Multiple signaling pathways regulate FGF-2-induced retinal ganglion cell neurite extension and growth cone guidance

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Introduction

Growth cones use cues in their environment in order to grow in a directed fashion to their targets. In *Xenopus laevis*, fibroblast growth factors (FGFs) participate in retinal ganglion cell (RGC) axon guidance in vivo and in vitro. The main intracellular signaling cascades known to act downstream of the FGF receptor include the mitogen-activated protein kinase (MAPK), phospholipase Cγ (PLCγ) and phosphotidylinositol 3-kinase (PI3K) pathways. We used pharmacological inhibitors to identify the signaling cascade(s) responsible for FGF-2-stimulated RGC axon extension and chemorepulsion. The MAPK, PI3K and PLCγ pathways were blocked by U0126, LY249002 and U73122, respectively. D609 was used to test a role for the phosphotidylcholine–PLC pathway. We determined that the MAPK and two PLC pathways are required for FGF-2 to stimulate RGC neurite extension in vitro, but the response of axons to FGF-2 applied asymmetrically to the growth cone depended only on the PLC pathways.

Results

The MAPK and PC–PLC Pathways are necessary for optic tract extension in vivo

To investigate this we used pharmacological inhibitors to block FGFR signaling pathways in in vitro and in vivo preparations designed to examine axon extension and guidance. Specifically, the MAPK, PLCγ and PI3K pathways, as well as a novel pathway downstream of the FGFR, the phosphotidylinositol phospholipase C (PC–PLC) pathway, were targeted. PI3K appeared unnecessary for RGC axon extension, but the other three pathways acted in a convergent fashion to mediate FGF-stimulated axon extension in vitro. In contrast, only the PLC pathways were required for the turning response of growth cones to an FGF-2 gradient.
33/34, the optic tract has crossed the optic chiasm to reach the contralateral forebrain. At this stage, the skin and dura from this side of the brain were removed and the appropriate inhibitors were added to the bathing media. Since the optic tract grows close to the pial surface, the axons were exposed to the inhibitors as they extended through the diencephalon to the optic tectum. RGC axons were anterogradely labeled with HRP at stage 40, when the optic tract has normally reached its target, and the brains were processed subsequently as whole-mounts with DAB. Using this assay system, we tested whether the MAPK, PC–PLC or PI3K pathways are needed for RGC axon extension and/or pathfinding. The PLCγ pathway was not examined because it is already known to be required for the extension of Xenopus RGC axons in vivo (Lom et al., 1998).

The optic tract developed normally when exposed to control medium (Fig. 1A), or medium treated with 12.5 or 25 μM of the PI3K inhibitor, LY294002 (Figs. 1B and 2A). These data argue that PI3K activity is not required for Xenopus RGC axon extension in vivo, given that comparable concentrations of LY294002 were effective in studies of Xenopus embryos and cultured cells (Carballada et al., 2001; Liou et al., 2003; Peng et al., 2004). To confirm that the PI3K pathway was indeed inhibited in vivo, whole brains exposed on one side to 25 μM LY294002 were dissected and processed for Western blot analysis with an antibody against the phosphorylated and active form of PI3K pathway downstream effector, Akt (pAkt) (Andersson et al., 2003; Atwal et al., 2000; Finkielstein et al., 2001; Zhou et al., 2004) (Fig. 2E). pAkt was notably reduced in the LY294002 brains, whereas levels of the di-phosphorylated and active form of extracellular signal-related kinase MAPK (ppERK) showed little change (Atwal et al., 2000; Carballada et al., 2001; Forcet et al., 2002; Nutt et al., 2001; Schohl and Fagotto, 2002). It should be noted that because Westerns were performed on whole brains, while only one side of the brain was exposed to the inhibitors, we did not expect to completely block the production of pAkt. Thus, LY294002 produced significant inhibition of the PI3K pathway, with no obvious effect on axon extension, which argues that this pathway is not important for formation of the optic projection in vivo.

