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Insulin-like growth factor-I is a potential trophic factor for amacrine cells

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Abstract

In this study we show that insulin-like growth factor (IGF)-I selectively promotes survival and differentiation of amacrine neurons. In cultures lacking this factor, an initial degeneration pathway, selectively affecting amacrine neurons, led to no lamellipodia development and little axon outgrowth. Cell lysis initially affected 50% of amacrine neurons; those remaining underwent apoptosis leading to the death of approximately 95% of them by day 10. Apoptosis was preceded by a marked increase in c-Jun expression. Addition of IGF-I or high concentrations (over 1 μM) of either insulin or IGF-II to the cultures prevented the degeneration of amacrine neurons, stimulated their neurite outgrowth, increased phospho-Akt expression and decreased c-Jun expression. The high insulin and IGF-II concentrations required to protect amacrine cells suggest that these neurons depend on IGF-I for their survival, IGF-II and insulin probably acting through IGF-I receptors to mimic IGF-I effects. Inhibition of phosphatidylinositol-3 kinase (PI 3-kinase) with wortmannin blocked insulin-mediated survival. Wortmannin addition had similar effects to IGF-I deprivation: it prevented neurite outgrowth, increased c-Jun expression and induced apoptosis. These results suggest that IGF-I is essential for the survival and differentiation of amacrine neurons, and activation of PI 3-kinase is involved in the intracellular signaling pathways mediating these effects.

Keywords: amacrine cells, apoptosis, insulin-like growth factor I, phosphatidylinositol 3-kinase (PI 3-kinase), retina.

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It is widely accepted that specific trophic factors control the survival of different neuronal cell types; their deficient supply to a given neuronal population might trigger neurodegenerative diseases by activation of programmed cell death (PCD) (Raff 1992; Raff *et al*. 1993). In the retina, extensive research has uncovered diverse trophic requirements of the different neuronal cell types. Thus, ganglion cells depend on brain-derived neurotrophic factor (BDNF) for their survival and on acidic fibroblast growth factor (FGF) for axon outgrowth (Barde *et al*. 1987; Lipton *et al*. 1988). Ciliary neurotrophic factor and basic-FGF improve photoreceptor survival and differentiation (Hicks and Courtois 1992; Fontaine *et al*. 1998; LaVail *et al*. 1998). Docosahexaenoic acid (DHA) is essential for delaying apoptosis and stimulating differentiation of photoreceptors (Rotstein *et al*. 1996, 1997, 1998). To date, only BDNF and neurotrophin-3 (De la Rosa *et al*. 1994; Bovolenta *et al*. 1996; Cellerino *et al*. 1998), have been reported to promote survival of some among the large number of amacrine cell subtypes occuring in the rat retina (McNeil and Masland 1998). However, the list of trophic factor requirements for most neuronal cell types grows increasingly larger and many more remain to be established.

Insulin and insulin-like growth factors (IGFs) appear to play a central role in building the cytoarchitecture of the retina (Hernández-Sánchez *et al*. 1995). Insulin has been included in the formulation of chemically defined media

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Abbreviations used: BDNF, brain-derived neurotrophic factor; DAPI, 4,6-diamidino-2-phenylindole; IGF, insulin-like growth factor; PBS, phosphate buffer saline; PCD, programmed cell death; PI 3-kinase, phosphatidylinositol 3-kinase.

(Bottenstein 1985) and it sustains long-term survival of mouse retinal neurons in culture (Politi *et al*. 1988). Insulin and its related factors decrease apoptosis and stimulate neuronal cell proliferation, differentiation, maturation and survival (De Pablo and De la Rosa 1995; Hernández-Sánchez *et al*. 1995; Singleton *et al*. 1996; Doré *et al*. 1997). Apoptosis contributes to neuronal death in retinas from experimentally induced diabetic rats and diabetic humans (Barber *et al*. 1998); moreover, IGF gene expression is reduced in neural tissues from diabetic rats and IGF administration protects against diabetic neuropathy (Ishii and Lupien 1995; Zhuang *et al*. 1997). In the neural retina, IGF-I promotes proliferation and differentiation (Frade *et al*. 1996), and has a neuroprotective effect on ganglion cells after transection of the optic nerve (Kermer *et al*. 2000). Hence, insulin and/or IGFs play a critical role during retinal development, and are crucial for the survival and/or differentiation of different neuronal cells.

In this work, we have investigated the effect of insulin and IGFs on retinal neurons in culture. Our results show that IGF-I selectively promoted the survival of amacrine neurons, which depended on this factor to avoid cell death, while high insulin and IGF-II concentrations mimicked IGF-I effects on amacrine cells. Activation of phosphatidylinositol 3-kinase (PI 3-kinase) was required for IGF-I-dependent effects.

Materials and methods

Materials

Plastic culture 35-mm-diameter dishes and multichambered slides (NUNC) were purchased from Metec SA (Buenos Aires, Argentina). Fetal calf serum was from Centro de Virología Animal (CEVAN, Buenos Aires, Argentina). Dulbecco's modified Eagle medium (Gibco, Rockville, MD, USA) was purchased from Life Technologies. Trypsin, trypsin inhibitor, transferrin, hydrocortisone, putrescine, insulin, polyornithine, selenium, gentamycin, 4,6-diamidino-2-phenylindole (DAPI), staurosporine, calphostin, fluorescein-conjugated secondary antibodies, IGF-I (human recombinant) and IGF-II, wortmannin (from *Penincillium fumiculosum*), propidium iodide and paraformaldehyde were from Sigma (St Louis, MO, USA). Vectastain, ABC Kit was from Vector Laboratories (Burlingame, CA, USA) and 3,3-diaminobenzidine tetrahydrochloride was from Polysciences Inc. (Warrington, PA, USA). Secondary antibodies, *Alexa 488*-conjugated goat anti-mouse and *Alexa 546*-conjugated goat anti-rabbit IgG were from Molecular Probes Inc. (Eugene, OR, USA). Monoclonal antibodies HPC-1 and Rho-4D2 were generous gifts from Dr C. Barnstable (Yale University) and Dr R. Molday (University of South Columbia), respectively. Polyclonal antibodies against phospho-Akt (Thr308), c-Jun and phosphorylated c-Jun (Ser63) II were from New England Biolabs. (Beverly, MA, USA). Docosahexaenoic acid was isolated from bovine retinas by a combination of chromatographic procedures (Rotstein *et al*. 1996). [^{32}P]phosphate and [γ-^{32}P]ATP were from NEN (New England Nuclear–Dupont, Boston, MA, USA). All other reagents were analytical grade.

Cultures of retinal neurons

Purified cultures of rat retinal neurons were prepared by methods previously described (Rotstein *et al*. 1996, 1997). Albino Wistar rats, 1–2 days old, bred in our own colony were used in all the experiments; all proceedings were in accordance with *Principles for Use of Animals and Guide for the Care and Use of Laboratory Animals* (NIH regulation). Neuronal cultures were incubated in chemically defined media (Rotstein *et al*. 1996, 1997) without insulin (controls), at different insulin, IGF-I or IGF-II concentrations, or with both insulin and IGF-I, as indicated in the text. About $60–80 \times 10^3$ cells per cm^2 were seeded on each plastic dish.

Addition of docosahexaenoic acid

To assess the effect of insulin on long-term survival of photoreceptor cells, neuronal cultures were supplemented with docosahexaenoic acid and incubated for different time periods without or with insulin (1.5 μM). Docosahexaenoic acid (6.7 μM), complexed with bovine serum albumin, was added at day 1 *in vitro*, as previously described (Rotstein *et al*. 1996).

Effect of wortmannin on the development of neuronal cells

Wortmannin was added to cultures in a DMSO solution at a final concentration of 100 nM (Guo *et al*. 1997), 2 h after seeding to allow adhesion of cells to substratum. The cultures were then incubated for 5 h to assess wortmannin effects on early neurite outgrowth, or up to 3 or 10 days to evaluate its long-term effects. To investigate the reversibility of its effects, wortmannin was washed out after 5 h or 3 days by rinsing the cultures twice with neuronal medium; fresh neuronal medium containing 1.5 μM insulin was then added. After different time periods, the cultures were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) (0.9% NaCl in 0.01 M NaH_2PO_4, pH 7.4), and analyzed by immunocytochemical methods as described below.

Phosphorylation assays

The pattern and levels of neuronal protein phosphorylation was investigated in 2-day cultures incubated without or with 1.5 μM insulin by using either [^{32}P]phosphate or [γ-^{32}P]ATP. In the first case, intact neuronal cells were incubated for 1 h, at 36°C, with 0.8 mL of culture medium containing [^{32}P]phosphate (sp. activity 1 Ci/mmol; 50 μCi per 35-mm culture dish). The cells were then rinsed twice with buffer Tris-Hepes-magnesium (THM) (40 mM Tris-Hepes, pH 7.4, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM KCl and 140 mM NaCl), with aprotinin (2 μg/mL), leupeptin (1 μg/mL) and PMSF (0.1 mM). Cells were then scraped with a Teflon spatula, transferred to eppendorf vials, centrifuged at 16 000 *g* for 10 min in a microfuge, and finally Laemmli buffer (Laemmli 1970) was added to the pellet.

