The Journal of Neuroscience, April 1992, 12(4): 1558–1566

IGF-I and IGF-II Protect Cultured Hippocampal and Septal Neurons against Calcium-mediated Hypoglycemic Damage

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Insulin and insulin-like growth factors I and II (IGF-I and IGF-II) have recently been shown to have biological activity in central neurons, but their normal functions and mechanisms of action in the brain are unknown. Since central neurons are particularly vulnerable to hypoglycemia that results from ischemia or other insults, we tested the hypothesis that growth factors can protect central neurons against hypoglycemic damage *in vitro*. IGF-I and IGF-II (3–100 ng/ml) each prevented glucose deprivation–induced neuronal damage in a dose-dependent manner in rat hippocampal and septal cell cultures. High concentrations of insulin (greater than 1 μg/ml) also protected neurons against hypoglycemic damage. Epidermal growth factor did not protect against hypoglycemic damage. Both IGFs and insulin were effective when administered 24 hr before or immediately following the onset of glucose deprivation. Direct measurements of intraneuronal calcium levels and manipulations of calcium influx demonstrated that calcium influx and sustained elevations in intraneuronal calcium levels mediated the hypoglycemic damage. IGF-I and IGF-II each prevented the hypoglycemia-induced elevations of intraneuronal free calcium. Studies with excitatory amino acid receptor antagonists and calcium channel blockers indicated that NMDA receptors did, and L-type calcium channels did not, play a major role in hypoglycemic damage. Taken together, these findings indicate that IGFs can stabilize neuronal calcium homeostasis and thereby protect against hypoglycemic damage.

Insulin and insulin-like growth factors I and II (IGF-I and IGF-II) are closely related polypeptides that are similar in their structures and are believed to have overlapping biological functions (Rinderknecht and Humbel, 1976, 1978a,b; Humbel, 1984; Czech, 1985; Froesch et al., 1985; Rechler and Nissley, 1985; Baskin, 1987; Baskin et al., 1988; Garofalo and Rosen, 1989; Knusel et al., 1990; Sara and Hall, 1990). Insulin regulates the uptake, cellular transport, and intermediary metabolism of small nutrient molecules such as glucose, amino acids, and fatty acids in muscle and adipose tissues. Recently, insulin and IGFs (and/or their mRNAs) have been localized in the CNS (Havrankova et al., 1978; Sara et al., 1982, 1986; Rechler and Nissley, 1985; Baskin et al., 1988; Rotwein et al., 1988), suggesting that they may have biological activity in the brain. In brain cell cultures, insulin, IGF-I, and IGF-II promote neuronal survival, neurite outgrowth, and gene expression (Bhat, 1983; Lenoir and Honegger, 1983; Mill et al., 1985; Recio-Pinto et al., 1986; Aizenman and DeVellis, 1987; Kyriakis et al., 1987; Avola et al., 1988; Knusel et al., 1990; Drago et al., 1991). Insulin, IGF-I, and IGF-II are each associated with a distinct cell surface receptor (Rechler and Nissley, 1985). The IGF-I receptor is structurally and functionally similar to the insulin receptor in that it possesses an α-subunit that binds the hormone-like agent and a β-subunit that has a tyrosine-specific protein kinase (Ebina et al., 1985; Ullrich et al., 1985, 1986). The IGF-II receptor is different in structure from the IGF-I receptor or insulin receptor and appears to be identical to an intracellular mannose-6-phosphate receptor (Morgan et al., 1986, 1987).

Insulin, IGF-I, and IGF-II can be produced in cultured neurons and have been reported to occur in the brain (Binoux et al., 1981; Weyhenmeyer and Fellows, 1983; Davies et al., 1986; Ballotti et al., 1987). mRNAs for both IGFs and insulin have been detected in many brain regions (Haselbacher et al., 1985; Young, 1986; Baskin et al., 1988; Rotwein et al., 1988). There is abundant evidence for the existence of receptors for insulin and IGFs in the brain (Raizada et al., 1982, 1988; Hill et al., 1986; Burgess et al., 1987; Mendlesohn, 1987; Waldbillig and LeRoith, 1987; Bohanon et al., 1988; Lesniak et al., 1988; Garofalo and Rosen, 1989). IGF-I receptor mRNA is widely distributed in the brain, whereas insulin receptor mRNA is restricted to certain areas and appears to be coexpressed with the IGF-I receptor mRNA (Baron-Van Evercooren et al., 1991). Receptors for IGF-I and IGF-II have been found to be located in hippocampus (Lesniak et al., 1988; Araujo et al., 1989). IGF-II receptor mRNA distribution in brain has not been examined.

A continuous supply of glucose is necessary for the normal functioning and survival of mammalian central neurons. Hypoglycemia results in increased utilization of endogenous substrates, depletion of ATP, membrane depolarization, extracellular accumulation of excitatory amino acids, loss of neuronal ion homeostasis, and ultimately neuronal death (Siesjo et al., 1988). Calcium normally serves physiologically important functions as a second messenger regulating neuronal plasticity (Lynch et al., 1983; Kater et al., 1988). However, excessive and sustained elevations in intracellular calcium are involved in neuronal degeneration caused by metabolic and environmental insults (Choi, 1988; Siesjo et al., 1988; Mattson, 1992). In the CNS, the excitatory neurotransmitter glutamate contributes to neuronal vulnerability to insults such as hypoglycemia and hyp-

Received Sept. 6, 1991; revised Nov. 19, 1991; accepted Nov. 22, 1991.