In the presence of 100 μM of the MAPK inhibitor, U0126, there was a significant decrease in optic tract length compared to control embryos (Figs. 1C–D and 2B). The amount of tract shortening was comparable to that observed previously with inhibition of the PLCγ pathway (Lom et al., 1998). Importantly, while 100 μM U0126 reduced effectively the levels of ppERK by Western analysis (Deacon et al., 2005; Nutt et al., 2001) (Fig. 2F), no similar reduction in pAkt levels was observed, arguing for specificity of U0126 actions on the MAPK pathway. In agreement with this, a second MAPK pathway inhibitor, PD98059, caused axon extension defects similar to those observed with U0126 (Figs. 1E and 2C). In culture, lower concentrations of U0126 are generally effective (Campbell and
Holt, 2003; Schmid et al., 2000). Presumably, the reason 100 μM U0126 was needed to produce defects in optic tract formation is that the growth cones extended 2–10 μm below the pial brain surface and actually saw a lower concentration of U0126. To ensure that high U0126 concentrations did not cause excessive cell death of brain neuroepithelial cells, we incubated brains exposed to U0126 between stages 33/34 and 40 with the vital dye trypan blue. On average, 26.7 ± 3.35 (n = 24) surface neuroepithelial cells in the entire forebrain and midbrain were unable to exclude trypan blue, only slightly higher than the numbers of trypan-blue-positive cell observed in control (8.86 ± 1.46, n = 21, P < 0.001, unpaired two-tailed Student’s t test). This small number of dead cells would be unlikely to have contributed to the RGC axon extension defects observed with 100 μM U0126.

As part of a screen to identify a role for other intracellular signaling pathways that may act downstream of the FGFR, we used a PC–PLC inhibitor, D609, in the exposed brain preparation. The PC–PLC pathway was interesting to us in that it had been implicated downstream of several growth factor-
mediated processes (Blanquet and Lamour, 1997; Cai et al., 1992; van Dijk et al., 1997), including FGF-2-dependent nitric oxide production in CHO-K1 cells (Arena et al., 2002). PC–PLC hydrolyzes phosphatidylycholine (PC), a major membrane phospholipid required for axonal growth, to generate diacylglycerol (DAG) and ultimately ceramide (Araki and Wurtman, 1997; Lozanski et al., 1997; Vance et al., 1991, 1995). While D609 is also known to inhibit phospholipase D and sphingomyelinase synthase, it does so at concentrations considerably higher than those used below (Kiss and Tomono, 1995; Luberto and Hannun, 1998; van Dijk et al., 1997). Brains exposed to 9.5 μM and 19 μM of D609 (Lozanski et al., 1997) exhibited significant optic tract extension defects (Figs. 1F and 2D). These data suggest that the MAPK and PC–PLC pathways, in addition to the PLCγ pathway (Lom et al., 1998), are required for *Xenopus* RGC axon extension within the diencephalon.

It was possible that the extension defects caused by the inhibitors were due not to their direct actions on RGC growth cones but either to the inhibitor being toxic to RGCs and/or inhibitor-induced changes in the brain neuroepithelium through which the RGC axons extended. Retinal sections of stage 40 embryos treated at stage 33/34 with either D609 or U0126 in the exposed brain preparation were immunolabeled with an antibody against caspase-3, an early effector of apoptosis (Campbell and Holt, 2003; Van Stry et al., 2004). Inhibitor treatment of the RGC axons did not increase the number of caspase-3-immunopositive cells in the RGC layer (control U0126 x = 6.2 ± 0.6 (SEM), n = 11 embryos; 100 μM U0126 x = 3.8 ± 0.6, n = 14; control D609 x = 5.9 ± 1, n = 11; 9.5 μM D609 x = 6.6 ± 1.1, n = 5). Further, brains exposed to the MAPK and PC–PLC inhibitors looked morphologically similar to control (see Figs. 1 and 3), and a number of neuroepithelial markers were expressed normally in the inhibitor-treated brains (Fig. 3). First, the polarity of the neuroepithelium appeared normal, as brains exposed to D609 and U0126 exhibited the expected islet-1-expressing ventral population of neurons in the forebrain (Figs. 3A–C). Moreover, immunolabeling with antibodies against general neuronal

