Characterization of a Neuronal Subtype of Insulin-like Growth Factor I Receptor*

(Received for publication, January 10, 1986, and in revised form, September 22, 1986)

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Primary neuronal cultures from fetal rat brain were utilized to investigate the possible role of insulin-like growth factor I (IGF-I) in neuronal growth and differentiation. 125I-IGF-I binding to intact cultured neurons was specific and saturable with an apparent $K_d$ of 7.0 ± 1.2 nM and a $B_{	ext{max}}$ of 1.8 ± 0.3 pmol/mg protein. Binding of 125I-IGF-I to neurons was inhibited by IGF-I, followed by IGF-II and insulin. 7 S nerve growth factor, but not 8-nerve growth factor, also inhibited 125I-IGF-I binding. A similar binding site was detected on brain membranes. Affinity cross-linking of 125I-IGF-I to intact cultured neurons revealed, under reducing conditions, a major binding moiety with an $M_r$ of 115,000 and a minor component at $M_r$ 260,000. The former represents a neuronal type of the IGF-I receptor subunit, whereas the latter probably represents an $a$ dimer. The $M_r = 115,000$ binding component for 125I-IGF-I was also present in membranes prepared from postnatal whole brain. In contrast, the binding moiety in cultured glial cells was of $M_r = 135,000$, which was identical to the IGF-I receptor $a$ subunit of placenta. Thus mature brain, despite its cellular heterogeneity, expresses a structural subtype of IGF-I receptor which appears to be unique to differentiated neural cells. Moreover, glial and neuronal cultures secreted a polypeptide which specifically bound IGF-I; the apparent $M_r$ of this binding protein was determined by affinity cross-linking to be approximately 35,000. The presence of neuronal IGF-I receptors and binding proteins suggested that IGF-I may exert neurotrophic effects on developing neurons. This possibility was supported by the observation that IGF-I markedly stimulated neuronal RNA synthesis.

The neuronal receptor for insulin-like growth factor I (IGF-I); somatomedin C) appears to provide a suitable model for an inquiry into the molecular responses to trophic factors which act on central nervous system neurons. Evidence is accumulating that an IGF-I neurotrophic and/or neuromodulatory system may exist within the developing brain (1–13). Significantly, the addition of either high concentrations of IGF-I to the anterior pituitary and hypothalamus supports the possibility that this peptide is involved in regulating central nervous system and pituitary function in mature mammals (11). IGF-I does indeed modulate release of somatostatin from hypothalamic tissue, apparently participating in negative feedback on growth hormone release (12) and may act as an endogenous satiety factor (13).

In this report, we characterize a structural subtype of IGF-I receptor present in mature brain and in enriched primary neuronal cultures and we contrast it with IGF-I receptors of glial cells and peripheral tissues. We also describe a soluble IGF-I binding protein produced by cultured neural cells. In addition, we present evidence that IGF-I is neurotrophic in this system because of stimulation of RNA synthesis after brief as well as prolonged exposure to the growth factor. Primary neuronal cultures may, therefore, represent a useful model for investigating the role of IGF-I in neuronal development.

**EXPERIMENTAL PROCEDURES**

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‡The abbreviations used are: IGF-I, insulin-like growth factor I; NGF, nerve growth factor; DSS, disuccinimidyl suberate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; MSA, multiplication-stimulating activity.

IGF-I was obtained from Amgen Biologicals Inc.; other growth factors including insulin, 7S and 8-NGF, IGF-II (MSA), and epidermal growth factor were purchased from Collaborative Research; DSS was supplied by Pierce Chemical Co.; culture media were obtained...
from Gibco; and Na\textsuperscript{125}I was obtained from Cinti-Chem. \textsuperscript{125}I-IGF-I was prepared by the chloramine-T method.

**Methods**

**Cell Cultures**—Braains of 16-day-old rat embryos were aseptically removed and freed of meninges, mechanically dissociated, and plated in poly-D-lysine-treated 24-well culture dishes in serum-free medium (basal medium of Eagle with Earle’s salts and L-glutamate/Ham’s F-12, 1:1, with glucose, glutamine, penicillin, streptomycin, transferrin, and 1 \mu M insulin) as previously described (16); these cultures after 5 days in *vitro* were >95% neuronal, judged by immunohistochemical staining for neurofilament protein. Astrogial cultures were prepared by the chloramine-T method. From 16-day-old rat embryonic cortex or neonatal cortex by replating poly-D-lysine-treated 24-well culture dishes in serum-free medium with the addition of 10% fetal calf serum. Morphological appearance and cell-type specific staining for glial fibrillary acidic protein or fibronectin indicated that these cultures were predominantly (>80%) astroglia or fibroblasts, respectively.

