not separated from PI3P and was commercially available). Radioactivity in P13P was quantitated by liquid scintillation spectrometry.

Immunoblotting of the p85 Subunit of PI3K. Control retinas as well as retinas taken several days after unilateral optic nerve crush (post-crush; PC) were lysed, followed by immunoprecipitation with anti-p85 antiserum as described above. After washing, immune complexes were resolved on 10% SDS-PAGE. Separated proteins were transferred to nitrocellulose paper and analyzed for the presence of p85 by immunoblotting with anti-p85 antiserum. Bound antibodies were visualized by an enhanced chemiluminescence (ECL) detection system, using horseradish peroxidase conjugated to anti-rabbit IgG as the secondary antibody (Amersham, Arlington Heights, IL).

RESULTS

PI3K Activity in Goldfish Retina. The presence of PI3K in goldfish retina was demonstrated by means of immunoprecipitation and western blotting, as well as by enzyme activity assays. As shown in Fig. 1A, western blotting of anti-p85 immunoprecipitates and ECL detection, revealed the presence of a p85-like protein in gold-

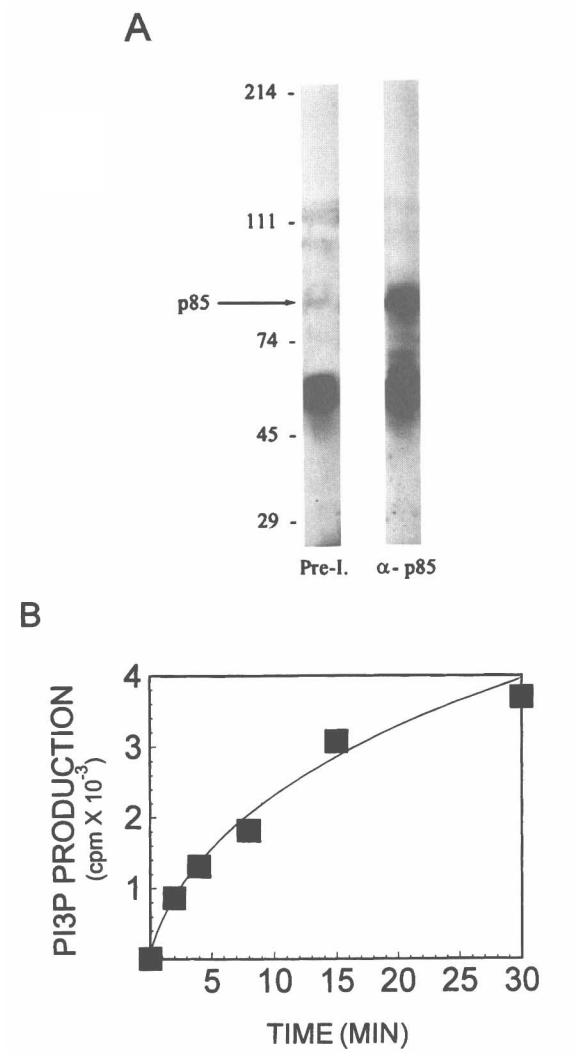

Fig. 1. Immunopurification of goldfish PI3K. A. Western blot with anti-p85 antiserum of goldfish retina immunoprecipitated with either anti-p85 antiserum or rabbit preimmune serum. The prominent spots above the 45 kDa marker represent IgG's. Detection by ECL. B. Assay of goldfish PI3K activity. One mg of anti-p85 antiserum immunoprecipitated enzyme and 50 µl of PI micelles were incubated in the presence of 20 µCi [γ-^{32}P] ATP. PI3P was separated by TLC and radioactivity was determined by liquid scintillation spectrometry. Data are from one of 3 experiment that gave similar results.