We thank S. Bose, J. Mattson, and B. Rychlik for technical help, and Drs. R. E. Rydel and J. Geddes for insightful comments on this work. This research was supported by NIH Grants NS29001 and AG05144, and the International Life Sciences Institute.

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oxia by enhancing calcium influx (Choi, 1988). The cellular and molecular mechanisms that normally protect neurons against such insults are largely unknown. In the present study we employed hippocampal and septal cell cultures to test the hypothesis that IGFs can stabilize neuronal calcium homeostasis and protect central neurons against hypoglycemic injury.

Materials and Methods

Hippocampal and septal cultures. The hippocampal and septal culture methods employed were similar to those of Mattson and Kater (1988) and of Hartikka and Hefti (1988), respectively. Briefly, rat hippocampi and septal areas were obtained from 18 d Sprague–Dawley fetuses and incubated for 15 min in a solution of 2 mg/ml trypsin in Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution buffered with 10 mM HEPES (HBSS; GIBCO). The hippocampi and septal areas were then rinsed once in HBSS, followed by a 5 min incubation in HBSS containing 1 mg/ml trypsin inhibitor (Sigma), and a final rinse in HBSS. Tissues were then dissociated by trituration through the narrowed bore of a fire-polished Pasteur pipette and were distributed to polylysine-coated plastic culture dishes (Corning) containing 2 ml of Eagle's minimum essential medium (GIBCO) buffered with 10 mM sodium bicarbonate and supplemented 10% (v/v) with fetal bovine serum (Sigma), 2 mM L-glutamine, 20 mM KCl, 1 mM pyruvate, and 40 mM glucose. The culture density was 80–120 cells/mm² of culture surface. Cultures were maintained at 37°C in a 6% CO_2/94% room air, humidified incubator. All experiments were done with neurons that had been in culture for 8–15 d. In some experiments, glial proliferation was halted by exposing cultures to either 10 μM cytosine arabinoside (Ara-C) or 20 μM 5-fluoro-2′-deoxyuridine for 2–3 d (culture days 3–5).

Assessment of neuronal survival. Neuronal damage was assessed by our well-established morphological criteria, which correlate well with vital dye staining methods (Mattson et al., 1988). Briefly, cultures were visualized and photographed with a phase-contrast Nikon Diaphot inverted microscope. Neurons were scored as viable if they had neurites that were uniform in diameter and smooth in appearance, and somata that were smooth and round to oval in shape. In degenerating nonviable neurons, neurites were fragmented and beaded, and the soma was rough, swollen, vacuolated, and irregular in shape. Subsequent to these morphological changes, the degenerated neurons detached from the culture substrate. Viable neurons in premarked culture regions (four regions of approximately 1 mm²/culture) were counted immediately prior to and 18–24 hr following glucose deprivation. Statistical comparisons were done using pairwise Student's *t* tests.

Glucose deprivation and experimental treatments. Glucose deprivation was carried out by removing the culture maintenance medium and washing three times with glucose-free Locke's solution. Locke's solution contained (in mM) NaCl, 154; KCl, 5.6; $CaCl_2$, 2.3; $MgCl_2$, 1.0; $NaHCO_3$, 3.6; and HEPES buffer, 5. Calcium-deficient medium consisted of glucose-free Locke's solution lacking added calcium. Cultures were washed thoroughly (six 2 ml washes) with calcium-deficient Locke's immediately prior to glucose deprivation. Growth factors were prepared as 100–1000× stocks in water and were added directly to the cultures. Cultures were pretreated with growth factor for 24 hr prior to the onset of hypoglycemia, and the growth factors were included in the glucose-free medium during the period of hypoglycemia. Insulin (bovine) and calcitonin gene-related peptide (CGRP) were from Sigma, while IGF-I and IGF-II (human, recombinant) and epidermal growth factor (EGF; from mouse submaxillary glands) were from Boehringer Mannheim. D-γ-Glutamylglycine (DGG), DL-2-amino-5-phosphonovaleric acid (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 6,7-dinitroquinoxaline-2,3-dione (DNQX) were from Tocris Neuramin; these agents were prepared as stocks in Locke's solution. Nifedipine, nimodipine, verapamil, diltiazem, flunarizine, lidoflazine, and trifluoperazine (Sigma) were prepared as 20–500× stocks in dimethyl sulfoxide. Equivalent volumes of vehicle were added to control cultures and did not affect neuronal survival.

Fura-2 measurements of intraneuronal calcium levels. For these studies cells were grown in glass-bottom dishes (Mat Tek, Inc.) coated with 0.05% polyethylenimine. Intraneuronal free calcium levels were measured 14–16 hr after the onset of glucose deprivation. The procedures for fura-2 fluorescence ratio imaging were similar to those of our past work (Mattson et al., 1989). Briefly, the cells were loaded at 37°C for 40 min with 4 μM fura-2 acetoxymethyl ester (Molecular Probes). The loaded cells were then washed two times with Locke's solution containing 2.3 mM $CaCl_2$ (with or without glucose) and incubated an additional 60 min prior to imaging to allow deesterification of the fura-2. Cells were viewed on an inverted Nikon microscope with a fluoro 40×, NA 1.3 objective and an intensified CCD Camera (Quantex). A Quantex imaging system with QFM software was used to acquire and process the images. Intracellular free Ca^{2+} levels were determined from the ratio of the fluorescence emission using two different excitation wavelengths (350 nm and 380 nm). Background fluorescence at each wavelength (background images were taken from regions of culture dish not containing cells) was subtracted from the cell image at that wavelength. The system was calibrated according to the procedures described by Grynkiewicz et al. (1985). Measurements were taken in neuronal cell bodies, and values represent the average free calcium level therein. Statistical comparisons were made using Student's *t* test.