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**Fig. 3.** The MAPK and PC–PLC pharmacological inhibitors do not affect gross brain morphology or patterning. Twelve-micrometer cross-sections of the diencephalon of stage 40 embryos. The left side of the brains were exposed at stage 33/34 to either control media (A, D, G), 9.5 μM D609 (B, E, H) or 100 μM U0126 (C, F, I). At stage 40, the embryos were cryostat sectioned and processed for immunocytochemistry with neuroepithelial markers. Representative images are shown for the transcription factor, islet-1 (A–C), and general neuronal markers, Zn-12 (D–F) and NCAM (G–I). Islet-1 is expressed in a bilateral population of cells in the ventral diencephalon (arrows) and in the retina. No gross alterations in the expression of the various antigens were observed either in control or inhibitor-treated brains. Ex, exposed side of brain; UnEx, unexposed side of brain; Ve, ventricle; np, neuropil; Di, diencephalon; R, retina; D, dorsal; V, ventral. Scale bar in panel A is 25 μm.
markers, NCAM and Zn-12, was similar between control and inhibitor-treated brains (Figs. 3D–I). Thus, these inhibitors do not obviously alter the gross morphology or patterning of the developing *Xenopus* brain, which lends support to the idea that the inhibitor-induced defects in RGC axon extension were due to direct actions on the growth cones. In addition, they argue that the U0126 and D609 concentrations we used were not toxic to *Xenopus* RGCs and neuroepithelial cells.

The MAPK, PC–PLC and PLC-γ signaling pathways mediate RGC axon extension in vitro

In the exposed brain experiments, the pharmacological inhibitors would have had access to the RGC growth cones and the neuroepithelial cells over which they extend. To determine if the pharmacological inhibitors directly affected RGC growth cones, in vitro axon extension and guidance assays were used on RGC axons growing on a laminin substrate. These experiments were also necessary to address whether or not the different signal transduction pathways acted downstream of FGF signaling, because in vivo the inhibitors could have affected signaling downstream of several growth and guidance cues. We used drug concentrations that are commonly used in the literature, and likely to be specific for their intended targets.

Previously, we showed that FGF-2 stimulated RGC neurite extension in vitro (McFarlane et al., 1995; Webber et al., 2003). To identify the intracellular signal transduction pathway(s) involved, different inhibitors were applied to stage 24 dissociated retinal cultures treated with or without 20 ng/ml human recombinant FGF-2. In addition to the MAPK and PC–PLC pathway inhibitors, a PLC-γ inhibitor, U73122, was used at concentrations used previously in *Xenopus* neuron cultures (Du and Poo, 2004; Liou et al., 2003; Xiang et al., 2002). Importantly, studies of rat macrophage and cat smooth muscle cells show that the D609 and U73122 inhibitors are specific for the PC–PLC and PLC-γ pathways, respectively, and have different effects on these cells (Giron-Calle et al., 2002; Sohn et al., 1997). U73122 directly inhibits PLC-γ, in contrast to the DAG lipase inhibitor that had previously been used to show a role for the PLC-γ pathway in *Xenopus* RGC axon extension (Lom et al., 1998). To address a role for PLC-γ in FGF-mediated chemorepulsion (see below), we needed to first confirm that U73122 produced a similar RGC axon extension phenotype as previously reported for DAG lipase inhibition (Lom et al., 1998). This was not possible in vivo, as we found U73122 was toxic to *Xenopus* embryos at concentrations reported to be effective in vitro, so instead U73122 was tested for its ability to inhibit FGF-2-stimulated neurite extension in vitro. The PI3K inhibitor was not examined in the in vitro studies, because it had no effect on axon extension or guidance in vivo, despite significantly inhibiting phosphorylation of the downstream effector Akt (Figs. 1, 2A and 2E).