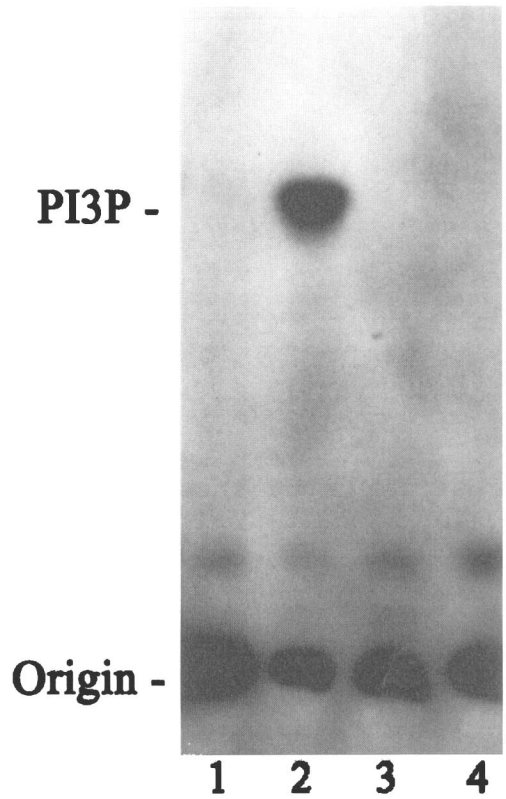

Fig. 2. Specificity of goldfish anti-p85 immunoprecipitation. The immunoprecipitates were assayed for PI3K activity by autoradiography following TLC separation as described in the text. Lane 1, preimmune serum immunoprecipitates; Lane 2, anti-p85 immunoprecipitates; lane 3, anti-p85 plus 0.5% NP-40; lane 4, anti-p85 plus 100 nM WT.

fish retina. Control preimmune rabbit serum immunoprecipitates did not contain detectable PI3K protein. PI is a convenient substrate for PI3K (24), and was therefore employed for enzyme activity studies. The reaction was found to be roughly linear for up to 15 min (Fig. 1B) and proportional to retinal protein added (not shown). These kinetic parameters, used for the WT studies, are similar to those described in mammalian cells by Serunian et al. (22). We next characterized PI3K activity in the anti-p85 immunopurified preparation. As indicated in Fig. 2, PI phosphorylation to PIP was detected in anti-p85 immunoprecipitates, but not in preimmune rabbit serum immunoprecipitates. In order to examine the possible copresence of PI 4-kinase (PI4K) activity in the preparations, we performed the phosphorylation reaction in the presence of NP-40, a detergent known to inhibit PI3K activity and to stimulate PI4K activity (25). Addition of 0.5% NP-40 to the reaction mixture completely inhibited the formation of ^{32}P-labeled lipid, indicating the absence of detectable PI4K activity in the preparation.

WT is a potent and highly selective inhibitor of broad-specifity mammalian PI3K (2). However, the yeast VPS34 homolog and the mammalian PI-specific PI3K are resistant to this toxin. Thus we tested whether WT is able to inhibit PI3K activity derived from the goldfish retina. We found that pretreatment for 15 min of anti-p85 immunoprecipitates with WT (100 nM), inhibit the phosphorylation of PI by 86%. The dose-dependent effect of WT on PI3K activity is shown in Fig.

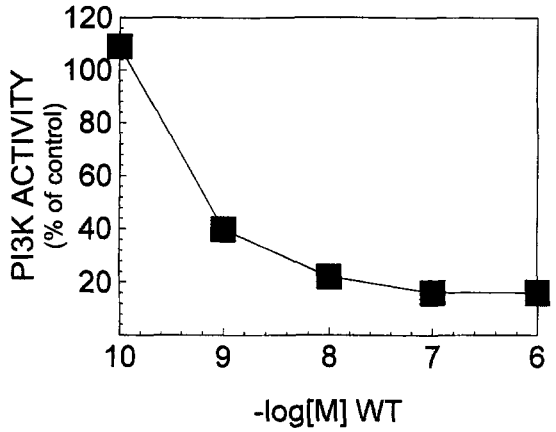

Fig. 3. Dose-dependent effect of WT on PI3K activity. Anti-p85 immunoprecipitates and PI micelles were preincubated with or without varying doses of WT for 15 min at room temperature. PI3K activity was measured as in Fig. 1B. The IC_{50} value was calculated to be 2×10^{-9} M. Data are from one of 3 experiments that gave similar results.

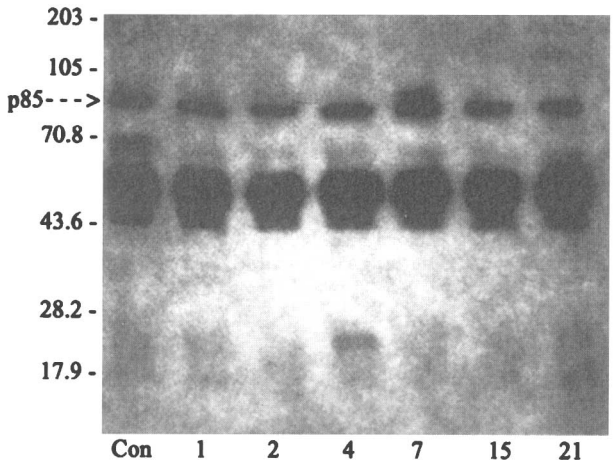

Fig. 4. PI3K levels in goldfish retina. Western blot of anti-p85 antiserum immunoprecipitation of retina from goldfish at various times PC and in controls (unlesioned). Lane 1, control; other lanes indicate the number of days between nerve crush and explantation. Amounts of retinal PI3K were not changed following optic nerve crush.