Results

IGF-I, IGF-II, and insulin protect hippocampal and septal neurons against hypoglycemic damage

Incubation of rat hippocampal and septal cultures in glucose-free culture medium resulted in highly significant neuronal damage and death during 18 hr (hippocampal) or 24 hr (septal) exposure periods when compared with cultures maintained in medium containing 5–40 mM glucose (Fig. 1, Table 1). Approximately 85–95% of rat hippocampal and septal neurons degenerated during these glucose deprivation periods (n = 4–10 separate experiments for each treatment condition, 3–4 cultures/experiment). When rat hippocampal and septal cultures were pretreated for 24 hr with 100 ng/ml of either IGF-I or IGF-II and then deprived of glucose for 18–24 hr, there was a dramatic reduction in neuronal damage compared to glucose-deprived cultures not receiving a growth factor (Fig. 1, Table 1). Neuronal survival was increased to approximately 70% in IGF-treated cultures as compared with 5–15% in the untreated glucose-deprived cultures (n = 4–10 separate cultures/treatment group; $p < 0.001$). Both IGF-I and IGF-II were able to sustain hippocampal and septal neurons for up to 40 hr in the absence of glucose (n = 4 separate cultures). Insulin at 100 ng/ml did not protect neurons against hypoglycemic damage (Table 1). The data presented in the dose–response curve in Figure 2 demonstrate that very low concentrations of IGF-I and IGF-II (1–10 ng/ml, approximately 150–1500 pM) can significantly protect against neuronal damage in both hippocampal and septal cultures. Insulin at concentrations up to 300 ng/ml (approximately 60 nM) did not protect against hypoglycemic damage. However, higher concentrations of insulin (1 μg/ml or greater) did significantly protect both hippocampal and septal neurons against hypoglycemic damage (Figs. 1, 2). EGF from 1 to 100 ng/ml failed to prevent neuronal damage after glucose deprivation. Similarly, no effects were observed with 100 nM CGRP.

Since both rat hippocampal and septal cultures contained glia (predominately type I astrocytes; cf. Mattson et al., 1988), we determined whether reducing the number of glial cells in the culture would influence the protective effects of IGFs against glucose deprivation. Glial cell proliferation was inhibited by the addition of 10 μM Ara-C to the cultures (Fig. 3). Our preliminary experiments showed that adding Ara-C to hippocampal cultures reduced the number of astrocytes to less than 5–10% of the total cell number without significantly affecting the number of neurons. IGF-I and IGF-II were found to protect against neuronal death induced by glucose deprivation in glia-depleted cultures (Fig. 3). Similar results were obtained in septal cultures (data not shown). These data suggested that the neuroprotective actions of IGF-I and IGF-II were not mediated by glia, although