Control or FGF-2-treated dissociated retinal cultures were grown in the presence of the MAPK, PC–PLC or PLC-γ inhibitor for 18–24 h. The longest uninterrupted neurite of RGCs, identified by morphology and an antibody against neurofilament-associated antigen, was measured. As demonstrated previously, FGF-2-treated RGC neurites were significantly longer than those measured in control cultures (McFarlane et al., 1995; Webber et al., 2003) (Fig. 4). A dose response was performed (data not shown) to first establish a concentration for each drug which itself did not affect basal outgrowth on a laminin substrate (Fig. 4A, 5 μM U1026; Fig. 4B, 1.9 μM U73122)
D609; Fig. 4C, 5 μM U73122). Yet, at these concentrations all three inhibitors abolished the ability of FGF-2 to stimulate RGC neurite extension. Since each signaling cascade was required for FGF-2 to stimulate neurite outgrowth, these pathways may converge to regulate axon extension in vitro.

**FGF-2-induced repulsion of RGC growth cones acts through the PC–PLC, PLCγ and MAPK signaling pathways**

Previously, we used an in vitro growth cone turning assay to show that FGF-2 directly repels *Xenopus* RGC growth cones in vitro (Webber et al., 2003). This assay was used here to investigate which signaling pathway(s) mediates this response. Stage 24 eye primordia were cultured for 18–24 h in control media before the growth cone turning assay was performed on individual growth cones that had extended from the explant. Left to develop in situ for this same time period, these RGC axons would have been extending through the diencephalon towards the optic tectum (Holt, 1989). Growth cones were first recorded by using time-lapse videomicroscopy for 30 min. Subsequently, control medium with or without one of the inhibitors was added to the dish, and an FGF-2 gradient was established from a pipette placed 100 μm distant and at a 45° angle to the extending growth cone. The growth cone trajectory was then recorded over a 45-min period. The control bathing solution contained DMSO at the concentration used to solubilize the inhibitor being tested. Experimental solutions consisted of the control media supplemented with either 10 μM U0126, 9.5 μM D609 or 2.5 μM U73122 to inhibit the MAPK, PC–PLC and PLCγ pathways, respectively. None of the inhibitors themselves altered the trajectory of RGC growth cones when a control solution was present in the pipette (n > 5 for each inhibitor, data not shown).