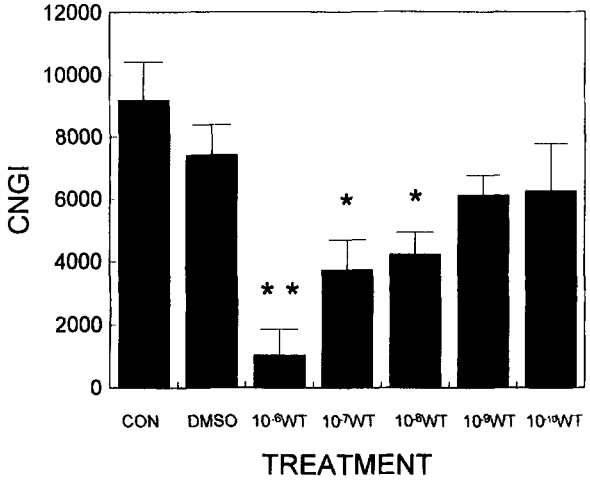

Fig. 5. The dose-dependent effect of WT on neurite outgrowth from 7 day PC goldfish retinal explants. Retinal explants received daily medium changes with or without 0.02% (v/v) DMSO with or without varying doses of WT. Neurite outgrowth was determined after 7 days by computer automated quantitation of digitized video images yielding a pixel count (CNGI). Results are averaged from 4 experiments. Error bars indicate S.E. Doses greater than 10^{-9} M are significantly different from DMSO control (* = $p < 0.05$, ** = $p < 0.005$ when compared to DMSO control).

3. WT (10^{-7} M) inhibits the retinal enzyme completely and partial inhibition was detected with concentration as low as 6×10^{-9} M. The IC_{50} value was calculated to be 2×10^{-9} M. These values are similar to those reported in other studies (3).

Estimation of PI3K in Retina. To evaluate whether retinal PI3K is altered following optic nerve crush, we examined levels of the p85 subunit by means of western blotting with anti-p85 antibody. The amount of p85 protein does not appear to change over a 7 day period (Fig. 4) although an additional band, possibly a p85 isoform, appears in 7 day PC retinas. PI3K catalytic activity was also found to be unchanged during this period (data not shown). The same assay, following anti-phosphotyrosine rather than anti-p85 antibody immunoprecipitation, indicated that the p85 subunit was not detectably subjected to tyrosine phosphorylation in control or PC retinas (data not shown).

Wortmannin Inhibits Neurite Outgrowth in Retinal Explants of Goldfish Undergoing Optic Nerve Regeneration. Explants derived from 7 days PC goldfish retina extend neurites in culture for about 1 week, while retinas removed from an intact visual system show little or no outgrowth. Neurite outgrowth is thus a useful in vitro indicator of nerve regeneration and can be quantitated by means of a manual (NGI) or automated (CNGI) procedure. In initial experiments, WT was added to the medium only once, at the time of explantation. There was little effect observed, with concentrations as high as 10^{-6} M. In subsequent studies, retinal explants were allowed to attach to the culture dishes for 24 h prior to WT addition. WT was then added daily for the entire period in culture. At 100 nM, WT inhibited neurite outgrowth by 53%, and at 1 μM WT, inhibition was 93% (Fig. 5). Neurites that grew out in the presence of the toxin appeared to be shorter and less abundant. Dark-field and phase photomicrographs of representative cultures are shown in Fig. 6.