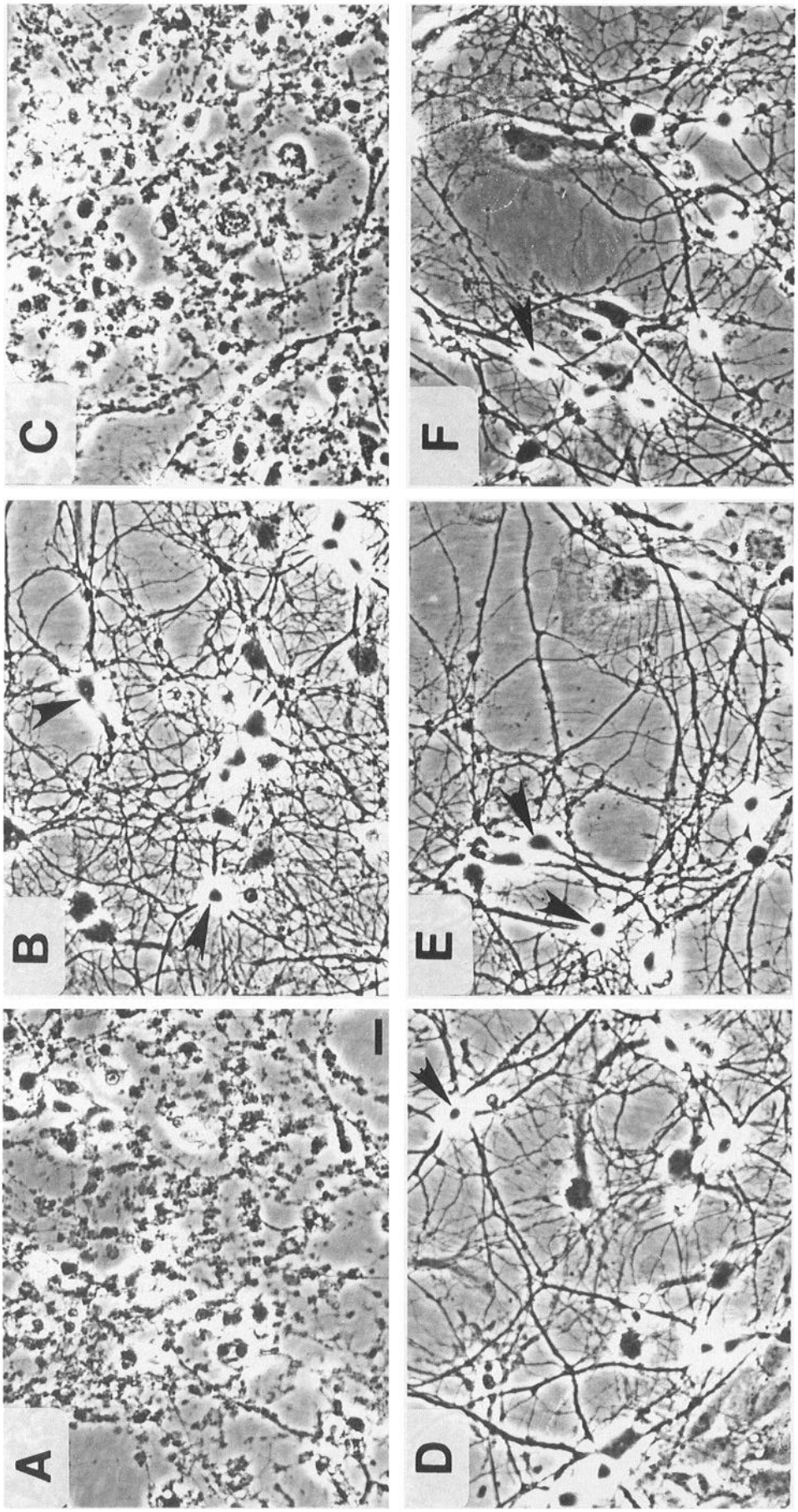

Figure 1. Hypoglycemic neuronal damage can be prevented by IGF-I, IGF-II, and insulin: phase-contrast micrographs from cell cultures of rat hippocampal neurons. *A*, After 18 hr of incubation in glucose-free medium; note massive damage to somata and neurites. *B*, After 18 hr of incubation in medium containing 20 mM glucose, somata (*arrowheads*) and neurites appear undamaged. *C*, A culture that had been pretreated with 100 ng/ml EGF for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. *D*, A culture that had been pretreated with 1 μg/ml insulin for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. *E*, A culture that had been pretreated with 100 ng/ml IGF-I for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. *F*, A culture that had been pretreated with 100 ng/ml IGF-II for 24 hr prior to the onset of glucose deprivation is shown after 18 hr of glucose deprivation. Scale bar, 10 μm.

Table 1. Effects of growth factors and calcium removal on neuronal damage induced by glucose deprivation in cultured rat hippocampal and septal neurons

	Neuronal survival (% of initial number)	
	Hippocampus	Septum
0 glucose	6.2 ± 2.6	9.84 ± 2.5
5 mM glucose	74.82 ± 9.3*	80.82 ± 6.7*
20 mM glucose	77.91 ± 8.4*	79.38 ± 7.2*
40 mM glucose	83.75 ± 9.4*	87.56 ± 10.2*
0 glucose + 100 ng/ml IGF-I	63.49 ± 7.7*	81.22 ± 9.8*
0 glucose + 100 ng/ml IGF-II	59.89 ± 6.3*	74.89 ± 6.7*
0 glucose + 100 ng/ml insulin	13.38 ± 4.5	12.31 ± 4.4
0 glucose + 100 ng/ml EGF	14.29 ± 4.8	11.37 ± 3.8
0 glucose + 0 $[Ca^{2+}]_o$	75.62 ± 7.3*	81.48 ± 5.9*
0 glucose + 100 nM CGRP	3.50 ± 1.1	4.92 ± 2.4

Cultures were incubated in the presence of growth factors for 24 hr prior to exposure to glucose-free medium. Neuronal survival was assessed 18 hr (hippocampus) or 24 hr (septum) following the onset of glucose deprivation. Values represent the mean ± SEM of determinations made in 4–10 separate cultures per treatment group.

* $p < 0.001$, as compared to corresponding values for treatments with 0 glucose, 0 glucose + insulin, 0 glucose + EGF, or 0 glucose + CGRP.

the possibility that the few remaining glia played a role in the action of the IGFs cannot be ruled out.

IGFs protect neurons against hypoglycemic damage by preventing a loss of cellular calcium homeostasis

Neuronal damage that occurs as the result of ischemia and excitotoxic insults results largely from aberrant elevations in intracellular calcium levels (Choi, 1988; Siesjo et al., 1988; Mattson, 1992). We therefore determined whether hypoglycemic neuronal damage was calcium dependent, and whether IGFs and insulin modified hypoglycemia-induced calcium responses. Hippocampal and septal cultures were incubated in medium lacking extracellular calcium during the period of glucose deprivation in order to prevent calcium influx through the plasma membrane (cf. Mattson et al., 1988). Hypoglycemic damage was significantly reduced in the cultures maintained in the calcium-deficient medium as compared to cultures maintained in the normal medium that contained 2.3 mM Ca^{2+} (Fig. 3, Table 1). These data indicated that calcium influx was necessary for hypoglycemic damage and suggested that glucose deprivation might result in a loss of neuronal calcium homeostasis. We therefore employed the calcium indicator dye fura-2 to determine the effects of hypoglycemia and growth factors on intraneuronal calcium levels.

Glucose deprivation caused a highly significant three- to five-fold elevation in intraneuronal calcium levels in both hippocampal and septal neurons during 14–16 hr periods (Table 2). IGF-I and IGF-II (100 ng/ml) each prevented the glucose deprivation–induced intraneuronal calcium increase. EGF did not prevent increase in intraneuronal calcium after glucose deprivation (Table 2). The results demonstrated that a loss of neuronal calcium homeostasis accompanied the hypoglycemic damage, and that IGFs stabilized intracellular calcium levels.

