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Two types of receptor for insulin-like growth factors in mammalian brain

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Two types of receptor for insulin-like growth factors (IGFs) have been identified on adult rat and human brain plasma membranes by competitive binding assay, affinity labelling, receptor phosphorylation and interaction with antibodies to insulin receptors. The type I IGF receptor consists of two species of subunits: α-subunits (mol. wt. ~115 000), which bind IGF I and IGF II with almost equal affinity and β-subunits (mol. wt. ~94 000), the phosphorylation of which is stimulated by IGFs. The α-subunits of type I IGF receptors in brain and other tissues differ significantly (mol. wt. ~115 000 *versus* 130 000), whereas the β-subunits are identical (mol. wt. ~94 000). The type II IGF receptor in brain is a monomer (mol. wt. ~250 000) like that in other tissues. Two antibodies to insulin receptors, B_2 and B_9, interact with type I but not with type II IGF receptors. B_2 is more potent than B_9 in inhibiting IGF binding and in immunoprecipitating type I IGF receptors, in contrast to their almost equal effects on insulin receptors. This pattern is characteristic for IGF receptors in other cells. The presence of two types of IGF receptor in mammalian brain suggests a physiological role of IGFs in regulation of nerve cell function and growth. Since IGF II, but not IGF I, is present in human brain, we propose that IGF II interacts with both types of IGF receptor to induce its biological actions.

Key words: brain cell growth/insulin-like growth factors/receptor phosphorylation/receptor structure/somatomedins

Introduction

Insulin-like growth factors (IGFs) I and II and insulin are structurally related peptides (Blundell *et al.*, 1983), which also show some similarities in their hormonal actions on metabolism and growth of mammalian cells (Kahn *et al.*, 1981; Zapf *et al.*, 1981). In the rat central nervous system (CNS) insulin exerts growth activity (Raizada *et al.*, 1980; Roger and Fellows, 1980) and this effect is mediated *via* receptors widespread in rat CNS (Havrankova *et al.*, 1978a; Gammeltoft *et al.*, 1984a). In contrast, the role of IGFs in the CNS is unknown. Recently, IGF II and its putative precursors were identified in human brain (Haselbacher *et al.*, 1985) and receptor binding of IGF I and IGF II has been demonstrated on brain membranes from the human fetus and adult rat (Sara *et al.*, 1983; Goodyer *et al.*, 1984).

In many cells IGFs and insulin have separate receptors which bind their respective ligands with high affinity (Zapf *et al.*, 1978; Rechler *et al.*, 1980). Analysis of the protein structure has revealed that receptors for IGF I and insulin are oligomers composed of two types of subunit: α-subunits (mol. wt. ~130 000) with the hormone-binding site and β-subunits (mol. wt. ~94 000), the phosphorylation of which is stimulated by the hormone. In contrast, IGF II receptors are monomeric proteins of mol. wt. ~250 000 without detectable kinase activity (Chernausek *et al.*, 1981; Kasuga *et al.*, 1981, 1982; Massagué *et al.*, 1981; Massagué and Czech, 1982; Van Obberghen *et al.*, 1983; Jacobs *et al.*, 1983; Mottala and Czech, 1983).

In the CNS, insulin receptors are also composed of two types of subunit, but the size of the α-subunit is significantly smaller (mol. wt. ~115 000), whereas the β-subunit (mol. wt. ~94 000) is similar to that of other cell types (Heidenreich *et al.*, 1983; Gammeltoft *et al.*, 1984b; Rees-Jones *et al.*, 1984). Here we report the identification of two types of IGF receptor on nerve cell membranes from the murine and human CNS based on their binding specificity, subunit structure, kinase activity and interaction with antibodies to insulin receptor. The type I IGF receptors have an oligomeric structure similar to the brain insulin receptors with α-subunits (mol. wt. ~115 000) and β-subunits (mol. wt. ~94 000) and the type II IGF receptors have a monomeric structure (mol. wt. ~250 000). The presence of these receptors on nerve cell plasma membranes suggests a physiological role of IGFs in the mammalian CNS.