**Binding of IGF-I to Intact Cells**—Binding was carried out with cells still adhering to culture dishes. Cells were washed twice with PBS containing 0.1% BSA, pH 7.4 (PBS/BSA). \textsuperscript{125}I-IGF-I (25,000 cpm/10\textsuperscript{5} cells/well; 112 \mu Ci/\mu g IGF-I), with or without the appropriate concentrations of competing peptides, were added in this buffer in a final volume of 200 \mu l. Incubation was at 15 °C for 80 min followed by 4 × 30 s washes with iced-cold buffer. Cells were harvested in 200 \mu l of 1 N NaOH and bound \textsuperscript{125}I-IGF-I was measured in a \gamma counter. Nonspecific binding was defined as that obtained in the presence of 250 nM IGF-I.

**Binding of IGF-I to Membranes**—Crude membranes were prepared from human placenta (18) or from whole brain of Sprague-Dawley rats after removal of nuclei and myelin. They were suspended in PBS/BSA at 0.5 mg of protein/ml and 100-\mu l aliquots were incubated with labeled ligand (25,000 cpm/assay; 112 \mu Ci/\mu g). After 60-min incubation at room temperature, the reaction mixture was layered over an air cushion over 0.3 M sucrose with 0.1% BSA and 25 mM HEPES in 400-\mu l Eppendorf tubes and centrifuged at 40,000 \times g for 20 min. The pellet was obtained by cutting off the tips of the tubes and bound radioactive ligand was determined.

**Affinity Cross-linking of \textsuperscript{125}I-IGF-I to Intact Cells and Membranes**—Intact cultured cells (10\textsuperscript{6} cells/well) or membranes from placenta or brain (50 \mu g of protein/well) were labeled with \textsuperscript{125}I-IGF-I (500,000 cpm/assay; 112 \mu Ci/\mu g) with or without competing peptides, and unbound ligand was removed, using the procedures described in the two preceding paragraphs. Disuccinimidyl suberate (DSS), freshly dissolved in dimethyl sulfoxide, was added as a 100 \mu M solution (except when indicated otherwise) in a final concentration of 1 mM (except when indicated otherwise) in serum-free medium containing 0.1% BSA, pH 7.4 (PBS/BSA). \textsuperscript{125}I-IGF-I (500,000 cpm/assay, 25 \mu M DSS) (Fig. 3A). Unlabeled IGF-I prevented the cross-linking of the radiolabeled ligand at the appropriate concentrations (Fig. 3A). The cross-linking reaction was quenched after 15 min at 4 °C by the addition of 50 \mu l of 50 mM Tris-HCl, pH 7.7, then after two washes cells or membranes were suspended in 100 \mu l of SDS sample buffer containing 40 mM dithiothreitol. Samples were boiled for 3 min and analyzed by SDS-polyacrylamide gel electrophoresis using 7.5% acrylamide gels (19) followed by autoradiography with Kodak Blue Brand or X-AR film.

**Characterization of IGF-I Binding Protein**—Conditioned medium (50 \mu l) from neurons grown 5 days in serum-free medium was used directly for cross-linking studies; the procedure was as described above, except that the washing steps were omitted. Partial purification of the binding protein was obtained by concentrating conditioned medium 10-fold using Amicon columns (M\textsubscript{r} = 10,000 cutoff) followed by chromatography on 10-ml A24 44 columns using PBS as elution buffer. One-mL fractions were tested at 1:1 dilution in PBS/BSA for interference with IGF-I binding on intact neurons, as compared with 5 and 10% concentrated conditioned medium.

**Incubation of \textsuperscript{3H}Uridine by Cultured Neurons**—Cells were grown for 3 days in defined medium. In some wells, the medium was replaced with fresh insulin-free medium. The cultures were grown for three additional days with or without the addition of IGF-I, then [\textsuperscript{3H}]uridine (5 \mu Ci/well) was added to the medium. Incubation was continued at 37 °C for 2 h. Cells were washed extensively with cold PBS and incorporated radioactivity measured; no change in specific incorporation into RNA was obtained if cultures were further washed with acid alcohol. Alternatively, the short term effects of IGF-I were examined by adding it together with [\textsuperscript{3H}]uridine followed by a 2-h incubation.