As shown previously, 50–70% of the RGC growth cones in control medium were repelled by the pipette containing FGF-2 (Figs. 5A–B, C, E and 6A) (Webber et al., 2003). However, the growth cones did not repel from the FGF-2 source when either the PC–PLC or PLCγ inhibitors were present in the bathing solution (Figs. 5D and F). The mean turning angles for the PC–PLC (control, θ = −9.88° ± 5.2 (SEM), n = 12; D609, θ = 2.09° ± 1.9 (SEM), n = 11) and PLCγ (control, θ = −18.86 ± 6.97 (SEM), n = 14; U73122, θ = 0.29° ± 5.2 (SEM), n = 11) inhibitor baths were significantly different (P < 0.05, unpaired two-tailed Student’s t test) than those measured in their respective control bath. In contrast, the MAPK inhibitor, U0126, had no effect on the FGF-2-induced chemorepulsion at a concentration (10 μM) that we showed blocked FGF-2-stimulated neurite outgrowth (U0126, θ = −19.25 ± 9.52 (SEM) n = 8; control, θ = −15.40 ± 6.21 (SEM) n = 10; P > 0.05, unpaired two-tailed Student’s t test) (Figs. 6A–B). It has been reported that a concentration of 50 μM U0126 is required to block FGF-2-stimulated activation of the MAPK pathway in cultured rat lens epithelial cells and the induction of the MAPK pathway in *Xenopus* oocytes (Lovicu and McAvoy, 2001; Watanabe et al., 2003). To address directly whether 10 μM U0126 inhibited effectively the MAPK pathway in *Xenopus* RGCs, 1-day-old stage 24 dissociated retinal cultures were pre-incubated with 10 μM U0126 prior to the addition of 20 ng/ml FGF-2 for 1 h, and then immunostained with an antibody against ppERK (Campbell and Holt, 2003; Schohl and Fagotto, 2002). A significant number of RGCs were ppERK positive in cultures that saw FGF-2 alone (52.9% of RGCs had immunofluorescence intensity significantly above background yolk fluorescence, n = 70), but not in cultures that were pre-treated with 10 μM U0126 (2.5%, n = 40) (Fig. 7). These data suggest that the MAPK pathway is largely blocked by 10 μM U0126 and is not
signaling cascades converge on a common mediator of RGC axon extension and guidance in vitro. Extension in vivo requires activation of the MAPK, PC–PLC and PLCγ pathways, but possibly not the PI3K pathway. In vitro, our results suggest that the MAPK, PC–PLC and PLCγ pathways converge to mediate an FGF-2-induced increase in RGC neurite extension, but that only the PLC pathways are required for FGF-2-mediated RGC growth cone repulsion. Collectively, these data argue that the MAPK pathway is only necessary for FGF-2-dependent axon extension and not chemorepulsion. Similarly, distinct signaling pathways are involved in the various actions of FGF-2 on cultured hippocampal neurons (Katsuki et al., 2000).

Axon extension is thought generally to depend on microtubules, while guidance depends on the actin cytoskeleton within the filopodia (Dent and Gertler, 2003; Henley and Poo, 2004). Together the PC–PLC, PLCγ and p42/44 MAPK pathways likely regulate microtubule polymerization downstream of bath-applied FGF-2. Since the PLC pathways are required for FGF-2 repulsion of RGC growth cones, the normal actions of these pathways likely result ultimately in actin depolymerization on the side of the growth cone closest to the FGF-2. Inhibition of either PLC pathway would be expected to only partially block FGF-2-stimulated axon outgrowth. For instance, blockade of any one of three signal transduction pathways only partially impaired nerve growth factor’s (NGF) capacity to induce neurite outgrowth from pheochromocytoma (PC12) cells (Inagaki et al., 1995). However, we found each inhibitor completely abolished the FGF-2 effect on neurite extension, without affecting baseline extension on laminin. Convergence of the pathways could occur at the level of the components of the signaling cascades. For example, both the PC–PLC and PLCγ pathways can work through the production of DAG, and growth factor activation of PC–PLC can result in activation of the MAPK pathway (Cai et al., 1993; Nofer et al., 1997; van Dijk et al., 1997). Alternatively, convergence may occur further downstream, for instance at the level of the Rho family of small guanosine triphosphatases (GTPases), which regulate the actin cytoskeleton (Dickson, 2001; Huber et al., 2003). Indeed, the Rho family member Rac1 is an important mediator of *Xenopus* RGC axon outgrowth in vivo (Ruchhoeft et al., 1999), and is known to be activated downstream of FGF in non-neuronal cells (Bleloch et al., 1999; Maddala et al., 2003).