Involvement of excitatory amino acid receptors in hypoglycemic damage

Ischemic neuronal damage *in vivo* (Simon et al., 1984) and *in vitro* (Goldberg et al., 1987) involves excitatory amino acid

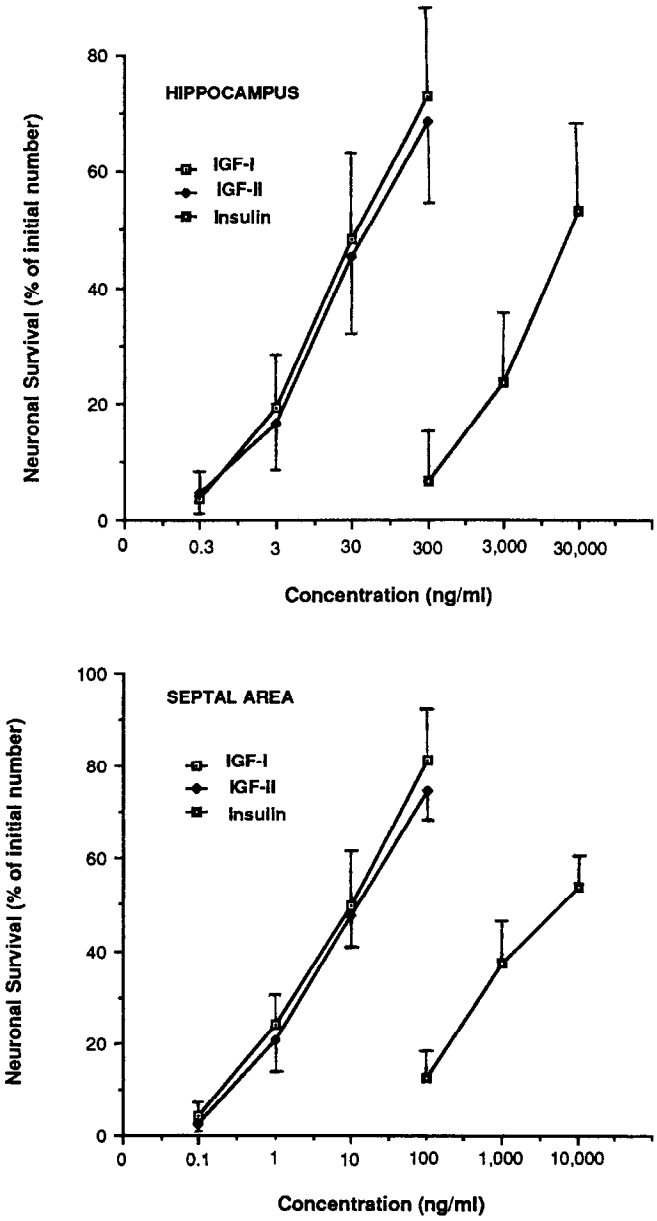

Figure 2. Dose–response curves for the protective effects of growth factors on rat hippocampal (*upper*) and septal (*lower*) cultures deprived of glucose. Cultures that had been pretreated for 24 hr with different concentrations of growth factors were exposed to glucose-free medium, and neuronal survival was assessed 18 hr (hippocampal cultures) or 24 hr (septal cultures) later. Values represent the mean and SEM of determinations made in three or four separate experiments.

receptor activation resulting in calcium influx. Since calcium influx was involved in the hypoglycemic damage in the present study, we assessed the involvement of excitatory amino acid receptors in the degenerative process. The NMDA receptor antagonist APV (100 μM) and the broad-spectrum glutamate antagonist DGG (100 μM) each prevented the elevation in intraneuronal calcium levels and neuronal damage induced by glucose deprivation (Fig. 4). The kainate/AMPA receptor–specific antagonists CNQX (100 μM) and DNQX (100 μM) did not protect against hypoglycemic damage in either hippocampal or septal cultures. APV, DGG, CNQX, and DNQX alone had no significant effect on neuronal survival in cultures maintained in

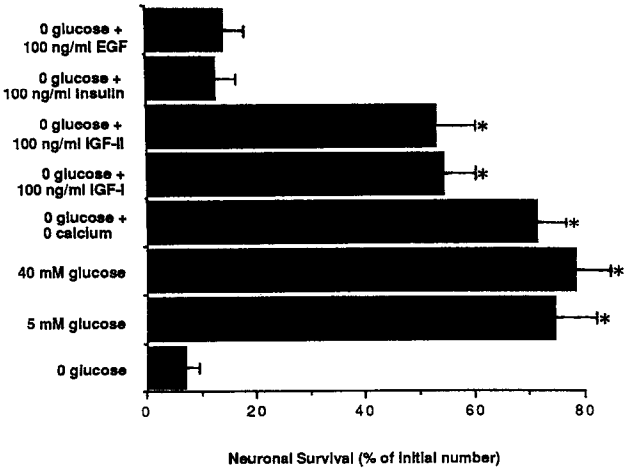

Figure 3. Evidence supporting a direct action of IGF-I and IGF-II in rat hippocampal neurons. Non-neuronal cell division was halted by a 3 d exposure to 10 μM Ara-C. Cultures were then exposed to a growth factor for 24 hr followed by an 18 hr exposure to medium lacking glucose. Values represent the mean and SEM of determinations made in three or four separate cultures. *, $p < 0.001$ compared with 0 glucose alone, 0 glucose + insulin, and 0 glucose + EGF.

glucose-containing medium (data not shown; cf. Mattson et al., 1988; Facci et al., 1990).