Results

Plasma membrane binding

The receptor binding of ^{125}I-labelled IGF I, IGF II and insulin is shown in Figure 1A-C. The type I IGF receptors showed little cross-reactivity with porcine insulin, proinsulin or coypu insulin (relative affinities 0.3-2%), whereas IGF II was bound with a relative affinity of 45% compared with IGF I. The type II IGF receptors were more specific because neither insulins from pig and coypu nor proinsulin showed inhibitory effects (relative affinities <0.01%) and IGF I was bound with a relative affinity of 22%. Finally, insulin receptors showed some cross-reactivity with IGF I and IGF II (relative affinities of 1% and 6%). This value for human IGF II is identical to that recently determined for rat multiplication stimulating activity i.e. rat IGF II (Gammeltoft *et al.*, 1984a). The binding parameters of the two types of IGF receptor were estimated by Scatchard analysis of the data in Figure 1. Type I IGF receptor is present as 120 pmol/g membrane protein and binds IGF I with K_d 2.4 nmol/l. The amount of type II IGF receptors is 40 pmol/g membrane protein, and the K_d of IGF II binding is 1 nmol/l.

Eight brain regions bound varying amounts of ^{125}I-labelled IGF I, IGF II and insulin (Table I). The binding of IGFs was significantly higher than that of insulin, and remarkably, the olfactory bulb showed the highest content of IGF receptors, a phenomenon which has been observed previously for insulin (Havrankova *et al.*, 1978a; Gammeltoft *et al.*, 1984a). In conclusion, IGF I and II interact with their own type of receptor

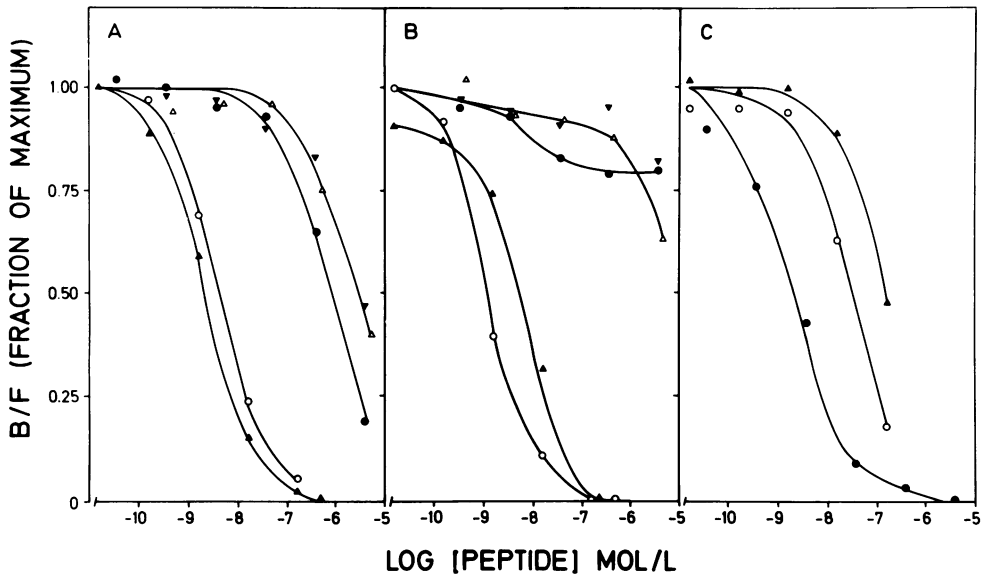


Fig. 1. Receptor binding of ^{125}I-labelled IGF I, IGF II and insulin. Rat brain cortical plasma membranes were incubated for 2 h at 15°C with either [^{125}I]-IGF I (**A**), [^{125}I]IGF II (**B**) or [^{125}I]insulin (**C**) in concentrations of 100 pmol/l plus addition of IGF I (▲), IGF II (○), porcine insulin (●), copyu insulin (▼) or porcine proinsulin (△) at the indicated concentrations. The receptor binding was expressed as the ratio between bound and free tracer in fraction of maximum i.e., the value with tracer alone. Points are mean of three experiments.