In experiments using [γ-^{32}P] ATP, cells incubated without or with 1.5 μM insulin were scraped and pelleted as described before. To investigate protein phosphorylation, cell pellets were rinsed and resuspended in 25 μL of a lysis buffer containing 20 mM Tris HCl, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1% Triton X-100, with or without 10 mM KF as a protein phosphatase inhibitor. Incubations were started by the addition of [γ-^{32}P]ATP (2 μCi, 10 μM), performed at 30°C, and stopped by adding Laemmli buffer. To study the involvement of protein kinase C,

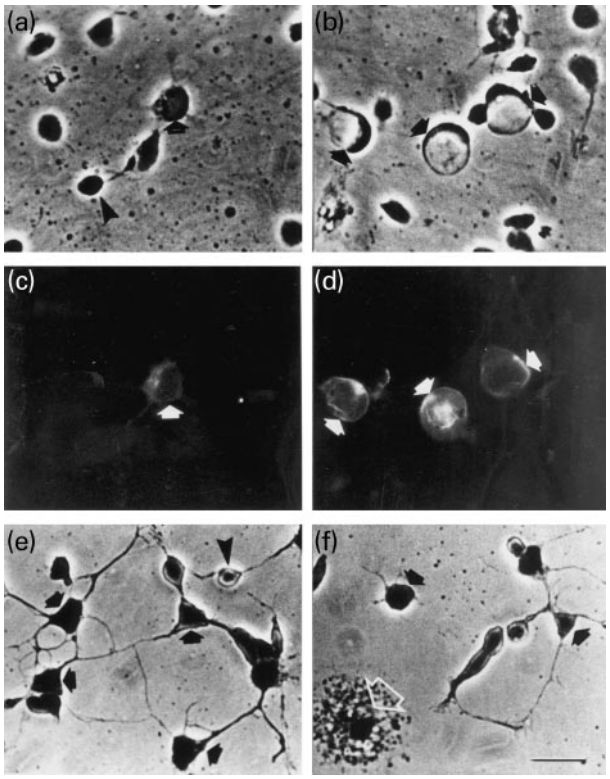

Fig. 1 Neuroprotective effect of insulin on amacrine cells. Phase (a, b, e and f) and fluorescence (c and d) photomicrograph of 2-day-old (a–d) and 3-day-old (e and f) amacrine neurons (short arrows) and photoreceptors (arrowheads), with (a, c and e) or without (b, d and f) insulin, added at day 0 to the culture medium. In insulin-lacking cultures, cell swelling of amacrine neurons is evident by day 2 (short arrows in b and d) and selective lysis occurs during the first 3 days (empty, white arrowhead in f). Amacrine neurons were recognized by their immunoreactivity with HPC-1 monoclonal antibody and by their distinct morphology. The bar indicates 10 μm.

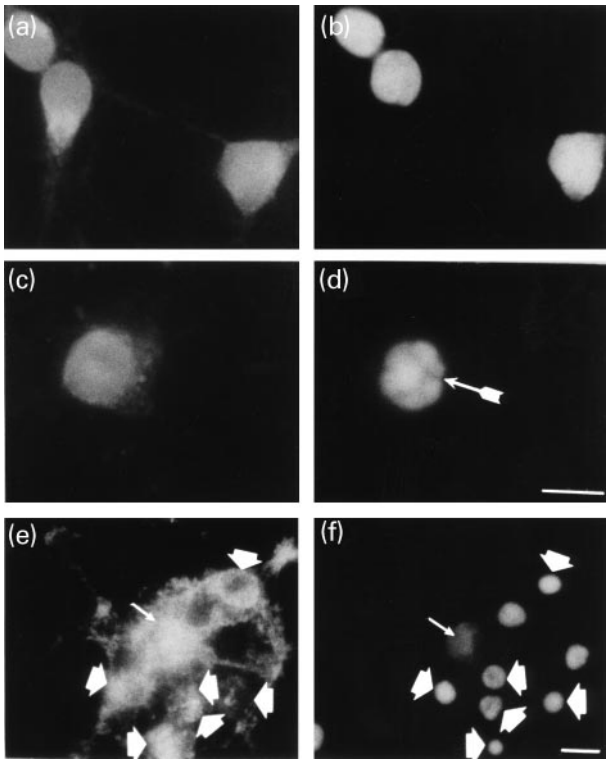

Fig. 2 Apoptosis of amacrine neurons in cultures lacking insulin and IGF-I. Fluorescence photomicrography of 5-day amacrine neurons incubated with (a and b) or without (c, d, e and f) insulin, and double labeled with HPC-1 monoclonal antibody (a, c and e) and with the nuclear marker DAPI (b, d and f). Amacrine neurons show intact nuclei (b) in cultures incubated with insulin, while in cultures devoid of insulin many of these nuclei are pycnotic or show evident signs of fragmentation, characteristic of apoptotic cells (arrow in d and wide arrows in F). The thin arrows in (e) and (f) point to an amacrine neuron still showing an intact nucleus. The bars indicate 4 μm in (a) to (d) and 15 μm in (e) and (f).

cultures grown with insulin (1.5 μM) were incubated with or without the PKC inhibitor staurosporine (10 μg/mL) as previously described (Politi *et al*. 1998). The cells were then pelleted and incubated with [γ-^{32}P] ATP for 30 min, as described above.

In all experiments, proteins in Laemmli buffer were boiled for 5 min and subjected to electrophoresis on 12.5% polyacrylamide gels (SDS-PAGE). Phosphoproteins were visualized by autoradiography. Sibling cultures were scraped to determine their protein content (Bradford 1976) or fixed with 2% paraformaldehyde to quantify cell number by immunocytochemistry, as indicated below.

Akt activation and c-Jun expression

Cells were permeated by a 15-min treatment with 0.2% Triton X-100 in PBS. Expression of phosphorylated Akt was determined using an anti-phospho-Akt monoclonal antibody. Expression of c-Jun and c-Jun phosphorylated at serine 63 were detected with anti-c-Jun or anti-phospho-c-Jun antibodies. *Alexa 546*-conjugated goat anti-rabbit IgG was used as a secondary antibody.

Immunocytochemical analysis and cell counting

Neuronal cell types were identified by fluorescence microscopy (Rotstein *et al*. 1996), or by the 'ABC' method, using the monoclonal antibodies HPC-1, for amacrine cells, and Rho-4D2, for photoreceptor cells (Barnstable 1980; Hicks and Barnstable 1987; Kljavin *et al*. 1994). Controls for immunocytochemistry were carried out by omitting either the primary or the secondary antibody. Dead cells were identified by fluorescence microscopy, incubating the cultures with 0.5 μg/mL (final concentration in culture) of propidium iodide for 1 min just before fixation (Jordán *et al*. 1997); similar results were obtained when analysing cell integrity by phase microscopy. Nuclei integrity was determined by fluorescence microscopy, using DAPI. The number of amacrine neurons with neurites longer than 1 cell body diameter was determined by phase microscopy. Ten fields per sample were analyzed in each case. Each value represents the average of at least three experiments ± SD performed by triplicate. Statistical significance was determined by Student's two-tailed *t-test*.

Table 1 c-Jun expression and apoptosis during amacrine cell development

Condition	Apoptosis (%)	c-Jun (%) Total	c-Jun (%) phosphorylated
Day 4			
– Insulin	26.5 ± 5.3	13.5 ± 3.1	6.3 ± 1.3
+ Insulin	0.7 ± 0.8[a]	5.6 ± 3.2[a]	2.0 ± 0.8[a]
+ Wortmannin	29.8 ± 2.5[a]	23.6 ± 3.4[a]	20.2 ± 3.8[a]
Day 6			
– Insulin	28.4 ± 2.7	75.5 ± 6.4	67.1 ± 10.2
+ Insulin	0.5 ± 0.5[a]	18.3 ± 2.9[a]	17.5 ± 6.5[a]
+ Wortmannin	51.4 ± 4.3[a]	73.5 ± 3.4	71.0 ± 3.4
Day 10			
– Insulin	42.0 ± 4.7	83.6 ± 8.3	80.0 ± 6.3
+ Insulin	0.6 ± 0.4[a]	35.5 ± 4.2[a]	27.5 ± 6.9[a]
+ Wortmannin	–	–	–

Neuronal cultures were incubated with or without 1.5 μM insulin or with 1.5 μM insulin plus 100 nM wortmannin. Apoptosis, total c-Jun and phosphorylated c-Jun expression were determined by cytochemistry, using DAPI or specific antibodies, respectively, as described in Methods. Results represent the percentage ± SD of apoptotic or c-Jun expressing cells referred to the total number of amacrine cells present in the cultures. Ten fields per sample were analyzed in each case. Each value represents the average of at least three experiments performed by triplicate. Apoptosis could not be determined in wortmannin-treated cultures by day 10 because of the extensive amacrine neuron degeneration. [a]$p < 0.05$, by Student's two-tailed *t-test*.