DISCUSSION

The inhibition of goldfish retina PI3K by WT permitted us to assess the involvement of this key enzyme in nerve regeneration by studying neurite outgrowth in retinal explants that had been activated by prior crush of the optic nerve in vivo. An advantage of using selective interventive agents such as WT is that one may directly address, in a complex biological tissue, the question of whether the metabolic step that has been blocked plays a critical biological role. Thus, in the present experiments, we show that WT blocks one or more essential steps in neurite outgrowth associated with optic nerve regeneration in the goldfish, a conclusion that could not have been drawn from measurement of the amounts of retinal PI3K in control and postcrush retinas. It is not yet clear whether the inferred critical step(s) occurs in the RGC body, or locally, at the site of elongation. At concentrations up to 100 nM, WT does not directly inhibit many enzyme activities thus so far investigated, including PI4K, tyrosine kinases and protein kinase C (11), myosin light-chain kinase or protein kinase A (26), many of which are induced in nervous system development, and might similarly be induced in neurite outgrowth during regeneration. Potent effects of WT on phospholipase A_2 (PLA_2) have been shown in bombesin-stimulated Swiss 3T3 cells (27), but it has not yet been demonstrated whether specific PLA_2 blockers inhibit neurite outgrowth in these cells or whether WT blocks PLA_2 in nervous system-derived cells. In serum-starved NS-20Y neuroblastoma cells, calmodulin-blocking agents prevent neurite outgrowth, while WT and the protein tyrosine kinase blocker genestein are ineffective (28). Of possibly greater relevance to the present studies are those of Kimura et al. (3) who found that WT blocked neurite outgrowth from PC12 cells. They further learned that WT must be replenished, since it is degraded in the cell culture with a half life of about 7 hours at 37.5°C. This corresponds to 22 h at 21°C, assuming a Q_{10} of 2 and could account for the observed effectiveness of daily application of WT in the present experiments. The question of the specificity of WT action was also addressed in these cells in a study in which NGF-stimulated neurite outgrowth in PC12 cells was blocked by overexpression of a defective p85 regulatory subunit of PI3K (29).

Assuming in the present instance, that WT does indeed act by blocking PI3K, and given the diverse involvements of the downstream products of PI3K, many additional possibilities for its site of action can be envisioned. PI3K was earlier found to have homology with $pp60^{c\text{-}src}$ (3) and a number of other protooncogenes have since been reported to change in the goldfish visual system during regeneration (30,31). $pp60^{c\text{-}src}$ expression is increased in the developing chick visual system (32) as well as in regenerating rat peripheral nerve (33). It is alternatively possible that WT blocks neurite outgrowth at or near the site of elongation. Of possible relevance, $pp60^{c\text{-}src}$ has been localized to growth cones of PC12 cells (34). The possible biochemical site(s) of WT action on neurite elongation is not presently known. PI3K has been shown to bind to tubulin (35), and also appears to be involved in membrane ruffling, known to occur at the growth cone (7). Growth cone extension appears to involve actin treadmilling and is modified by gelsolin and profilin (36), proteins that are regulated by $PI(4,5)P_2$. The outgrowth of neurites may then involve a balance of two regulatory pathways, the aforementioned PI3P pathway and its end products: $PI(3,4)P_2$ and $PI(3,4,5)P_3$, as well as the product of the PI4K pathway: $PI(4,5)P_2$. These two pathways diverge from the progenitor lipid PI, and possible regulatory interactions have been proposed (37,38).

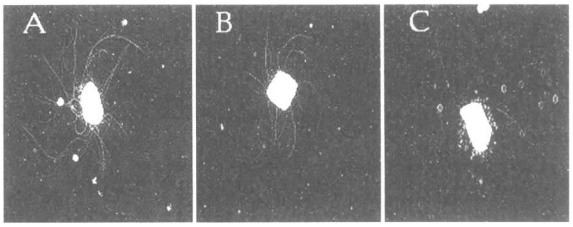

Fig. 6. The effect of WT on neurite outgrowth from 500 × 500 μM full thickness goldfish retinal explants. Digitized video images of dark-field microscopy of 7 day retinal explants are shown: A, DMSO control; B, 10^{-7} M WT; C, 10^{-6} M WT. Media were changed daily. The NGI and CNGI for these explants were, respectively, A, 4.0 and 6776 (pixels); B, 2.5 and 3865; C, 0.25 and 1052. For the entire groups (N = 16), the values (mean ± S.E.) were: A, 2.97 ± 0.063 and 7416 ± 970; B, 1.41 ± 0.41 and 3739 ± 939; C, 0.20 ± 0.133 and 1058 ± 804.

The explant preparation used in the present study as well as in vivo approaches to optic nerve regeneration in the goldfish will answer a number questions. For example, it will be of interest to ascertain whether PI3K synthesis is enhanced following nerve crush, and also whether the enzyme is rapidly or slowly transported in the newly regenerated nerve, since membranes are rapidly transported, while the cytoskeleton is slowly transported (39).