Table I. Distribution of receptors for IGF I, IGF II and insulin in rat brain

CNS region	Receptor-bound ^{125}I-labelled peptide (pmol/g protein)[a] mean ± SD[b]		
	IGF I	IGF II	Insulin
Cerebral cortex	3.2 ± 0.1	2.9 ± 1.0	0.8 ± 0.3
Olfactory bulb	8.7 ± 1.0	3.9 ± 0.4	1.9 ± 0.7
Olfactory tubercle	3.7 ± 0.9	1.4 ± 0.7	0.9 ± 0.3
Hippocampus	3.8 ± 0.6	2.6 ± 0.9	1.0 ± 0.2
Amygdala	3.1 ± 0.7	4.1 ± 1.2	0.4 ± 0.1
Corpus striatum	2.7 ± 0.6	1.9 ± 0.5	1.0 ± 0.4
Thalamus	2.3 ± 0.4	1.0 ± 0.3	0.4 ± 0.2
Hypothalamus	1.6 ± 0.4	1.4 ± 0.4	0.6 ± 0.1
Cerebellum	4.2 ± 0.6	2.6 ± 0.9	0.5 ± 0.2
Pons	0.8 ± 0.2	1.4 ± 0.5	0.2 ± 0.1

[a]Measured with 100 pmol/l of ^{125}I-labelled peptide.
[b]Number of determinations = 3.

in rat brain, although they display significant cross-reactivity with the other type of receptor.

Affinity labelling

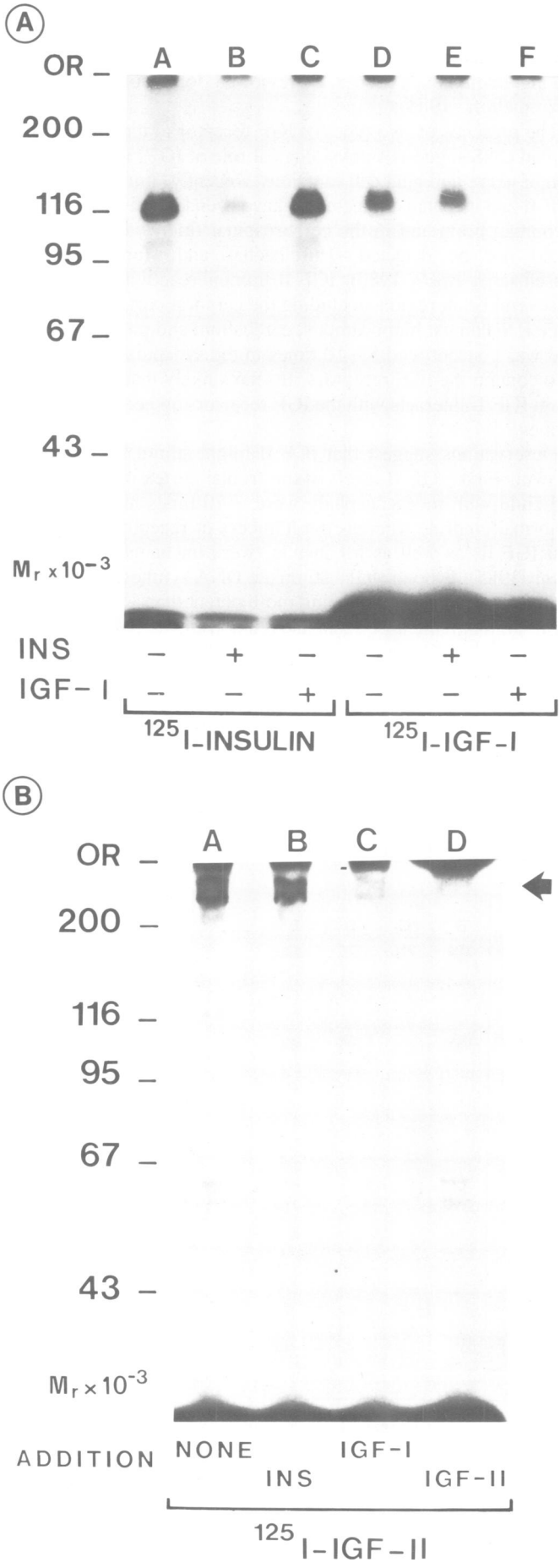
Binding of [^{125}I]IGF or [^{125}I]insulin followed by addition of disuccinimidyl suberate and SDS polyacrylamide gel electrophoresis of the radioactive proteins, showed specific labelling of the type I IGF and insulin receptor α-subunits with mol. wt. ~115 000 (Figure 2A). The labelling of the insulin receptor was completely prevented by 1 μmol/l insulin but not by 1 μmol/l IGF I. The opposite was seen with the [^{125}I]IGF I binding indicating the presence of a specific IGF I receptor α-subunit of the same molecular size as that of the insulin receptor α-subunit.