Results

Insulin and IGF-I effect on amacrine neuron survival

Figure 1 shows 2-day-old (a, b, c and d) and 3-day-old (e and f) cultures of rat retinal neurons incubated without (b, d and f) or with 1.5 μM insulin (a, c and e). In both conditions, amacrine neurons and photoreceptor cells were the major cell types in the cultures (Rotstein *et al*. 1996). In the absence of insulin or IGF-I, amacrine neurons showed evident signs of cell swelling by day 2 (Figs 1b and d), that ended up in the selective lysis of approximately 50% of these cells 1 day later (Figs 1f and 3). By day 4, the surviving amacrine neurons started an apoptotic process (Figs 2c–f and Table 1), which progressed rapidly: by day 10, the combination of apoptotic death with the initial cell lysis left only 5% of the original amacrine neurons in the culture (Fig. 3, upper panel), and approximately 42% of them were apoptotic (Table 1).

Addition of either 1.5 μM insulin or 0.5 μM IGF-I almost completely prevented amacrine neuron death (Figs 1e and 3, upper panel). After a slight initial reduction, the percentage of surviving neurons remained the same: approximately

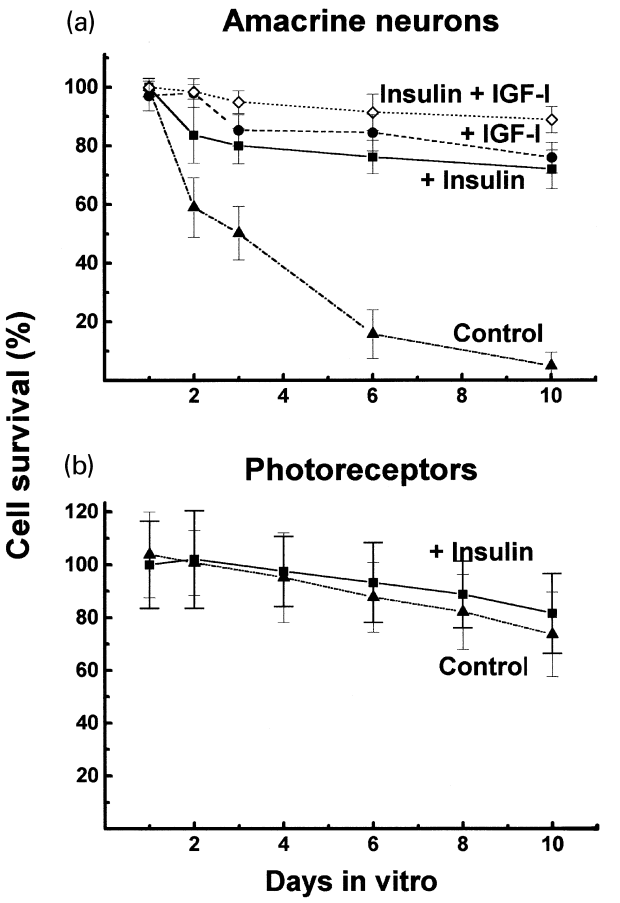

Fig. 3 Selective effect of IGF-I and insulin on amacrine cell survival. The figure depicts the percentage ± SD of surviving amacrine neurons (upper panel) and photoreceptors (lower panel) at different times *in vitro*, determined in (a) cultures incubated without (▲, control) or with (■) 1.5 μM insulin, 0.5 μM IGF-I (●) or 1.0 μM insulin plus 67 nM IGF-I (◇). In the absence of insulin or IGF-I, most amacrine neurons undergo cell death after 10 days in culture. Most of them could be rescued by adding 1.5 μM insulin, 0.5 μM IGF-1 or 1.0 μM insulin plus 67 nM IGF-I to the culture medium. (b) Cultures were incubated with docosahexaenoic acid (6.7 μM), added at day 1, to selectively promote photoreceptor survival as previously reported (Rotstein *et al.* 1996, 1997) with (■) or without (control, ▲) 1.5 μM insulin. Photoreceptors developed normally regardless of the presence or absence of insulin. Each value represents the average of at least three experiments performed by triplicate.

80% of them were still present by day 10 in culture (Fig. 3, upper panel).

Photoreceptor cells, the other major neuronal type in the cultures, require docosahexaenoic acid (DHA) for survival. Without this fatty acid, these cells start an apoptotic process by day 4, in both the presence or the absence of insulin, which is delayed by the addition of DHA, as we have previously shown (Rotstein *et al*. 1996, 1997, 1998). In DHA-supplemented cultures, photoreceptors developed in a

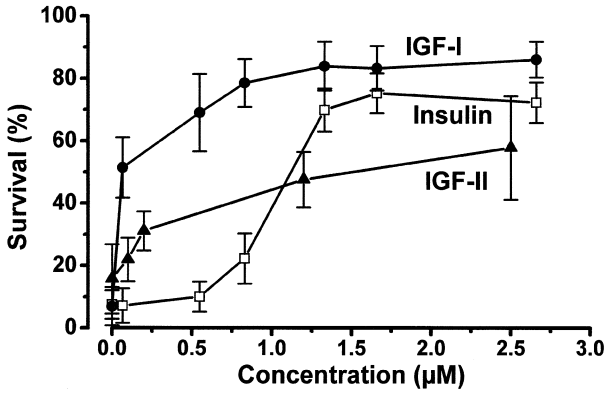

Fig. 4 Dose–response curves of IGF-I, insulin and IGF-II on neuronal survival. Retinal cultures were incubated with different concentrations of IGF-I (●), IGF-II (▲) or insulin (□), and the percentage ± SD of surviving amacrine neurons, referred to those present at day 0, was determined after 6 days. Each value represents the average of at least three experiments performed by triplicate. Note that incubation of cultures with 67 nM IGF-I could rescue approximately 50% of amacrine neurons, while it was necessary to add approximately 1 μM insulin or IGF-II to produce the same survival effect.

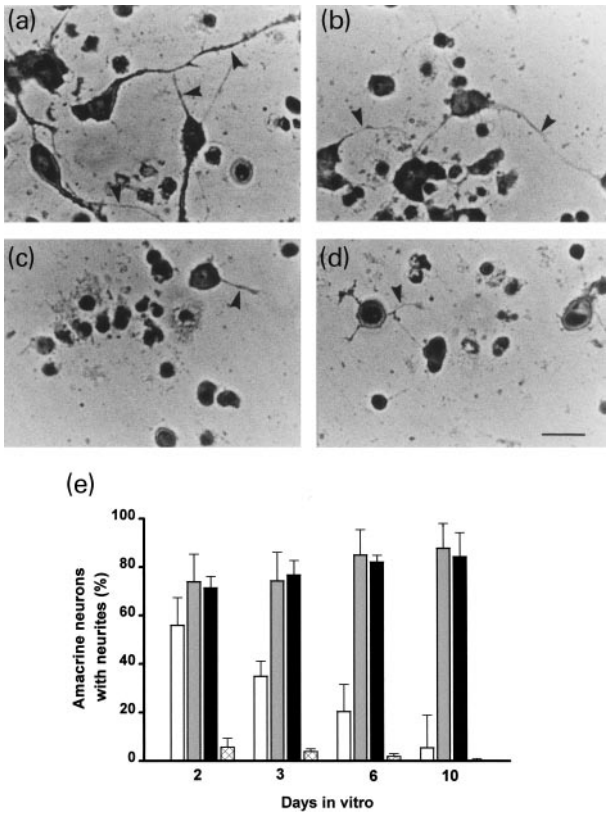

Fig. 5 Effect of insulin and IGF-I on amacrine neurite outgrowth. Retinal cultures were incubated without (c and white bars in e), with (a and gray bars in e) 1.5 μM insulin, 0.5 μM IGF-I (b and black bars in e) or 1.5 μM insulin plus 100 nM wortmannin (d and hatched bars in e). Conspicuous neurite outgrowth is observed in insulin and IGF-I-supplemented cultures (arrowheads in a and b), while neurites are almost absent or barely developed (arrowheads) in control (c) and wortmannin-treated (d) cultures. The percentage ± SD of retinal neurons bearing neurites longer than one cell body diameter was determined at different times . Each value represents the average of at least three experiments performed by triplicate. The bar indicates 20 μm.

similar fashion when incubated with or without insulin (Fig. 3, bottom panel); the slight reduction in their survival by day 10 was unaffected by insulin addition.

Hence, the absence of insulin or IGF-I in the culture medium induced amacrine cell death by two different degenerative pathways: an early cell lysis and a late apoptotic progression, without affecting the development of photoreceptor cells. The addition of insulin or IGF-I to the cultures selectively rescued most amacrine neurons.