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REFERENCES

1. Parker, P. J., and Waterfield, M. D. 1992. Phosphatidylinositol 3-kinase: a novel effector. Cell Growth and Differentiation 3:747–752.
2. Stephens, L. 1995. Molecules mediating signals. Biochem. Soc. Trans. 23:207–221.
3. Kimura, K., Hattori, S., Kabuyama, Y., Shizawa, Y., Takayanagi, J., Nakamura, S., Toki, S., Matsuda, Y., Onodera, K., and Fukui, Y. 1994. Neurite outgrowth of PC12 cells is suppressed by wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase. J. Biol. Chem. 269:18961–18967.
4. Varticovski, L., Harrison-Findik, D., Keeler, M. L., and Susa, M. 1994. Role of PI 3-kinase in mitogenesis. Biochim. et Biophys. Acta 1226:1–11.
5. Cheatham, B., and Kahn, C. R. 1995. Insulin action and insulin signaling network. Endocrine Reviews 16:117–142.
6. Kotani, K., Hara, K., Kotani, K., Yonezawa, K., and Kasuga, M. 1995. Phosphoinositide 3-kinase as an upstream regulator of the small GTP-binding protein Rac in the insulin signaling of membrane ruffling. Biochem. Biophys. Res. Commun. 208:985–990.
7. Ridley, A. J. 1994. Membrane ruffling and signal transduction. BioEssays 16:321–327.
8. Kaplan, D. R., Whitman, M., Schafthausen, B., Pallas, D. C., White, M., Cantley, L., and Roberts, T. M. 1987. Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity. Cell 50:1021–1029.
9. Carpenter, C. L., Duckworth, B., Auger, K. R., Cohen, B., Schaffhausen, B. S., and Cantley, L. C. 1990. Purification and characterization of phosphatidylinositol 3-kinase from rat liver. J. Biol. Chem. 265:19704–19711.
10. Morgan, S. J., Smith, A. D., and Parker, P. J. 1990. Purification and characterization of bovine brain type I phosphatidylinositol kinase. Eur. J. Biochem. 191:761–767.
11. Powis, G., Bonjouklian, R., Berggren, M., Gallegos, A., Abraham, R., Ashendel, C., Zalkow, L., Matter, W. F., Dodge, J., Grindley, G., and Vlahos, C. J. 1994. Wortmannin, a potent and selective inhibitor of phosphatidylinositol 3-kinase. Cancer Res. 54:2419–2423.
12. Grafstein, B. 1991. The goldfish visual system as a model for the study of regeneration in the central nervous system. Pages 190–205 *in* Development and Plasticity of the Visual System Cronly-Dillon, J.R., (ed.), Vol. II. Macmillan, London.
13. Murray, M., and Grafstein, B. 1969. Changes in the morphology and amino acid incorporation of regenerating goldfish optic neurons. Exper. Neurol. 23:544–560.
14. Heacock, A. M., and Agranoff, B. W. 1982. Protein synthesis and transport in the regenerating goldfish visual system. Neurochem. Res. 7:771–788.
15. Schwartz, M., Belkin, M., Harel, A., Solomon, A., Lavie, V., Hadani, M., Rachailovich, I., and Stein-Izsak, C. 1985. Regenerating fish optic nerves and a regeneration-like response in injured optic nerves of adult rabbits. Science 228:600–603.
16. Finklestein, S. P., Benowitz, L. E., Olson, A. J., Perrone-Bizzozero, N. I., Majocha, R. E., and Apostolides, P. J. 1987. Conditioned media from the injured lower vertebrate CNS promote neurite outgrowth from mammalian brain neurons in vitro. Brain Res. 413:267–273.
17. Schwalb, J. M., Boulis, N. M., Gu, M.-F., Winickoff, J., Jackson, P. S., Irwin, N., and Benowitz, L. I. 1995. Two factors secreted by the goldfish optic nerve induce retinal ganglion cells to regenerate axons in culture. J. Neurosci. 15:5514–5525.
18. Heacock, A. M., and Agranoff, B. W. 1976. Enhanced labeling of a retinal protein during regeneration of optic nerve in goldfish. Proc. Nat. Acad. Sci. U.S.A., 73, 828–832.
19. Kappeller, R., Toker, A., Cantley, L. C., and Carpenter, L. 1995. Phosphoinositide 3-kinase binds constitutively to α/β-tubulin and binds to γ-tubulin in response to insulin. J. Biol. Chem. 270: 25985–25991.