Affinity labelling of receptors with [^{125}I]IGF II followed by SDS-polyacrylamide gel electrophoresis showed a broad band of mol. wt. ~220 000–250 000 (Figure 2B). The labelling with [^{125}I]IGF II was not affected by an excess of insulin (1 μmol/l), but was inhibited partially by IGF I and completely by IGF II at concentrations of 1 μmol/l, in accordance with their affinity in the binding assay. A closer examination of the labelled type II IGF receptor revealed that the broad band appeared as a doublet, suggesting heterogeneity of the binding protein or proteolytic degradation of a unique binding subunit. As seen in Figure 2B, cross-linking of [^{125}I]IGF II to brain plasma membranes also resulted in a weak labelling of the IGF I receptor α-subunit (mol. wt. ~115 000) reflecting the cross-reactivity of IGF II with the type I IGF receptor (see Figure 1A). Labelling of receptors on human brain cortical membranes with ^{125}I-labelled IGF I, IGF II or insulin revealed that the mol. wts. of the respective subunits in human and rat brain are identical (data not shown).

The size of the type I IGF receptor α-subunits in brain (mol. wt. ~115 000) is significantly smaller than the value (mol. wt. ~130 000) described in several other tissues (Massagué and Czech, 1982; Kull *et al.*, 1983). This difference was confirmed by a direct comparison with affinity-labelled IGF I receptors from human placenta (data not shown). The insulin receptor α-subunit from rat brain also has a smaller mol. wt. (mol. wt. ~115 000) than in other tissues (mol. wt. ~130 000) (Yip *et al.*, 1980; Heidenreich *et al.*, 1983; Gammeltoft *et al.*, 1984b, 1985). Finally, a direct comparison between affinity-labelled IGF II receptors in brain cortex and liver revealed no difference in their molecular size (mol. wt. ~250 000) (data not shown). In conclusion, affinity labelling shows that the α-subunits of type I IGF and insulin receptors in rat and human brain have a similar molecular size (mol. wt. ~115 000) which is significantly smaller than in other tissues. In contrast, the type II IGF receptors in brain have an average molecular size (mol. wt. ~220 000–250 000) similar to that in other cells.

Phosphorylation

The β-subunit of the type I IGF receptors was identified by phosphorylation with [γ-^{32}P]ATP of solubilized, lectin-purified receptors from human brain cortex using immunoprecipitation with a specific monoclonal antibody to IGF I receptor, αIR-3 (Kull *et al.*, 1983). As shown in Figure 3, the antibody precipitated a phosphorylated protein of mol. wt. ~94 000, which is similar to the β-subunit of the IGF I receptor from placenta and IM-9 lymphocytes (Kull *et al.*, 1983; Jacobs *et al.*, 1983). The labelling was doubled by incubation with IGF I at



a concentration of 0.1 μmol/l. The β-subunit of lectin-purified insulin receptors from rat brain cortex (mol. wt. ~94 000) was precipitated with antibody to insulin receptor B_9, and its phosphorylation was clearly stimulated by insulin (data not shown) in agreement with our previous observations using a crude preparation of solubilized receptors (Gammeltoft *et al.*, 1984b). We cannot answer the question whether brain IGF II receptors are also phosphorylated because we had no antibody to IGF II receptor for immunoprecipitation. In conclusion, the type I IGF receptors in mammalian brain cortex are oligomers composed of α-subunits with a hormone binding site and β-subunits which can be phosphorylated.