Interestingly, not all the pathways that mediated FGF-2-stimulated axon extension were required for FGF-2-dependent growth cone turning. Inhibiting either the PC–PLC or the PLCγ pathway caused the growth cone to ignore FGF-2, indicating that both pathways needed to be active. In contrast, the p42/44 MAPK pathway inhibitor, U0126, had no effect on FGF-2-mediated repulsion. Given that ppERK levels were dramatically decreased in cultures treated acutely with the same concentration of U0126, it argues that the MAPK pathway is only necessary for FGF-2-dependent axon extension and not chemorepulsion. Similarly, distinct signaling pathways are involved in the various actions of FGF-2 on cultured hippocampal neurons (Katsuki et al., 2000).
observed in vivo when *Xenopus* pathways. Nonetheless, similar axon extension defects were observed in vivo when *Xenopus* axons expressed a dominant-negative FGFR (McFarlane et al., 1996). Interestingly, axon pathfinding within the diencephalon was not obviously affected by the MAPK, PLCγ and PC–PLC pathway inhibitors. The fact that multiple cues likely mediate each guidance decision made by an axon provides a possible explanation for this observation. If the various guidance cues act through different intracellular signaling cascades, the use of a single pathway inhibitor would eliminate some but not all of the guidance information required by an axon. We could address this possibility by simultaneously inhibiting two of the signaling pathways, which may then result in axon guidance defects. Axon pathfinding would be difficult to study, however, if more severe axon extension defects occurred. A molecular approach, involving trkA receptor mutants unable to activate different signaling pathways, was used successfully to address the role of the different pathways in NGF-mediated neurite outgrowth and axon guidance in vitro (Inagaki et al., 1995; Ming et al., 1999). In the future, a similar approach, involving transfection of RGCs with pathway-specific FGFR mutants, could be used to directly address the role of the different signal transduction mechanisms in vivo (Cross et al., 2000; McFarlane et al., 1996; Mohammadi et al., 1992; Peters et al., 1992).

We identified the PC–PLC pathway as one of several transduction mechanisms that underlie FGFR signaling in RGC growth cones. PC hydrolysis by PC–PLC leads to the activation of protein kinase C, which in turn results in the production of various other signaling molecules, including DAG and ceramide (Zamorano et al., 2003). The literature suggests that FGFs can either activate or inhibit PC–PLC signaling depending upon the biological context. For instance, FGF-2 activated PC–PLC to stimulate nitric oxide production in CHO-K1 cells (Arena et al., 2002), whereas FGF removal from the culture media of vascular endothelial cells led to PC–PLC activation (Du et al., 2003; Miao et al., 1997). An alternative possibility is that PC–PLC regulates FGF-2-stimulated RGC extension in an FGFR-independent fashion through the shared PC–PLC and PLCγ pathway constituent, DAG (Klint and Claesson-Welsh, 1999; Monick et al., 1999). In this case, D609 inhibition of active PC–PLC would lower growth cone DAG levels and impact FGFR-mediated signaling through the PLCγ pathway. Indeed, D609 and U73122 had similar effects on axon extension and guidance in vitro. While PC–PLC has also been shown to act upstream of activation of the MAPK pathway in growth factor signaling (Cai et al., 1993; van Dijk et al., 1997), it is unlikely that this is the case in *Xenopus* RGC growth cones in that the MAPK and PC–PLC inhibitors had distinct effects on growth cone turning in response to FGF-2. While it remains to be determined by which means PC–PLC is involved in RGC growth cone FGF signaling, the fact that the two PLC inhibitors had similar effects in all of the assays argues that they act through a common mechanism, such as inhibition of DAG (Cai et al., 1993).

The present study took a pharmacological approach to investigate intracellular signaling downstream of the FGFR in RGC growth cones. To conclusively demonstrate the involvement of the different signaling pathways in FGF-dependent RGC axon extension and guidance in vivo, however, genetic and molecular approaches specific for FGFR signaling in RGCs will need to be used in the future (Chang et al., 2004; Cross et al., 2000; Kanazawa et al., 2002; Kim et al., 2004).

**Experimental procedures**

**Animals**

Eggs obtained from adult female *Xenopus laevis* injected with human chorionic gonadotrophin (Intervet) were fertilized in vitro to generate embryos. Embryos were kept in 0.1× Marc’s modified ringer’s solution (MMR; 0.1 M NaCl, 2 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM HEPES, pH 7.5), with the temperature varying between 14°C and 27°C to control their speed of development. Embryos were staged according to Nieuwkoop and Faber (1994).