Effects of calcium channel blockers and kinase inhibitors on hypoglycemic neuronal damage

Calcium influx through voltage-sensitive channels (Siesjo et al., 1988) and overactivation of protein kinases (Favaron et al., 1990; Mattson, 1991) have been implicated in ischemic/excitotoxic neuronal damage. Previous work indicated that blockers of L-type calcium channels could protect neurons against excitotoxicity (Weiss et al., 1990). It was therefore of interest to determine whether voltage-sensitive calcium channels and/or calcium-dependent protein kinases played a role in glucose deprivation–induced neuronal damage. The dihydropyridine nifedipine (100 μM), and verapamil (10 μM), did not protect hippocampal neurons against death caused by glucose deprivation (Fig. 5). Similarly, two other L-type calcium channel blockers, diltiazem (10 μM) and nimodipine (20 μM), were ineffective in protecting hippocampal neurons against hypoglycemic damage (data not shown). These concentrations of calcium channel blockers did not significantly affect neuronal survival in hippocampal cultures incubated in the presence of glucose ($n = 4$ separate cultures). Similar results were obtained in septal cultures with these calcium channel blockers (data not shown). Taken together with the data above, these results indicated that calcium influx was responsible for the hypoglycemic neuronal damage, and that the damaging calcium influx occurred through the NMDA receptor channel and/or through non-L-type channels.

In order to determine whether calcium/calmodulin-dependent protein kinases were involved in the hypoglycemic damage, we employed calmodulin inhibitors that had previously been shown to protect neurons in several different paradigms of neuronal death (Rich and Hollowell, 1990; Mattson, 1991). Flunarizine (10 μM) did not afford significant protection against hypoglycemic damage (Fig. 5). Two other calmodulin inhibitors, lidoflazine (10 μM) and trifluoperazine (100 nM), also did not protect neurons against hypoglycemic injury (data not shown). Since recent evidence indicated that overactivation of protein kinase C (PKC) can result in neurodegeneration (Favaron et al., 1990; Mattson, 1991), we determined whether the PKC inhibitor H-7 would modify hypoglycemic damage. H-7 (5 μM) did not prevent neuronal damage after 18 hr of glucose deprivation in hippocampal cell cultures (Fig. 5). Flunarazine, lidoflazine, and H-7, at the same concentrations as were added to glucose-deprived cultures, did not significantly affect neuronal survival in hippocampal cultures incubated in the presence of glucose; however, trifluoperazine did cause significant neuronal degeneration (data not shown). These data indicate that blockade of calcium/calmodulin-dependent kinases and PKC will not prevent glucose deprivation-induced neuronal death.

Table 2. IGF-I and IGF-II prevent the increase in intraneuronal free calcium caused by glucose deprivation

	Intraneuronal calcium concentration (nM)	
	Hippocampus	Septum
0 glucose	346 ± 23	320 ± 20
5 mM glucose	91 ± 13*	84 ± 9*
20 mM glucose	81 ± 9*	84 ± 8*
40 mM glucose	89 ± 10*	80 ± 10*
0 glucose + 100 ng/ml IGF-I	68 ± 8*	78 ± 9*
0 glucose + 100 ng/ml IGF-II	72 ± 12*	83 ± 7*
0 glucose + 100 ng/ml EGF	265 ± 26	324 ± 17
0 glucose + 0 $[Ca^{2+}]_o$	82 ± 10*	79 ± 8*

Cultures were incubated in the presence of growth factors for 24 hr prior to exposure to glucose-free medium. Intraneuronal calcium levels were measured after 14–16 hr of incubation in the indicated conditions. Values represent the mean ± SEM of determinations made in 20–40 neurons.

* $p < 0.001$, as compared with cultures maintained in 0 glucose or 0 glucose + EGF.

Discussion

An increasing number of growth factors are being identified that protect central neurons against environmental insults. Previous work demonstrated protective effects of basic fibroblast growth factor (bFGF) and NGF against physical, ischemic, and/or excitotoxic damage (Hefti et al., 1985; Anderson et al., 1988; Mattson et al., 1989; Cheng and Mattson, 1991). The present data demonstrated the potent protective effect of IGFs against hypoglycemic damage in hippocampal and septal cell cultures. Furthermore, we provided evidence that the neuroprotective action of IGFs results from their ability to stabilize neuronal calcium homeostasis. Since central neurons are particularly vulnerable to ischemic insults, these data suggest that IGF may play a neuroprotective role under conditions of reduced energy supply.