Insulin, IGF-I and IGF-II dose–response curves

Retinal neurons were incubated from day 0 with increasing concentrations of insulin, IGF-I or IGF-II, and the number of surviving amacrine neurons at day 6 was determined in each case. IGF-I had a greater survival effect than IGF-II and insulin (Fig. 4): it was necessary to incubate with 67 nM IGF-I to rescue 50% of amacrine neurons, while approximately 1 μM IGF-II or insulin were required to obtain a similar effect (Fig. 4). This insulin concentration was higher than the required for other reported effects of this factor (Waldbillig *et al.* 1987; Waldbillig and Chader 1988). The combined addition of suboptimal concentrations of both IGF-I and insulin rescued approximately 90% of amacrine neurons (Fig. 3). Altogether, these results suggest that amacrine neurons strongly depended on IGF-I for their survival and high concentrations of IGF-II or insulin could mimic IGF-I survival effects.

Insulin capability to promote amacrine neuronal survival depended on the time of development at which it was added

Table 2 Time-dependence of insulin addition on amacrine cell survival

Day of insulin addition	Survival of amacrine neurons (%)
Day 0	79.6 ± 13.4
Day 1	49.0 ± 7.9[a]
Day 2	34.0 ± 9.2[a]
No insulin addition	5.4 ± 4.6[a]

Insulin (1.5 μM) was added to neuronal cultures at days 0, 1 or 2, and the percentage ± SD of surviving amacrine neurons, referred to those present at day 0, was determined after 10 days . Each value represents the average of at least three experiments perfomed by triplicate. [a]$p < 0.05$, by Student's two-tailed *t-test*.

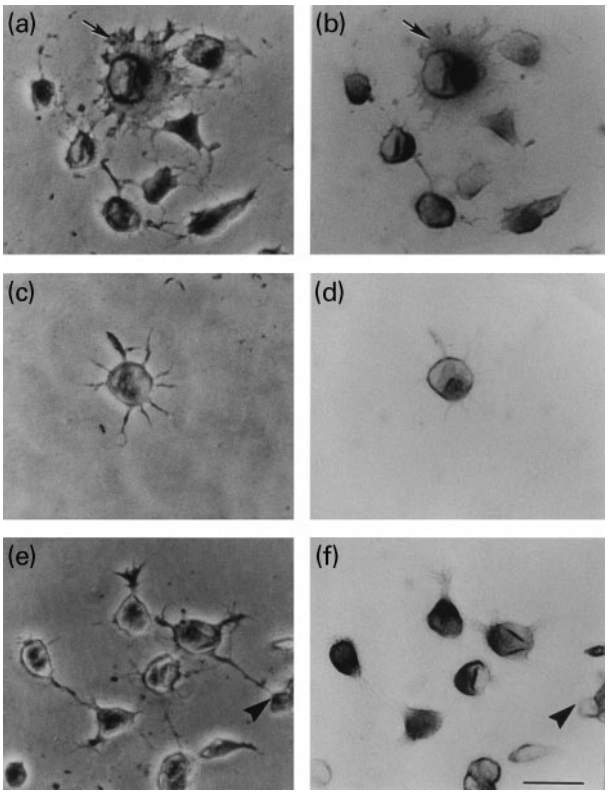

Fig. 6 Inhibition of amacrine lamellipodia development by wortmannin. Phase (a, c and e) and bright field (b, d and f) photomicrographs of 1 day cultures incubated with 1.5 μM insulin (a and b), with 1.5 μM insulin plus 100 nM wortmannin (c and d), or for 5 h with wortmannin, followed by addition of fresh insulin-containing media after removing wortmannin-containing media (e and f). The identity of amacrine neurons showed in the left panels was assessed by their immunoreactivity to HPC-1 monoclonal antibodies by the 'ABC' method (right panels). Photoreceptors were not stained with this antibody (arrowhead in e and f). The extensive lamellipodia development in cultures incubated with insulin (arrows in a and b) contrasts with the complete absence of lamellipodia in wortmannin-treated cultures (c and d). After washing out wortmannin, followed by incubation with insulin (e and f), the surviving amacrine neurons continued their development, growing again their neurites. The bar indicates 10 μm.

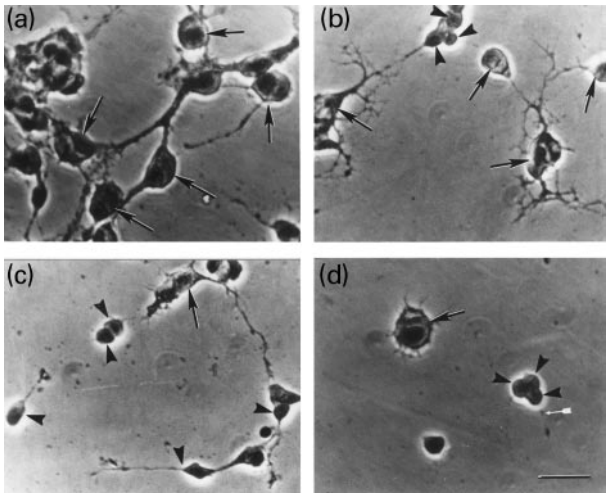

Fig. 7 Effect of wortmannin on amacrine cell death. Cultures were incubated for 6 days with (a) or without (c) 1.5 μM insulin, with 1.5 μM insulin plus 100 nM wortmannin (d) or with wortmannin for 5 h followed by washing out the incubation medium and replacing it with insulin-containing medium for 6 days (b). Amacrine neurons (arrows) survived and differentiated normally in cultures incubated with insulin. Cultures devoid of insulin (c) showed a drastic reduction in the number of amacrine neurons without apparent damages to photoreceptor survival (arrowheads in c). Long-term treatment of cultures with wortmannin also affected amacrine neuron survival and neurite outgrowth (arrow in d). In addition, wortmannin either prevented photoreceptor axon outgrowth (black arrowheads in d) or caused abnormal axon development (white arrow in d). Washing wortmannin after 5 h incubation, followed by addition of insulin (b), rescued approximately 50% of amacrine neurons by day 6. The bar indicates 10 μm.

to the cultures (Table 2). Maximal survival effect at day 10 could be observed when insulin was added at day 0 *in vitro*. Its addition at days 1 or 2 had a much lower effect because the lytic death of amacrine neurons had already started; however, those remaining could still be rescued. Addition of insulin after 3 days had little or no effect on amacrine neuron survival (not shown).

Insulin dependence of lamellipodia development and neurite outgrowth in amacrine cells

In control cultures, neurite outgrowth was severely affected (Fig. 5c): only approximately 55 and 35% of amacrine neurons developed neurites after 2 and 3 days *in vitro*, respectively (Fig. 5e). Of those neurons developing neurites, approximately one-third had single, long axons instead of the extensive neurite outgrowth characteristic of amacrine neurons (Fig. 5c). The sparse neurite outgrowth might be related to the fact that, in control cultures, amacrine neurons lacked their characteristic lamellipodia (Figs 6a–d). The percentage of neurons bearing neurites decreased steadily with culture time; only 5% of the surviving amacrine neurons had neurites by day 10 (Fig. 5e). Insulin and IGF-I efficiently promoted neurite outgrowth (Figs 5a and b). In insulin-supplemented cultures many amacrine neurons showed conspicuous lamellipodia during the first 24 h of development (compare Figs 6a and c) and more than 70% of them already had neurites as early as day 2, a percentage that increased to approximately 85% by day 10 (Fig. 5e). A similar effect was observed when cultures were incubated with IGF-I (Figs 5b and e). Therefore, insulin and IGF-I not only rescued developing amacrine neurons but also stimulated their lamellipodia development and neurite outgrowth.

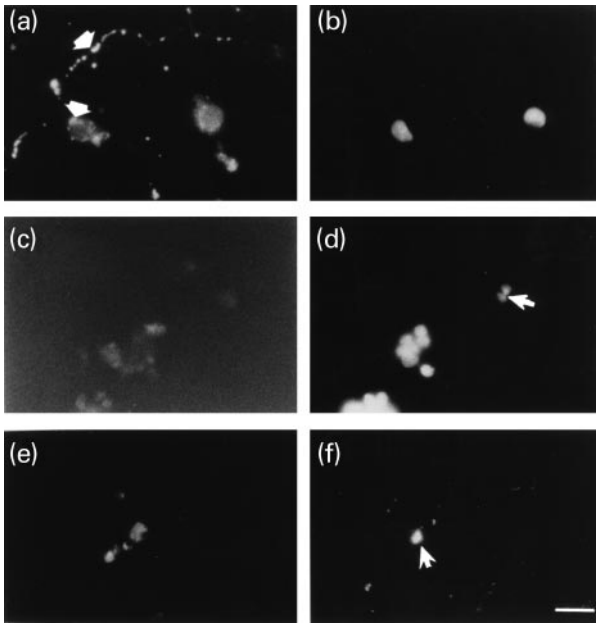

Fig. 8 Enhancement of phospho-Akt expression by insulin. Fluorescence photomicrographs of 9-day cultures incubated with (a and b) or without (c and d) 1.5 μM insulin or with (e and f) 1.5 μM insulin plus 100 nM wortmannin showing phospho-Akt expression (a, c and e) and nuclear staining with DAPI (b, d and f). Note phospho-Akt expression in amacrine cell neurites and bodies in insulin-supplemented cultures (wide arrows in a), corresponding to intact nuclei (b). Attenuation of this expression in (c) and (e) was consistent with the presence of pycnotic or fragmented nuclei (arrows in d and f). The bar indicates 20 μm.