**Retinal cultures**

Eye primordia were dissected from stage 24 *Xenopus* embryos. For neurite measurements eye primordia were plated as dissociated cells on poly-L-ornithine/laminin (10 μg/ml; Sigma)-coated coverslips (as per Harris and Messersmith, 1992). For the growth cone turning assay, eye primordia were grown as explants on 50 μM fibronectin (Roche). Culture media consisted of 60% L-15-glutamine (Invitrogen) and 0.01% bovine serum albumin (BSA; Sigma). Several different pharmacological inhibitors were added to dissociated cultures grown either in the presence or absence of 20 ng/ml human recombinant FGF-2 (Invitrogen), including 5–10 μM of the MAPK pathway inhibitor, U0126 (1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene; Sigma); 1.9–19 μM of the PC–PLC inhibitor, D609 (tricyclodecan-9-yl-xanthate; Calbiochem); and 0.5–10 μM of the PLCγ inhibitor, U73122 (1-[6-((17B-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; Calbiochem). Control solutions contained the same concentration of dimethyl sulfoxide (DMSO) as the highest dose of pharmacological inhibitor being tested.

**Exposed brain preparation**

The exposed brain preparation was performed as described previously (Chien et al., 1993; Webber et al., 2002). Briefly, embryos were anesthetized in modified Barth’s saline (MBS) supplemented with 0.4 mg/ml tricaine, 50 mg/ml gentamicin sulfate (Sigma) and 10 mg/ml Phenol Red (Sigma). The embryos were pinned in a Sylgard dish (Dow Corning), and the skin and dura covering the left side of the brain, as far caudal as the posterior optic tectum, were removed. To determine which intracellular signaling cascade(s) was important for optic tract development, different inhibitors were added to the control MBS solution: 50 μM and 100 μM U0126; 0.4–19 μM D609; and 12.5 μM and 25 μM PI3K inhibitor, LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Calbiochem). The control brains were treated with the same DMSO concentration as the highest dose of pharmacological inhibitor being tested. For the 100-μM U0126 experiments, a trypan blue exclusion assay was used to label dead cells.
cells. After exposure of the optic projection for 20 h, stage 40 embryos were incubated in the vital dye trypan blue (0.4%, diluted in MBS). Embryos were rinsed well, fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) and brains dissected. Blue cells, that had failed to exclude trypan blue, were counted within the pial surface of the forebrain, midbrain and hindbrain in whole-mount preparations.

Visualization of the optic projection

To visualize the optic projection, RGC axons were anterogradely labeled using horseradish peroxidase (HRP, type IV; Sigma) as described previously (Cornell and Holt, 1992). Briefly, at stage 40 the lens of the right eye was surgically removed and HRP, dissolved in 1% lyssolecithin (Sigma), was placed in the eye cavity to restrict labeling to RGC axons. After allowing time for labeling, embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS. The HRP-labeled optic tract was visualized by reacting with diaminobenzidine (DAB; Sigma). Subsequently, the brains were dehydrated through a graded series of alcohol and cleared in 2:1 benzyl benzoate–benzyl alcohol. A camera lucida attachment on a Zeiss microscope was used to draw the outlines of brains and their labeled optic tracts. The length of the optic tract was measured by using macro software in NIH Image (Chien et al., 1993). Samples were processed statistically by using an ANOVA, followed by a Dunnett’s post hoc test (InStat 2.0).

Immunocytochemistry

Fixed embryos were quick-frozen at −20°C in optimal cutting temperature (OCT) compound (Baxter), and 12 μm transverse cryostat sections through the embryonic diencephalon and midbrain were cut. Samples were incubated overnight at 4°C in primary antibody diluted in PBT (PBS, 0.1% BSA, 0.5% Triton (BDH)) with 5% goat serum (Invitrogen). Rhodamine Red X (RRX) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.; 1:50) were applied for 1 h at room temperature. (RRX) secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.; 1:50) were applied for 1 h at room temperature.