The protective effects of IGFs and insulin against glucose deprivation were concentration dependent and specific. Both IGF-I and IGF-II were effective in reducing neuronal death at concentrations 1–3 ng/ml (approximately 150–450 pM), with half-maximally effective concentration of approximately 20 ng/ml (approximately 3 nM). The dose–response curves for the two IGFs were remarkably similar. In contrast, insulin was only effective in protecting neurons against hypoglycemic damage when administered at levels 100–1000-fold greater than the IGFs (1–3 μg/ml; approximately 200–500 nM). These data are consistent with the possibility that the protective effect of insulin

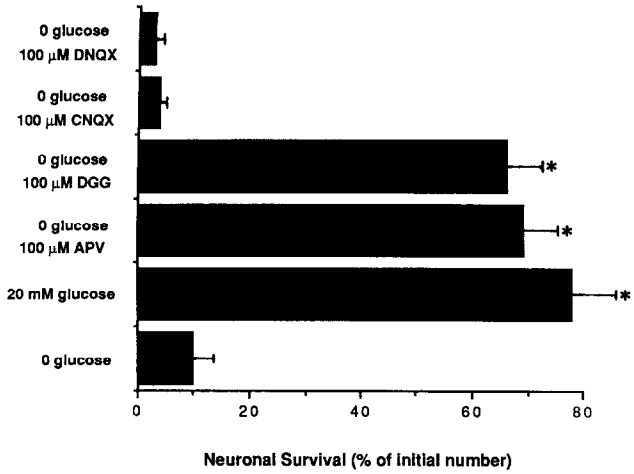

Figure 4. Effects of NMDA and non-NMDA receptor antagonists on hippocampal neuronal death due to glucose deprivation. Cultures were exposed to the indicated treatments and neuronal survival was assessed 18 hr following the onset of glucose deprivation. APV is an NMDA receptor–specific antagonist, CNQX and DNQX are non-NMDA receptor–specific antagonists, and DGG is a broad-spectrum glutamate receptor antagonist. Values represent the mean and SEM of determinations made in three or four separate experiments. *, $p < 0.001$ compared to 0 glucose, 0 glucose + CNQX, and 0 glucose + DNQX.

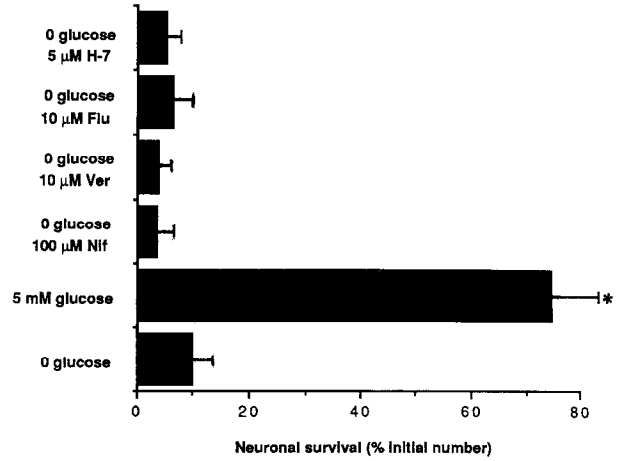

Figure 5. Effects of calcium channel blockers and kinase inhibitors on hippocampal neuronal death resulting from glucose deprivation. Inhibitors were included in the culture medium during an 18 hr period of glucose deprivation. Inhibitors were nifedipine (*Nif*), verapamil (*ver*), flunarizine (Flu), and H-7. Values represent the mean and SEM of determinations made in three or four separate cultures. Nifedipine, verapamil, flunarizine, and H-7 did not protect against the neuronal death ($p > 0.05$).

was mediated by IGF-I receptor since previous studies have demonstrated an approximately 1000-fold lower affinity of insulin (as compared to IGFs) for IGF-I receptor (Ullrich et al., 1985, 1986). Since insulin does not appear to bind to IGF-II receptor (Baskin et al., 1988), the growth or protective effects of insulin on CNS cells may be due to its binding to the IGF-I receptor. Autoradiographic studies indicated that there are IGF-I and IGF-II receptors, but no insulin receptors, in the hippocampus (Bohanon et al., 1988; Lesniak et al., 1988). IGF-I receptors are concentrated in strata radiata and oriens, particularly in region CA3, suggesting that they may be present on pyramidal neurons. *In situ* hybridization studies indicated that insulin and IGF-I receptor mRNAs are present in hippocampus (Baron-Van Evercooren et al., 1991). We found that IGF-I and IGF-II were equipotent in protecting neurons against hypoglycemia. When taken together with the fact that IGF-I has a higher affinity for IGF-I receptors than does IGF-II (and vice versa), our data suggest that hippocampal and septal neurons have both IGF-I and IGF-II receptors.

Rat hippocampal cultures contain essentially all non-cholinergic neurons, whereas septal cultures contain a large population of cholinergic neurons (Hefti et al., 1989). Previous studies showed that IGFs and insulin elevated ChAT activity in septal cultures and dopamine uptake in mesencephalic cultures (Knusel et al., 1990). No data were previously available concerning actions of IGFs and insulin in hippocampal neurons. Our data indicate that IGFs are likely to influence a rather large number of neuronal types in the brain. As with the IGFs, bFGF has been shown to support cell survival and neurite outgrowth in cultured neurons (including cholinergic and non-cholinergic) from various brain regions (Morrison et al., 1986; Walicke et al., 1986; Unsicker et al., 1987; Hatten et al., 1988; Walicke, 1988). Intracerebral administration of bFGF prevents degenerative changes of lesioned cholinergic neurons of the basal forebrain (Anderson et al., 1988). In contrast to IGFs and FGF, the biological actions of NGF in the CNS seem to be limited to certain populations of primarily cholinergic neurons (Thoenen et al., 1987; Hefti et al., 1989; Snider and Johnson, 1989). On the other hand, we recently found that NGF and bFGF protected both rat hippocampal and human cortical neurons against hypoglycemic damage (Cheng and Mattson, 1991), indicating that NGF, in addition to actions on peripheral neurons and central cholinergic neurons, can also directly affect central non-cholinergic neurons. Taken together, the available data indicate that IGF-I and IGF-II, as well as bFGF and probably NGF, affect both cholinergic and non-cholinergic central neurons. The extent to which the signal transduction systems for these different growth factors are different or overlapping remains to be determined.