**Results**

**Binding of IGF-I to Neurons in Primary Culture**—\textsuperscript{125}I-IGF-I bound to intact neuronal cells from 16-day-old embryonic brains cultured for 7 days, and up to 70% of the binding could be displaced by unlabeled ligand. The specific binding reached equilibrium after 40 or 60 min at 21 °C or 4 °C, respectively. Increasing concentrations of \textsuperscript{125}I-IGF-I yielded saturable binding with a B\textsubscript{max} of 1.8 ± 0.3 pmol/mg protein and an apparent K\textsubscript{D} of 7.0 ± 1.2 nM at 15 °C (Fig. 1 and a B\textsubscript{max} of 1.7 pmol/mg and a K\textsubscript{D} of 3.3 nM at 4 °C (data not shown). The corresponding values, obtained when brain membranes were evaluated, were 0.18 pmol/mg protein and 1.1 nM (data not shown).

**Specificity of IGF-I Binding**—\textsuperscript{125}I-IGF-I binding to intact neurons was inhibited not only by IGF-I, but also by relatively low concentrations of 7 S NGF, whereas IGF-II and insulin were less effective (Fig. 2A). Inhibition of IGF-I binding to brain membranes by related peptides was also in the order IGF-I > 7 S NGF > MSA > insulin (Fig. 2B). With the exception of 7 S NGF, these cross-reactivities are consistent with previously characterized IGF-I receptors. Since the interaction of NGF was unexpected, attempts were made to rule out possible artifacts, such as binding of IGF-I to NGF. NGF apparently did not act as a binding protein for IGF-I, because preincubation of labeled IGF-I with NGF did not alter the elution pattern of \textsuperscript{125}I-IGF-I from an A24 44 column (not shown). However, we cannot exclude other potential artifacts. Although 7 S NGF displaced \textsuperscript{125}I-IGF-I binding with a K\textsubscript{D} of less than 5 nM, \textsuperscript{125}I-IGF-I obtained from the same commercial source (Collaborative Research Inc.) had no displacing activity at 100 nM (not shown).

**Affinity Cross-linking of \textsuperscript{125}I-IGF-I to Neurons and Other Cells**—Whereas \textsuperscript{125}I-IGF-I labeled the \alpha subunit of placent al IGF-I receptor at M\textsubscript{r} = 135,000, cross-linking of the ligand to receptors from cultured neurons labeled a polypeptide corresponding to M\textsubscript{r} = 115,000, after subtraction of the M\textsubscript{r} = 7,500. The affinity of cross-linked IGF-I binding to the M\textsubscript{r} = 115,000 polypeptide at 21 °C was determined by the addition of [\textsuperscript{3H}]IGF-I to intact neurons (Fig. 3A). An M\textsubscript{r} = 260,000 polypeptide was also labeled by \textsuperscript{125}I-IGF-I. Although this
FIG. 2. Competition for IGF-I binding by related peptides. A, displacement of $^{125}$I-IGF-I on intact primary neurons (as in Fig. 1) by varying concentrations of IGF-I (solid circles), NGF (open circles), IGF-II (MSA) (solid squares), and insulin (open squares) after incubation for 1 h at 21°C. Data are presented as percent of total binding (100%) in the absence of competing peptides, without correction for nonspecific binding and are the mean values ± S.E. of triplicate determinations. B, displacement of $^{125}$I-IGF-I on brain membranes by IGF-II (MSA), NGF, and IGF-I. Brain membranes from 1-week-old rats were incubated for 1 h at 21°C with $^{125}$I-IGF-I in the presence or absence of MSA, NGF, and IGF-I. Data points represent mean ± S.E. for four values.

corresponds in size to the receptor for IGF-II, the fact that binding was inhibited by insulin, which does not interact with the IGF-II receptor, but not by low concentrations of MSA itself (not shown), suggests that it may be a cross-linked dimer. In nonreducing conditions, the major labeled species was at $M_r$ ~ 400,000 (data not shown).