20. Landreth, G. E., and Agranoff, B. W. 1979. Explant culture of adult goldfish retina: a model for the study of CNS regeneration. Brain Res. 161:39–53.
21. Landreth, G. E., and Agranoff, B. W. 1976. Explant culture of adult goldfish retina: effect of prior optic nerve crush. Brain Res. 118:299–303.
22. Serunian, L. A., Auger, K. R. and Cantley, L. C. 1991. Identification and quantification of polyphosphoinositides produced in response to PDGF stimulation. Methods Enzymol. 198:811–816.
23. Ford-Holevinski, T. S., Dahlberg, T. A., and Agranoff, B. W. 1986. A microcomputer-based image analyzer for quantitating neurite outgrowth, Brain Res. 368:339–346.
24. Berggren, M. I., Gallegos, A., Dressler, L. A., Modest, E. J., and Powis, G. 1993. Inhibition of the signalling enzyme phosphatidylinositol 3-kinase by antitumor ether lipid analogues. Cancer Res. 53:4297–4302.
25. Carpenter, C. L., and Cantley, L. C. 1990. Phosphoinositide kinases. Biochemistry. 29:11147–11156.
26. Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., and Matsuda, Y. 1992. Wortmannin, a microbial product inhibitor of myosin light chain kinase. J. Biol. Chem. 267:2157–2163.
27. Cross, M. J., Stewart, A., Hodgkin, M. N., Kerr, D. J., and Wakelam, M. J. H. O. 1995. Wortmannin and its structural analogue dimethoxyviridin inhibit stimulated phospholipase A_2 activity in Swiss 3T3 cells. J. Biol. Chem. 270:25352–25355.
28. Uemera, K., and Taketomi, T. 1995. Inhibition of neurite outgrowth in murine neuroblastoma NS-20Y cells by calmodulin inhibitors. J. Biochem. 118:371–375.
29. Jackson, T. R., Blader, I. J., Hammonds-Odie, L. P., Burga, C. R., Cooke, F., Hawkins, P. T., Wolf, A. G., Heldman, K. A., and Theibert, A. B. 1996. Initiation and maintenance of NGF-stimulated neurite outgrowth requires activation of a phosphoinositide 3-kinase. Cell Science 109:289–300.
30. Stein-Izsak, C., Cohen, I., and Schwartz, M. 1987. Expression of the proto-oncogenes *fos* and *myc* and optic nerve regeneration. Neurosci. Res. Comm. 1:87–95.
31. Herdegen, T., Bastmeyer, Bahr, M., Stuermer, C., Bravo, R., and Zimmermann, M. 1993. Expression of JUN, KROX, and CREB transcription factors in goldfish and rat retinal ganglion cells following optic nerve lesion is related to axonal sprouting. J. Neurobiol. 24:528–543.
32. Sorge, L. K., Levy, B. T., and Maness, P. F. 1984 $pp60^{c\text{-}src}$ is developmentally regulated in the neural retina. Cell. 38:249–257.
33. LeBeau, J. M., Tedeschi, B., and Walter, G. 1991. Increased expression of $pp60^{c\text{-}src}$ protein-tyrosine kinase during peripheral nerve regeneration. J. Neurosci Res. 28:299–309.
34. Sobue, K., and Kanda, K. 1988. Localization of $pp60^{c\text{-}src}$ in growth cone of PC12 cell. Biochem. Biophys. Res. Comm. 157:1383–1389.
35. Kapeller, R., and Cantley, L. C. 1994. Phosphatidylinositol 3-kinase. BioEssays 16:565–576.
36. Janmey, P. A., Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. 1994. Ann Rev. Physiol. 56:169–191.
37. Lavie, Y., and Agranoff, B. W. 1996. Inhibition of neuroblastoma cell phosphatidylinositol 3-kinase by CDP-diacylglycerol and phosphatidate. J. Neurochem. 66:811–816.
38. Lavie, Y., and Agranoff, B. W. 1996. Carbachol inhibits insulin-stimulated phosphatidylinositol 3-kinase activity in SH-SY5Y Neuroblastoma cells. J. Neurochem. 1996. 62:1245–1251.
39. Hammerschlag, R., Cyr, J. L., and Brady, S. T. 1994. Axonal transport and the neuronal cytoskeleton. Pages 545–571, *in* Basic Neurochemistry Siegel, G. J., et al. (eds.), 5th edition, Raven Press, New York.