Insulin and IGFs are known to affect glial cells (Lenoir and Honegger, 1983; Avola et al., 1988). Glial cells, in turn, are known to provide trophic support for neurons (Mattson and Rychlik, 1990). In the present study, however, treatment of the cultures with Ara-C to prevent cellular proliferation only slightly reduced the neuroprotective effects of IGFs. This suggests that the neuroprotective effects of IGFs were mainly the results of direct actions on the hippocampal and septal neurons, although a role for glia was not completely ruled out.

The mechanism of hypoglycemic neuronal damage is not completely understood, but appears to involve a loss of neuronal calcium homeostasis. In support of this mechanism, we found that removal of extracellular calcium prevented hypoglycemic damage. Contributing to the loss of calcium homeostasis was calcium influx triggered by activation of NMDA receptors since APV, a specific blocker of NMDA-type calcium channels, significantly reduced hypoglycemic damage. This possibility is consistent with previous data obtained in mouse cortical and rat cerebellar cultures wherein hypoglycemic damage was reduced by NMDA receptor antagonists (Monyer et al., 1989; Facci et al., 1990). Non-NMDA receptor antagonists (CNQX and DNQX) did not reduce hypoglycemic neuronal damage in the present study, indicating that activation of non-NMDA recep-

tors is probably not necessary for hypoglycemic damage. In addition, dihydropyridine blockers of L-type calcium channels (nimodipine and nifedipine), as well as verapamil and diltiazem did not reduce hypoglycemic damage, indicating that calcium influx through the L-type channel was not a major factor contributing to the loss of calcium homeostasis. Taken together, these findings suggest that calcium influx through the NMDA receptor channel was a major contributor to the degenerative effects of glucose deprivation. However, we cannot yet rule out the possibility that calcium influx through non-L-type calcium channels plays a role in hypoglycemic neuronal damage, since a substantial portion of high-threshold calcium current is resistant to dihydropyridines and ω-conotoxin (Regan et al., 1991).

Both IGF-I and IGF-II prevented hypoglycemia-induced loss of neuronal calcium homeostasis. The specific system(s) for calcium homeostasis affected by IGFs is not clear. Previous work provided evidence that neuronal growth factors can stabilize neuronal calcium homeostasis. For example, NGF influenced the expression of calcium channels and calcium-extruding systems in PC12 cells (Takahashi et al., 1985; Chalazonitis et al., 1987; Masiakowski and Shooter, 1988; Streit and Lux, 1990). In addition, bFGF protected cultured rat hippocampal neurons against excitatory amino acid neurotoxicity (Mattson et al., 1989). The protective effect of bFGF is mediated at least in part by a suppression of the expression of an NMDA receptor protein by this growth factor (Mattson et al., 1991; Michaelis et al., 1991). In addition, bFGF may enhance the ability of neurons to reduce intracellular calcium levels following an excitatory challenge (Mattson and Rychlik, 1990). In a recent related study we found that NGF and bFGF protected rat hippocampal and human cerebral cortical neurons against neuronal damage caused by glucose deprivation (Cheng and Mattson, 1991). In the latter study we found that both NGF and bFGF prevented the loss of neuronal calcium homeostasis that normally mediated hypoglycemic damage. Growth factors may also stabilize intracellular calcium levels in peripheral neurons. For example, in cultured sympathetic neurons NGF appears to influence neuronal systems for calcium homeostasis and these effects of NGF are correlated with its trophic action (Koike and Tanaka, 1991). Taken together, the available data suggest that a general feature of growth factor action may be to stabilize neuronal intracellular free calcium levels.

Overactivation of protein kinases has been suggested to be involved in the neuronal damage that occurs in a number of neurodegenerative conditions. For example, ischemic brain damage is associated with altered PKC activity (Louis et al., 1988), and administration of a PKC inhibitor was found to reduce ischemic damage (Joo et al., 1989). Overactivation of calcium/calmodulin-dependent kinase(s) and PKC have been implicated in the neurofibrillary degeneration that occurs in Alzheimer's disease and related disorders (Mattson, 1990, 1991). In the present study, calmodulin inhibitors (flunarizine, lidoflazine, and trifluoperazine) and the PKC inhibitor H-7 did not protect hippocampal or septal neurons against hypoglycemic damage. Thus, we were not able to provide evidence supporting a role for calmodulin-dependent protein kinases or PKC in hypoglycemic damage. These data suggest that these calcium-regulated kinases may not be involved in hypoglycemic damage. However, since we did not directly assess kinase activity, we cannot rule out the possibility that the kinase inhibitors used did not completely block kinase activities. Clearly, further work will be required to understand the specific events triggered by glucose deprivation that lead to a loss of neuronal calcium homeostasis and cell death.

The protective effects of IGFs against hypoglycemic damage demonstrated here are consistent with the possibility that IGFs play a neuroprotective role *in vivo*. In the developing nervous system IGFs may play a role in determining which neurons survive during the period of naturally occurring neuronal death, and in the process of synaptic organization. From a pathological standpoint, IGFs may also play a role in preventing neuronal death. The hippocampus and septal area are brain regions that are particularly vulnerable in acute (e.g., stroke) and chronic (e.g., Alzheimer's disease) neurodegenerative disorders. The present data may therefore have implications for approaches to preventing neuronal damage in these disorders.

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