Wortmannin inhibition of insulin effects

Many insulin and IGF-I effects require of the activation of PI 3-kinase (reviewed in Shepherd *et al*. 1998). To test if this enzyme was involved in the effects of these factors on amacrine neurons, cultures were incubated for 10 days with 100 nM wortmannin, a specific inhibitor of PI 3-kinase activity at this concentration (Guo *et al*. 1997; Pong *et al*. 1998; Shepherd *et al*. 1998). Its addition completely blocked the action of insulin (Figs 6c and d, and Fig. 7d), entirely preventing neurite outgrowth (Figs 5d and e); this was an early effect, since lamellipodia development was already blocked after 5 h of incubation (Figs 6c and d). Wortmannin addition also triggered apoptosis; by day 4, the percentage of apoptotic amacrine neurons was approximately 30% in both insulin-lacking and wortmannin-treated cultures (Table 1). Apoptosis then progressed more rapidly in wortmannin-treated cultures than in those lacking insulin. Experiments in which cell cultures were treated for only 3, instead of 10, days with wortmannin, and then incubated with fresh, insulin-containing medium, showed almost the same results (not shown). Nevertheless, wortmannin deleterious effects could be halted and reversed by washing it out after a 5-h incubation and then adding fresh, insulin-supplemented neuronal medium (Figs 6e and f, and Fig. 7b). Under these conditions, the remaining amacrine neurons again rapidly started growing their neurites and lamellipodia (Figs 6e and f), showing no signs of apoptosis.

It is noteworthy that photoreceptors treated with wortmannin for 6 days also showed an abnormal pattern of development and axon outgrowth, as compared with those incubated in an insulin-depleted media for a similar period (Figs 7c and d). This suggests that PI 3-kinase might be involved, at least to some extent, in the cascade of events leading to photoreceptor development.

Akt activation

The serine/threonine protein kinase Akt (also known as protein kinase B) is activated by diverse trophic factors via a PI 3-K-dependent pathway (Burgering and Coffer 1995; Dudek *et al*. 1997). To evaluate its participation in IGF and insulin-mediated survival of amacrine neurons, we investigated the expression of its active, phosphorylated form, in control and insulin-supplemented cultures. Expression of phosphorylated Akt was observed in insulin-supplemented cultures, and this expression was enhanced at long culture times (Fig. 8a). On the contrary, phosphorylated Akt was hardly visible in insulin-lacking or wortmannin-treated cultures (Figs 8c and e), where amacrine cell nuclei showed either the pycnotic or highly fragmented appearance characteristic of the late stages of apoptosis (Figs 8d and f).

C-Jun expression

An increase in transcription factor c-Jun expression has been involved in the regulation of cell death (Estus *et al*. 1994; Ham *et al*. 1995) and its phosphorylation has been proposed to be required for induction of apoptosis upon trophic factor withdrawal (Watson *et al*. 1998). Hence, we investigated c-Jun and phospho-c-Jun expression in amacrine neurons in culture (Fig. 9). In the absence of IGF-I and insulin, c-Jun expression in amacrine neurons increased with culture time (Fig. 9h, Table 1). By day 4, 13.5% of these neurons expressed total c-Jun and half of it was phosphorylated (Table 1). These percentages dramatically increased by day 6, when over 75% of amacrine neurons expressed c-Jun and almost all of it was phosphorylated. These values were slightly higher by day 10. Most of the label was found in the cytoplasm (Fig. 9h), although at day 10 expression of phosphorylated c-Jun was found in the nuclei of 13.8 ± 2.0% of the amacrine cells in cultures devoid of insulin. The evolution of apoptosis in insulin-lacking cultures was slower than the increase in c-Jun expression; by day 10, apoptosis extended to 42% of these cells, while approximately 84% of them expressed c-Jun (Table 1). Addition of 1.5 μM insulin markedly reduced both c-Jun expression and apoptosis (Figs 9b and c): at day 4, less than 6% of amacrine neurons expressed c-Jun and only 2% phosphorylated c-Jun (Table 1). These percentages

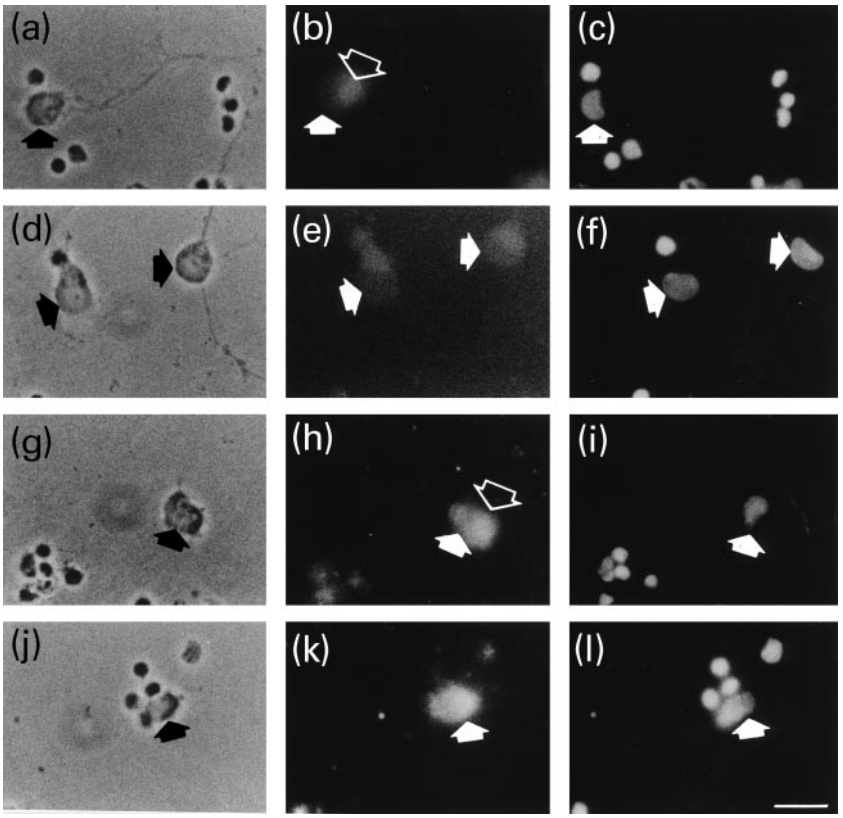

Fig. 9 c-Jun and phospho c-Jun expression: insulin and wortmannin effects. Phase (a, d, g and j) and fluorescence photomicrographs depicting c-Jun (b) and phospho c-Jun (e, h and k) expression and nuclear staining with DAPI (c, f, i and l) of 10-day cultures incubated with (a–f) 1.5 μM insulin; without (g–i) insulin or with (j–l) 1.5 μM insulin plus 100 nM wortmannin. Note that while cytoplasmic (open arrow in h) and nuclear (white arrows in h and k) localization of phospho c-Jun is observed in insulin-lacking and wortmannin-treated cultures, mainly cytoplasmic localization of c-Jun (open arrow in b) and phospho c-Jun (e) is detected in insulin-supplemented cultures. Also notice that amacrine cell nuclei, intact in insulin-supplemented cultures (white arrows in c and f), are fragmented in control or wortmannin-treated cultures (i and l). The bar indicates 20 μm.

increased with culture time: however, only 35% of these cells were c-Jun positive by day 10, less than half of those expressing c-Jun at the same time in insulin-lacking cultures. In insulin-supplemented cultures, less than 1% of the amacrine neurons were apoptotic at every incubation time (Table 1). It is noteworthy that almost all phospho-c-Jun was found in amacrine cell cytoplasm in insulin-supplemented cultures by day 10 (Fig. 9e); only a negligible 0.1% ± 0.1% was detected in cell nuclei.

Evolution of c-Jun expression in wortmannin-treated cultures was similar to that observed in amacrine neurons grown without insulin (Fig. 9k, Table 1); although more amacrine neurons expressed c-Jun by day 4 in the former than in the latter condition, both values were approximately the same by day 7 in culture. Therefore, an increase in c-Jun expression correlated with amacrine neuron degeneration, and preceded nuclear fragmentation.

Effects of insulin on protein phosphorylation

Protein composition and the pattern of protein phosphorylation in cultures incubated with or without insulin were also analyzed. By day 2, protein amounts and composition were similar in both culture conditions. A significant increase in protein content occured in insulin-containing cultures by day 5: while cells grown without insulin had 31.0 ± 5.5 μg protein per culture dish, those incubated with 1.5 μM insulin had 80.4 ± 10.9 μg protein per dish. The higher protein content was consistent with the increased survival and differentiation (i.e. higher number of neurites) of amacrine neurons in insulin-supplemented cultures.