Cultures were pre-treated for 1 h with either a control solution or 10 μM U0126 before application of 20 ng/ml human recombinant FGF-2 for 1 h. Cultures were then fixed in 4% paraformaldehyde and processed as above with a mouse monoclonal antibody that recognizes ppERK (Clone, MAPK-YT, Sigma; 1:1000). Cultures were viewed by using a Zeiss Axioplan II microscope, and images were captured with fixed shutter times by using a Spot II camera and Spot Advanced software (Diagnostics Instruments). The average fluorescence intensity of RGC somata were measured by using NIH Image (shareware), with a value of 0 being most intense and a value of 255 being the dimmest.

Growth cone turning assay

Stage 24 retinal explant cultures were used in the growth cone turning assay 18–24 h after plating (de la Torre et al., 1997; Webber et al., 2003). The responses of actively extending growth cones to an applied FGF-2 concentration gradient were recorded for 45 min using a Cohu CCD video camera and Scion Image capture software (shareware). Stable FGF-2 gradients were formed by pulsatile ejection of recombinant human FGF-2 from a 0.5- to 1-μm tip glass capillary pipette placed at a 45° angle from the growth cone (Zheng et al., 1994). A standard pressure pulse of 3 psi was applied (Picospritzer III General Valve) for 20 ms to the pipette at a frequency of 2 Hz using a pulse generator (SD9, Grass Instruments). By this method, the growth cone sees a concentration approximately 1000-fold lower than in the pipette (Lohof et al., 1992; Zheng et al., 1994). The pipette solution consisted of 0.1 μg/ml FGF-2 in 10 mM Tris (pH 8.0) in 1× PBS. Experimental bathing solutions consisted of 10 μM U0126, 9.5 μM D609 or 2.5 μM U73122 to inhibit the MAPK, PC–PLC and PLCy pathways, respectively. The control bath included the same concentration of DMSO as the experimental condition. Only actively extending growth cones were analyzed. Experiments and analysis were performed blind. For analysis, the trajectories of the growth cones were traced onto a graph and the turning angles (degree of turning from the initial trajectory made over the 45-min exposure to the FGF-2 concentration gradient) were recorded. Samples were analyzed statistically using a two-tailed, unpaired Student’s t test.

Western analysis

The brains of embryos exposed to either control DMSO containing solution, 100 μM U0126 or 25 μM LY294002, were dissected and placed immediately on dry ice. Isolated brains were homogenized in 10-μl homogenization buffer/brain (137 mM NaCl, 0.1% Triton, pH 8.0, 1% NP40, 1.5 mM sodium vanadate, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol and a 1:100 dilution of Roche protease inhibitor mix). This mixture was centrifuged for 10 min at 4°C, and the concentration of protein in the supernatant determined using a Bicinchoninic Acid Kit (Pierce). SDS (4×) sample buffer (4% SDS, 2% mercaptethanol, 20% glycerol, bromophenol blue) was added, and the mixture boiled for 5 min. Eight micrograms of each sample was run on a 10% denaturing poly-acrylamide gel. Prestained molecular weight markers (Invitrogen) were run alongside for size determination. The samples were blotted onto PVDF membranes (Biorad). The
blots were rinsed briefly in TBS–Twy (Tris-buffered saline, 0.1% Tween 20) and blocked in TBS–Twy with 5% skim milk powder either at room temp for 1 h or overnight at 4°C. The blots were incubated in mouse anti-ppERK and rabbit anti-phospho-AKT (Ser 473) (Cell Signaling Technologies) diluted 1:1000 in block overnight at 4°C. Subsequently, blots were washed in TBS–Twy and incubated with the appropriate HRP-conjugated secondary antibodies. (Jackson Laboratories) diluted 1:500 in blocking solution, for 1 h at room temperature. Protein bands were detected with ECL solutions (Amersham Bioscience) as per manufacturer’s protocol. The blots were exposed to Hyperfilm (Amersham Bioscience).

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