$^{125}$I-IGF-I could also be specifically cross-linked to an $M_r$ = 115,000 polypeptide from brain membranes of rats older than 1 week (Fig. 3C). Under identical conditions, the IGF-I receptor from placenta yielded an $M_r$ = 135,000 polypeptide, in agreement with previous work on the cross-linking of $^{125}$I-IGF-I to the $\alpha$ subunit of the receptor (Fig. 3C). Interestingly, primary rat astroglial cultures (Fig. 3D) and rat fibroblasts (not shown) had receptors with the same apparent $M_r$ as the placental receptor. This was true whether glia were prepared from the same 16-day-old embryos, as were the neurons, or from neonatal cerebral cortex. The pattern of $^{125}$I-IGF-I cross-linking to cultured neurons was not altered following addition of 10% serum for 2 days, nor was the glial pattern altered by deletion of serum for 4 days, suggesting that serum factors such as protease inhibitors were not responsible for the differences between the neuronal and glial receptor $\alpha$ subunits. We have also found the high molecular weight receptor in brain of 15-day-old embryos, while neonatal brain appears to have an intermediate form (not shown).

Secretion of IGF-I Binding Protein by Cultured Cells—Since diminished binding of IGF-I was obtained in the presence of conditioned medium, it seemed that cultures might be producing either high concentrations of endogenous IGF-I or a binding protein which reduced the effective concentration of added IGF-I at the receptor. In fact, when partially purified conditioned medium was added back, $^{125}$I-IGF-I binding was reduced (Fig. 4) and the inhibitory moiety migrated on an AcA 44 gel permeation column with a $K_a$ consistent with an apparent $M_r$ of 30,000–50,000. The inhibitory component was also able to undergo a stable interaction with $^{125}$I-IGF-I, resulting in an apparent increase in the ligand size (Fig. 4).
These data suggested that the inhibitor was interacting with IGF-I itself rather than with the receptor, indicating the presence of an IGF-I-binding protein. This possibility was confirmed by the cross-linking of $^{125}$I-IGF-I to a soluble polypeptide with an apparent $M_r$ of 35,000. This protein was able to bind $^{125}$I-IGF-I selectively such that the binding was displaced by unlabeled IGF-I, but not by NGF or insulin (Fig. 3E). An $M_r$ = 35,000 polypeptide was also specifically cross-linked to $^{125}$I-IGF-I in the intact astroglial preparations (Fig. 3D) and might represent binding protein adhering to the glial membranes with relatively high affinity. The IGF-I-binding protein was produced in much greater quantities by astroglial cultures than by neuronal cultures. It is possible, in fact, that contaminating glia initially present in neuronal cultures are the source of the binding protein.

**IGF-I Stimulation of $^{3}$H]Uridine Uptake—Uptake of $^{3}$H]uridine into intact neurons which had been incubated from the third to the sixth day in vitro with IGF-I and/or fresh medium was enhanced (Table I). Concentrations of IGF-I as low as 1 nM increased RNA synthesis. The stimulatory effect of IGF-I was strongly enhanced in cultures which had fresh medium. This effect was probably due to the removal of IGF-I-binding protein which was secreted by the neuronal cultures, resulting in an increase in the effective concentration of the added and perhaps endogenous IGF-I. The stimulation of RNA synthesis could likewise be effected by a brief (2 h) exposure of the cells to IGF-I on the sixth day in culture (Table I).

**DISCUSSION**

Cultured dissociated neurons expressed relatively high levels of IGF-I receptors. Affinity cross-linking studies demonstrated that the $\alpha$ subunit of the IGF-I receptor of cultured dissociated neurons and adult brain has an $M_r$ of approximately 115,000, whereas the IGF-I receptor of glia and fetal brain resembles more the peripheral-type receptor with an apparent $M_r$ of 135,000. After these studies were completed, we learned that another group has also observed an $M_r$ = 115,000 IGF-I receptor $\alpha$ subunit in brain (20). The finding of the low molecular weight form of the receptor in mature brain seems surprising since glial cells comprise the majority of brain cells. This may be attributed to the lesser abundance of receptors on glia than on neurons (not shown). Moreover, the concentration of IGF-I receptors in intact purified primary neuronal cultures is about 5-fold greater per mg of protein than that in brain membranes; this comparison actually underestimates the contribution of neurons in brain membranes because the latter should be more enriched in membrane receptors than are intact whole cells. These data further suggest that neurons, rather than glia, account for the majority of receptors in brain membrane preparations.

The structural difference between the neuronal and glial receptors may serve as an important marker in the differentiation pathway from progenitor neuroblast. In this regard, it was previously reported that IGF-I receptor in human brain at mid-gestation (14-18 weeks (21)) is structurally identical to placental receptor and that high affinity IGF-I binding sites begin to appear just after mid-gestation (6). Since neuroblasts begin to differentiate at this time, these results may indicate that the appearance of a neuronal-type receptor may represent an important aspect of neuronal differentiation. Our finding that fetal brain and nondifferentiated neuroblastoma N4TG1 cells express the peripheral receptor type is consistent with this view.