Entire cells incubated with [^{32}P]phosphate were rapidly phosphorylated and several neuronal proteins were labeled (Fig. 10a, lanes d and e). This labeling was enhanced by insulin: after 1 h of incubation, proteins were more heavily labeled in insulin-supplemented than in insulin-lacking cultures.

Neuronal proteins were also rapidly and heavily phosphorylated in cell lysates incubated with [γ-^{32}P]ATP (Fig. 10b). The pattern of phosphorylation was selectively modified leading to an increase in the labeling of a 105–110-kDa band in cells obtained from insulin-supplemented compared with those from insulin-lacking cultures (Fig. 10b, lanes e and d). The different patterns of phosphorylation obtained in the entire system and in the lysates might be due to the presence of insulin in the culture medium during the phosphorylation reaction in the first case, and its absence in the latter. In this case, neurons were grown with insulin and then phosphorylation was performed in a medium without insulin. This suggests that the presence of insulin was required for an overall increase in phosphorylation.

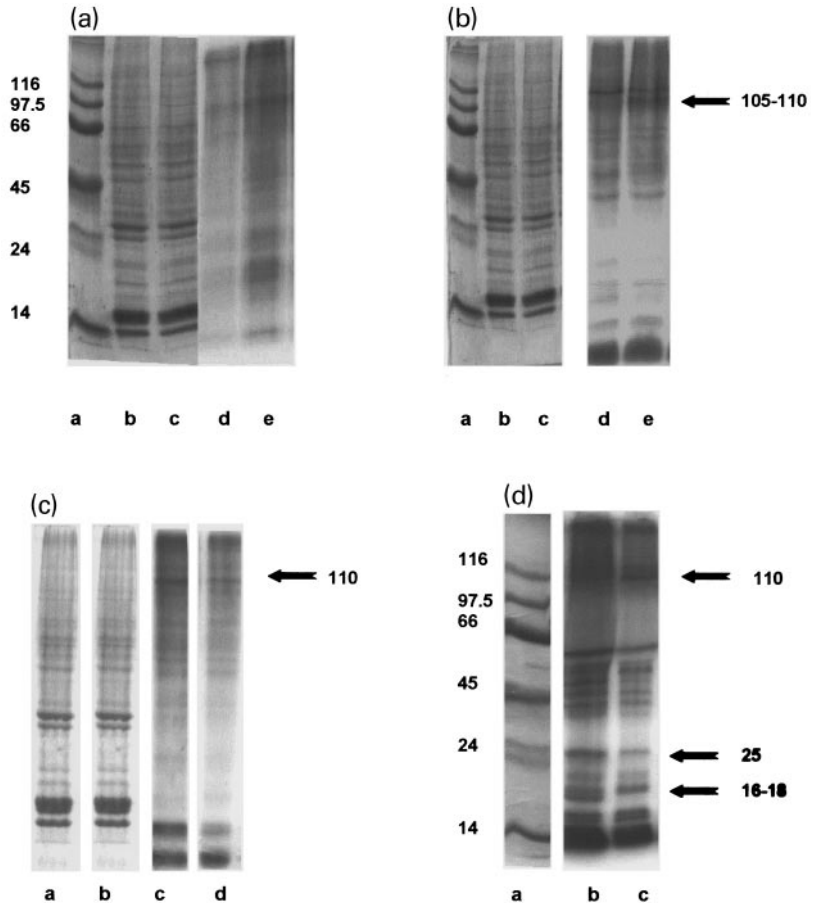

Fig. 10 Insulin effects on the phosphorylation of neuronal proteins. (a) Phosphorylation of neuronal proteins with [^{32}P] phosphate. Neurons were grown with or without insulin for 2 days and then [^{32}P]phosphate was added to the cultures for 30 min. Lane a – molecular weight standards; lanes b and c – neuronal proteins from cultures incubated without and with insulin, respectively, stained with Coomasie Blue; lanes d and e – phosphorylation pattern of the neuronal proteins shown in lanes b and c, respectively. (b) Phosphorylation of neurons with [γ-^{32}P] ATP, in the presence of 10 mM KF as a phosphatase inhibitor. Lane a – molecular weight standards; lanes b and c – neuronal proteins from cultures incubated without and with insulin, respectively, stained with Coomasie Blue; lanes d and e – autoradiograms from lanes b and c, respectively. (c) Protein pattern of 2 days neurons without or with insulin: lanes a and b, respectively, incubated with [γ-^{32}P] ATP in the absence of phosphatase inhibitors; lanes c and d – autoradiogram of lanes a and b, respectively. (d) Phosphorylation with [γ-^{32}P] ATP of neuronal proteins incubated in insulin-supplemented medium in the presence of staurosporin. Lane a – molecular weight standards; autoradiogram of lane b, control condition (with DMSO) or lane c with staurosporin. In all cases, samples were normalized to load the same amount of proteins (~15 μg per well) in each lane.

Phosphatases were among the plethora of proteins activated by insulin. In the experiments described above, the incubation media contained phosphatase inhibitors. When incubation with [γ-^{32}P]ATP was performed without any phosphatase inhibitor, cells obtained from 2-day insulin-supplemented cultures showed a striking decrease in protein labeling (Fig. 10c, lanes c and d). Most proteins were dephosphorylated in these conditions and the band at 105–110 kDa was markedly reduced. On the contrary, in cells from insulin-lacking cultures the pattern and intensity of protein labeling was approximately the same either with or without phosphatase inhibitors. This suggests that insulin was required for phosphatase activity.

Previous work from our laboratory has shown that PKC is involved in amacrine axon outgrowth (Politi *et al*. 1998). To investigate the role of this kinase in neuronal protein phosphorylation, insulin-supplemented 2-day cultures were pre-incubated with staurosporine, a PKC inhibitor, and then incubated with [γ-^{32}P]ATP (Fig. 10d) for 30 min. The presence of staurosporine led to a reduction in the labeling of the 24–25 kDa and 105–110 kDa bands and to the disappearance of the lower of the double bands observed at 16–18 kDa (Fig. 10d).

Discussion

IGF-I as a trophic factor for amacrine neurons

The present report shows that amacrine neurons selectively depended on IGF-I for their survival and differentiation, strongly suggesting that this molecule might be a natural trophic factor for amacrine neurons. Insulin and IGF-II in a high concentration had a similar survival effect. Our results show for the first time that deprivation of these molecules led to the selective activation of apoptosis in rat amacrine neurons. Apoptosis had been shown to occur in the inner nuclear layer of retinas from diabetic rats and humans (Barber *et al*. 1998), and attenuation of apoptotic death by

insulin had been demonstrated in embryonic chick retinas (Diaz *et al*. 1999); however, the affected neuronal type had not been previously identified.

Amacrine cells showed apoptosis after 4 or 5 days , in the absence of IGFs and insulin. Addition of IGF-I prevented the onset of apoptosis in these cells more efficiently than insulin or IGF-II. Retina insulin receptors have a higher affinity for IGF-I than for insulin (Waldbillig *et al*. 1988), and IGF-II has been reported to act via activation of IGF-I receptors, inducing a similar response with a lower affinity (Nielsen *et al*. 1991). This is consistent with our results and suggests that IGF-I might be the trophic factor required by amacrine neurons, while the effects of insulin and IGF-II were probably mediated through IGF-I receptors.

Interestingly, amacrine cells in culture also underwent early cell lysis, which usually suggests a necrotic rather than a PCD. However, the latter cannot be ruled out because PCD can be executed by different routines, including necrotic appearances (Nicotera *et al*. 1999). These two modes of cell death suggest that insulin and IGF-I probably have different roles in different subpopulations of amacrine neurons.

Trophic factors should not only support survival but also be coordinately expressed both anatomically and temporally during development in the tissues where the target neurons develop. IGF-I fulfils these requirements because this molecule and its receptors are concentrated in the neural retina (Waldbillig *et al*. 1987; Rodrígues *et al*. 1988; Waldbillig and Chader 1988) and are expressed in a highly developmentally regulated and coordinated manner (Hernández-Sánchez *et al*. 1995; Lee *et al*. 1992).

Molecular mechanisms involved in insulin and IGF-I effects

Our results show that insulin affected both protein phosphorylation and dephosphorylation, leading to a general increase in protein phosphorylation when cultures were incubated with [^{32}P]phosphate. In neuron cell lysates incubated with [γ-^{32}P]ATP, only a 105–110-kDa protein was more labeled in insulin-supplemented than in control conditions. The marked decrease in the labeling of this and other proteins in the presence of staurosporine, a PKC inhibitor, shows that their phosphorylation was PKC-dependent to some extent. This kinase is involved in the regulation of laminin-dependent axon outgrowth in amacrine neurons (Politi *et al*. 1998), suggesting that stimulation of amacrine axon outgrowth by IGF-I and insulin might also involve PKC activity. As experiments lacking phosphatase inhibitors showed, dephosphorylation of most proteins was also heavily dependent on insulin, strongly suggesting that insulin is required for activation of phosphatases. By controlling phosphorylation/dephosphorylation mechanisms, IGF-I or insulin might regulate the activity of key proteins involved in the survival and differentiation of amacrine neurons.