Insulin receptors in brain are also of distinctly lower molecular weight than those in other tissues (22-25) which may be due to brain-specific differences in glycosylation (22, 23, 27); associated functional differences unique to brain insulin

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**Table I**

| IGF-I | 3-day IGF-I exposure | 2-h IGF-I exposure |
|-------|---------------------|-------------------|
|       | Conditioned medium | Fresh medium       | Conditioned medium |
| Control | 100         | 142 ± 41          | 100               |
| 0.1 nM  | 135 ± 3     | 211 ± 39          | 125 ± 1           |
| 1.0 nM  | 166 ± 45   | 226 ± 17          | 167 ± 12          |
| 10.0 nM | 204 ± 34   | 331 ± 57          | 207 ± 12          |

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$^2$ S. K. Burgess, S. Jacobs, and P. Cuatrecasas, unpublished data.
Neuronal IGFR-I Receptors

receptor include lack of down-regulation (24, 26) and lack of insulin-stimulated glucose transport (24). The functional relevance of the neuronal subtype of IGFR-1 receptor is not known, nor has the molecular mechanism which is involved in producing a structurally modified receptor been determined. Developmentally regulated changes in post-translational processing have been proposed to account for the presence of structurally modified forms of N-CAM (28, 29) and c-src (30) in post-mitotic neurons. However, these changes in processing might be secondary to differences in primary sequence of the proteins; for example, due to tissue-specific gene processing which yields alternative mRNAs from a single gene. Recent evidence suggests that the gene for c-src is subject to developmentally regulated alternative processing (31). While IGFR-I receptor and c-src might be structurally related (32, 33), the possibility that a similar scheme could account for the appearance of neuron-specific IGFR-I receptors can be experimentally approached only when the IGFR-I receptor gene is characterized.

It has been previously reported that NGF inhibits IGFR-I receptor binding in developing chick brain (34). The data here show that 7 S NGF, but not 2.5 S yNGF, inhibits IGFR-I binding to the rat brain IGFR-I receptor, possibly indicating that either the entire 7 S complex is required or the α or γ subunits are the active species. However, since β-NGF is the species that binds to its own receptor in brain (35) and since it is the species that has some structural similarity with the insulin-like family of peptides, this finding is surprising and its significance is unclear. Inhibition does not appear to result from the formation of a complex between IGFR-I and 7 S NGF since 7 S NGF did not alter the mobility of IGFR-I on an Aca 44 column (data not shown). Nevertheless, other artifactual explanations are possible. Although 7 S NGF and unlabeled IGFR-I were equimolar in a pool of mouse in vitro binding of 125I-IGFR-I, on a mass basis, much greater amounts of NGF were necessary. Therefore, the possibility that a minor contaminant was responsible for the inhibitory action cannot be ruled out.

Neuronal cultures secreted a binding protein(s) for IGFR-I in sufficiently large amounts that no IGF-I binding could be detected in the presence of conditioned medium. Since this binding protein was much more abundant in purified astroglial cultures (in serum-free medium) than in enriched neuronal cultures, it is quite possible that glial cells which initially contaminated the enriched neuronal cultures are the cellular source for IGFR-1-binding protein. IGF-I-binding proteins have been described for various cell types (26), including cultured neural cells (3, 8). Such proteins may serve as storage forms for extracellular IGFR-I. Cross-linking of labeled IGFR-I to conditioned medium from cultured neurons revealed a major specific binding species of Mr = 55,000; the predominant IGF-I binding species on intact cultured astroglia had the same apparent Mr. The ability of developing brain to synthesize an IGF-I-binding protein as well as possibly IGFR-I itself (8), suggests a dual mechanism by which the effective concentration of IGF-I can be regulated. Furthermore, a possible interaction between neurons and the binding protein itself has not been ruled out.

The implications of the presence on cultured neurons of an IGFR-I receptor remain to be elucidated. However, the stimulation of RNA synthesis after either brief or prolonged exposure to IGF-I suggests a neurotrophic role for this molecule. Investigating the mode of induction of neuronal RNA synthesis and the possible involvement of IGF-I receptor tyrosine kinase activity (20, 37) may provide useful clues to neurotrophic mechanisms. In addition, clarification of the molecular events responsible for production of a neuron-specific IGFR-I receptor may provide insight into important principles of neuronal differentiation.

Acknowledgment—We are pleased to acknowledge the intellectual contributions of Steven Blanchard to the substance of this manuscript.

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