PI 3-kinase plays a central role in insulin and IGF-I signaling pathways, its activation being necessary and in many cases sufficient to trigger many insulin-stimulated signaling pathways (Kapeller and Cantley 1994; Singleton *et al*. 1996; Yenush and White 1997; Shepherd *et al*. 1998). In the present work, inhibition of this enzyme with wortmannin completely blocked insulin-promoted survival and resembled insulin and IGF-I deprivation. A well-known downstream effector required for PI 3-kinase dependent neuronal survival is Akt (Dudek *et al*. 1997; Crowder and Freeman 1998). Its activation was stimulated in insulin-supplemented cultures and almost completely prevented upon wortmannin addition, providing further support for PI 3-kinase involvement in amacrine neuron survival. Apoptosis of amacrine cells in wortmannin-treated cultures progressed more rapidly than that induced by insulin and IGF-I deprivation. In insulin and IGF-I-lacking cultures, a small amount of these factors might still be available by autocrine/paracrine manner, as previously suggested (Spaventi *et al*. 1990), thus leading to a slight activation of PI 3-kinase and, subsequently, to a slower progression of apoptosis. Addition of wortmannin would completely block PI 3-kinase activation, enhancing the advance of apoptosis. Removal of wortmannin after a brief exposure restored neurite outgrowth in the surviving cells and prevented further cell death; however, after a 3-day treatment, wortmannin effects could no longer be reversed. This is consistent with the absolute requirement for IGF-I and insulin addition before day 3 in culture, during the early stages of amacrine neuron development to prevent their death. As a whole, these results suggest that insulin and IGF-I effects depended on the activation of PI 3-kinase. Inhibition of this enzyme leads to apoptosis in several neuronal systems and its overexpression prevents cell death (Yao and Cooper 1995; D'Mello *et al*. 1997; Crowder and Freeman 1998). It is also implicated in many cellular responses to insulin and IGF-I, such as cell growth, proliferation and differentiation (reviewed in Shepherd *et al*. 1998). Addition of IGF-I or insulin to the culture medium prompted lamellipodia development and led to extensive neurite outgrowth. PI 3-kinase is required for process formation (Kobayashi *et al*. 1997; Toker and Cantley 1997) and mediates the enhancement in growth cone motility promoted by IGF-I (Feldman *et al*. 1997). Hence, the lack of activity of this kinase, due to either insulin deprivation or wortmannin addition, might lead to the notorious decrease in lamellipodia development and neurite outgrowth. Photoreceptor cells were not affected by insulin or IGF-I deprivation, strongly depending on docosahexaenoic acid for their survival and differentiation (Rotstein *et al*. 1996, 1997, 1998). Both IGF-I and insulin have been previously shown to be unable to protect

photoreceptors from the damaging effect of constant light (LaVail *et al*. 1992). However, inhibition of PI 3-kinase led to some impairment in their survival and process formation; this suggests that photoreceptors and amacrine cells may share a common effector, PI 3-kinase, apparently regulated by different survival signals.

The c-Jun transcription factor, proposed to act downstream of PI-3 kinase (D'Mello *et al*. 1997; Okubo *et al*. 1998), may control both neuronal survival and death (Herdegen *et al*. 1997). An increase in its expression and subsequent phosphorylation is necessary for cell death upon trophic factor withdrawal in several systems (Estus *et al*. 1994; Ham *et al*. 1995; Watson *et al*. 1998; Eilers *et al*. 1998). In insulin-lacking cultures, the number of amacrine cells expressing c-Jun rapidly increased with time; once expressed, c-Jun was hastily phosphorylated suggesting a high basal activity of the c-Jun kinases. A similar result was observed in wortmannin-treated cultures by day 7; the differences in c-Jun expression in insulin-lacking and wortmannin-treated cultures by day 4 are consistent with the different evolution of apoptosis in both experimental conditions discussed above. It was unexpected to discover a higher proportion of c-Jun in the cytoplasm than in the nucleus; however, cytoplasmic detection of c-Jun has also been reported during PCD of spinal cord motoneurons (Ayala *et al*. 1999). The number of amacrine cells expressing c-Jun was much lower in insulin-supplemented cultures. Our results showed that a low c-Jun expression was characteristic of healthy amacrine neurons and an increased expression correlated with and preceded the evolution of apoptosis induced by either insulin deprivation or PI 3-kinase inhibition. This suggests that insulin might down-regulate c-Jun expression by a pathway that may depend on PI 3-kinase activation. Further studies are necessary to establish the role of c-Jun during amacrine cell apoptosis.

In conclusion, our results suggest that IGF-I acts as a trophic factor for amacrine neurons regulating their survival and differentiation. PI 3-kinase seems to be required for the intracellular signaling and regulation of the pathways leading to amacrine cell survival.

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References

Ayala V., Casas C., Ribera J., Caldero J., Oppenheim R. W. and Esquerda J. E. (1999) Specific association of c-Jun-like immunoreactivity but not c-Jun p39 with normal and induced programmed cell death in the chick embryo. *J. Neurobiol.* **38**, 171–190.

Barber A. J., Lieth E., Khin S. A., Antonetti D. A., Buchanan A. G. and Gardner T. W. (1998) Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. *J. Clin. Invest.* **102**, 783–791.

Barde Y., Davies A., Johnson J., Lindsay R. and Thoenen H. (1987) Brain derived neurotrophic factor. *Prog. Brain Res.* **71**, 185–189.

Barnstable C. J. (1980) Monoclonal antibodies which recognize different cell types in the rat retina. *Nature* **286**, 231–235.

Bottenstein J. E. (1985) Growth and differentiation of neural cells in defined media, in *Cell Culture in the Neurosciences* (Bottenstein J. E. and Sato G., eds), pp. 3–43. Plenum Press, NY and London.

Bovolenta P., Frade J. M., Marti E., Rodriguez-Peña M. A., Barde Y. A. and Rodriguez-Tebar A. (1996) Neurotrophin-3 antibodies disrupt the normal development of the chick retina. *J. Neurosci.* **16**, 4402–4410.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Burgering B. M. and Coffer P. J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599–602.

Cellerino A., Pinzon-Duarte G., Carroll P. and Kohler K. (1998) Brain-derived neurotrophic factor modulates the development of the dopaminergic network in the rodent retina. *J. Neurosci.* **18**, 3351–3362.

Crowder R. J. and Freeman R. S. (1998) Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. *J. Neurosci.* **18**, 2933–2943.

D'Mello S. R., Borodezt K. and Soltoff S. P. (1997) Insulin-like growth factor and potassium depolarization maintain neuronal survival by distinct pathways: possible involvement of PI 3-kinase in IGF-I signaling. *J. Neurosci.* **17**, 1548–1560.

De la Rosa E., Arribas A., Frade J. M. and Rodriguez-Tebar A. (1994) Role of neurotrophins in the control of neural development: neurotrophin-3 promotes both differentiation and survival of cultured retinal cells. *Neuroscience* **58**, 347–352.

De Pablo F. and De la Rosa E. J. (1995) The developing CNS: a scenario for the action of proinsulin, insulin and insulin-like growth factors. *Trends Neurosci.* **18**, 143–150.

Diaz B., Pimentel B., De Pablo F. and De la Rosa E. J. (1999) Apoptotic cell death in proliferating neuroepithelial cells in the embryonic retina is prevented by insulin. *Eur. J. Neurosci.* **11**, 1624–1632.

Doré S., Satyabrata K. and Quirion R. (1997) Rediscovering an old friend, IGF-I: potential use in the treatment of neurodegenerative diseases. *Trends Neurosci.* **20**, 323–331.

Dudek H., Datta S. R., Franke T. F., Birnbaum M. J., Yao R., Cooper G. M., Segal R. A., Kaplan D. R. and Greenberg M. E. (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275**, 661–665.

Eilers A., Whitfield J., Babij C., Rubin L. L. and Ham J. (1998) Role of c-June kinase pathway in the regulation of c-June expression and apoptosis in sympathetic neurons. *J. Neurosci.* **18**, 1713–1724.

Estus S., ZaksV., Freeman R. S., Gruda M., Bravo R. and Johnson E. M. (1994) Altered gene expression in neurons during programmed cell death identification of c-jun as necessary for neuronal apoptosis. *J. Cell Biol.* **127**, 1717–1727.

Feldman E. L., Sullivan K. A., Kim B. and Russell J. W. (1997) Insulin-

like growth factors regulate neuronal differentiation and survival. *Neurobiol. Dis.* **4**, 201–214.

Fontaine V., Kinkl N., Sahel J., Dreyfus H. and Hicks D. (1998) Survival of purified rat photoreceptors in vitro is stimulated directly by fibroblast growth factor-2. *J. Neurosci.* **18**, 9662–9672.

Frade J. M., Marti E., Bovolenta P., Rodríguez Pena M. A., Pérez García D., Rohrer H., Edgar D. and Rodríguez Tebar A. (1996) Insulin-like growth factor-I stimulates neurogenesis in chick retina by regulating expression of the alpha 6 integrin subunit. *Development* **122**, 2497–2506.

Guo X., Ghalayini A. J., Chen H. and Anderson R. E. (1997) Phosphatidylinositol 3-kinase in bovine photoreceptor rod outer segments. *Invest. Ophthalmol. Vis. Sci.* **38**, 1873–1882.

Ham J., Babij C., Whitfield J., Pfarr C. M., Lallemand D., Yaniv M. and Rubin L. L. (1995) A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron* **14**, 927–939.

Herdegen T., Skene P. and Bahr M. (1997) The c-Jun transcription factor – bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci.* **20**, 227–231.

Hernández-Sánchez C., Lopez-Carranza A. L., Alarcon C., De La Rosa E. J. and De Pablo F. (1995) Autocrine/Paracrine role of insulin-related growth factors in neurogenesis: local expression and effects on cell proliferation and differentiation in retina. *Proc. Natl Acad. Sci. USA* **92**, 9834–9838.

Hicks D. and Barnstable C. (1987) Different rhodopsin monoclonal antibodies reveal different binding patterns on developing and adult rat retina. *J. Histochem. Cytochem.* **35**, 1317–1328.

Hicks D. and Courtois Y. (1992) Fibroblast growth factor stimulates photoreceptor differentiation in vitro. *J. Neurosci.* **12**, 2022–2033.

Ishii D. N. and Lupien S. B. (1995) Insulin-like growth factors protect against diabetic neuropathy: effects on sensory nerve regeneration in rats. *J. Neurosci. Res.* **40**, 138–144.

Jordán J., Galindo M. F., Prehn J. H. M., Weicheselbaum R. R., Beckett M., Ghadge G. D., Roos R. P., Leiden J. M. and Miller R. J. (1997) p53 expression induces apoptosis in hippocampal pyramidal neuron cultures. *J. Neurosci.* **17**, 1397–1405.

Kapeller R. and Cantley L. C. (1994) Phosphatidylinositol 3-kinase. *Bioessays* **16**, 565–576.

Kermer P., Klocker N., Labes M. and Bahr M. (2000) Insulin-like growth factor-I protects axotomized rat retinal ganglion cells from secondary death via PI_3-K-dependent Akt phosphorylation and inhibition of caspase-3 in vivo. *J. Neurosci.* **20**, 722–728.

Kljavin I. J., Lagenaur C., Bixby J. L. and Reh T. A. (1994) Cell adhesion molecules regulating neurite growth from amacrine and rod photoreceptor cells. *J. Neurosci.* **14**, 5035–5049.

Kobayashi M., Nagata S., Kita Y., Nakatsu N., Ihara S., Kaibuchi K., Kuroda S., Ui M., Iba H., Konishi H., Kikkawa U., Saitoh I. and Fukui Y. (1997) Expression of a constitutively active phosphatidylinositol 3-kinase induces process formation in rat PC12 cells. *J. Biol. Chem.* **272**, 16089–16092.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **227**, 680–685.

LaVail M. W., Unoki K., Yasumura D., Matthes M. T., Yancopoulos G. D. and Steinberg R. H. (1992) Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc. Natl Acad. Sci. USA* **89**, 11249–11253.

LaVail M. W., Yasumura D., Matthes M. T., Lau-Villacorta C., Unoki K., Sung C. H. and Steinberg R. H. (1998) Protection of mouse photoreceptors by survival factors in retinal degenerations. *Invest. Ophthalmol. Vis. Sci.* **39**, 592–602.

Lee W. H., Javedan S. and Bondy C. A. (1992) Coordinate expression of insulin-like growth factor system components by neurons and neuroglia during retinal and cerebellar development. *J. Neurosci.* **12**, 4737–4744.

Lipton S., Wagner J., Madison R. and D'Amore P. (1988) Acidic fibroblast growth factor enhances regeneration of process by postnatal mammalian retinal ganglion cells in culture. *Proc. Natl Acad. Sci. USA* **85**, 2388–2392.

McNeil M. and Masland R. H. (1998) Extreme diversity among amacrine cells: implications for function. *Neuron* **20**, 971–982.

Nicotera P., Leist M. and Manzo L. (1999) Neuronal cell death: a demise with different shapes. *Trends Pharmacol. Sci.* **20**, 46–51.

Nielsen F. C., Wang E. and Gammeltoft S. (1991) Receptor binding, endocytosis and mitogenesis of insulin-like growth factors I and II in fetal rat brain neurons. *J. Neurochem.* **56**, 12–21.

Okubo Y., Blakesley V. A., Stannard B., Gutkind S. and Le Roith D. (1998) Insulin-like growth factor-I inhibits the stress-activated protein kinase/c-Jun N-terminal kinase. *J. Biol. Chem.* **273**, 25961–25966.

Politi L. E., Lehar M. and Adler R. (1988) Development of neonatal mouse retinal neurons and photoreceptors in low density cell culture. *Invest. Opthalmol. Vis. Sci.* **29**, 534–543.

Politi L. E., Insua F. and Buzzi E. (1998) Selective outgrowth and differential tropism of amacrine and photoreceptor axons to cell targets during early development in vitro. *J. Neurosci. Res.* **52**, 105–117.

Pong K., Xu R. Y., Baron W. F., Louis J. C. and Beck K. D. (1998) Inhibition of phosphatidylinositol 3-kinase activity blocks cellular differentiation mediated by glial cell-line-derived neurotrophic factor in dopaminergic neurons. *J. Neurochem.* **7**, 1912–1919.

Raff M. C. (1992) Social controls on cell survival and cell death. *Nature* **356**, 397–400.

Raff M. C., Barres B. A., Burne J. F., Coles H. S., Ishizaki Y. and Jacobson M. D. (1993) Programmed cell death and the control of cell survival: lessons from the nervous system. *Science* **262**, 695–700.

Rodrígues M., Waldbillig R. J., Rajagopalan S., Hackett J., LeRoith D. and Chader G. J. (1988) Retinal insulin receptors: localization using a polyclonal anti-insulin receptor antibody. *Brain Res.* **443**, 389–394.

Rotstein N. P., Aveldaño M. I., Barrantes F. J. and Politi L. E. (1996) Docosahexaenoic acid is required for the survival of rat retinal photoreceptors in vitro. *J. Neurochem.* **66**, 1851–1859.

Rotstein N. P., Aveldaño M. I., Barrantes F. J., Roccamo A. M. and Politi L. E. (1997) Apoptosis of retinal photoreceptors during development in vitro: protective effect of docosahexaenoic acid. *J. Neurochem.* **69**, 504–513.

Rotstein N. P., Politi L. E. and Aveldaño M. I. (1998) Docosahexaenoic acid promotes differentiation of developing photoreceptors in culture. *Invest. Ophthalmol. Vis. Sci.* **39**, 2750–2758.

Shepherd P. R., Withers D. and Siddle K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem. J.* **333**, 471–490.

Singleton J. R., Dixit V. M. and Feldman E. L. (1996) Type I insulin-like growth factor receptor activation regulates apoptotic proteins. *J. Biol. Chem.* **271**, 31791–31794.

Spaventi R., Antica M. and Pavelic K. (1990) Insulin and insulin-like growth factor I (IGF I) in early mouse embryogenesis. *Development* **108**, 491–495.

Toker A. and Cantley L. C. (1997) Signaling through the lipid products of phosphoinositide-3-kinase. *Nature* **387**, 673–676.

Waldbillig R. J. and Chader G. J. (1988) Anomalous insulin-binding activity in the bovine neural retina: a possible mechanism for regulation of receptor binding specificity. *Biochem. Biophys. Res. Commun.* **15**, 1105–1112.

Waldbillig R. J., Fletcher R. T., Chader G. J., Rajagopalan S., Rodrigues M. and LeRoith D. (1987) Retinal insulin receptors. 1. Structural heterogeneity and functional characterization. *Exp. Eye Res.* **45**, 823–835.

Waldbillig R. J., Fletcher R. T., Somers R. L. and Chader G. J. (1988) IGF-I receptors in the bovine neural retina: structure, kinase activity and comparison with retinal insulin receptors. *Exp. Eye Res.* **47**, 587–607.

Watson A., Eilers A., Lallemand D., Kyriakis J., Rubin L. L. and Ham J. (1998) Phosphorylation of c-jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J. Neurosci.* **18**, 751–762.

Yao R. and Cooper G. M. (1995) Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267**, 2003–2006.

Yenush L. and White M. F. (1997) The IRS-signaling system during insulin and cytokine action. *Bioessays* **19**, 491–500.

Zhuang H. X., Wuarin L., Fei Z. J. and Ishii D. N. (1997) Insulin-like growth factor (IGF) gene expression is reduced in neural tissues and liver from rats with non-insulin-dependent diabetes mellitus, and IGF treatment ameliorates diabetic neuropathy. *J. Pharmacol. Exp. Ther.* **283**, 366–374.