Interaction with antibodies to insulin receptor

Antibodies to insulin receptors from patients with severe insulin resistance, and acanthosis nigricans inhibit insulin binding and show insulin-like effects *in vitro* on glucose metabolism (Kahn *et al.*, 1981). The antibodies to insulin receptor cross-react with type I IGF receptors from several tissues (Jonas *et al.*, 1982; Kasuga *et al.*, 1983) indicating identities in antigenic determinants of the insulin and type I IGF receptors. We studied the interaction of two antibodies to insulin receptors, B_2 and B_9, with the IGF and insulin receptors in rat brain with respect to inhibition of affinity labelling and immunoprecipitation of receptors. Both antisera caused a partial inhibition of the labelling of receptors with either [^{125}I]IGF I or [^{125}I]insulin, but not with [^{125}I]IGF II. Antiserum B_2 was a more efficient inhibitor of IGF I binding than B_9, in contrast to their almost equal inhibition of insulin-receptor labelling (Figure 4). The antibodies immunoprecipitated solubilized receptors which have been covalently linked with either [^{125}I]IGF I or [^{125}I]insulin, but not receptors labelled with [^{125}I]IGF II. Again antiserum B_2 was more potent than B_9 in precipitating the [^{125}I]IGF I labelled receptor, whereas both antisera precipitated the same amount of insulin receptors (data not shown). The difference in the effects of these two antisera is in accordance with studies of type I IGF and insulin receptors in other cells (Jonas *et al.*, 1982; Kasuga *et al.*, 1983) and suggests similarities in antigenic determinants of the type I IGF receptors in brain and other tissues tested.

Discussion

We have shown the widespread distribution of two types of IGF receptor in the rat and human CNS. The IGF receptors in brain have been identified unequivocally by their binding specificity, subunit structure, protein kinase activity and interaction with antibodies to insulin receptor, which resemble the characteristics of IGF receptors in other tissues (Zapf *et al.*, 1978; Rechler *et al.*, 1980; Massagué and Czech, 1982; Kull *et al.*, 1983; Thibault *et al.*, 1984). One difference was observed regarding the lower molecular size of the α-subunit of type I IGF receptor in brain (mol. wt. ~115 000) compared wtih that in other tissues (mol. wt. ~130 000). The same is true of the brain insulin receptor (Yip *et al.*, 1980; Heidenreich *et al.*, 1983; Gammeltoft *et al.*,

Fig. 2. (**A**). Affinity labelling of insulin and type I IGF receptors. Rat brain cortical plasma membranes were incubated for 2 h at 20°C with [^{125}I]insulin (10 nmol/l) (**A**–**C**) or [^{125}I]IGF (10 nmol/l) (**D**–**F**) in the absence or presence of insulin or IGF I (1 μmol/l) as indicated. Cross-linking with disuccinimidyl substrate, solubilization of membranes in 3% SDS plus mercaptoethanol, polyacrylamide gel electrophoresis and autoradiography were performed (see Materials and methods). (**B**) Affinity labelling of type II IGF receptors. Rat brain membranes were incubated with [^{125}I]IGF II (10 nmol/l) in the absence or presence of insulin, IGF I and IGF II (1 μmol/l) as indicated. After cross-linking, the labelled proteins were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography.

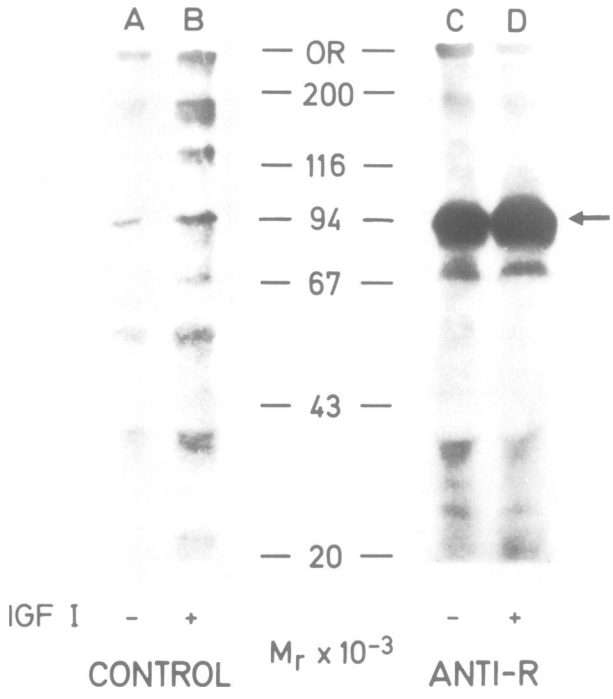


Fig. 3. Phosphorylation of type I IGF receptors. Plasma membranes from human brain cortex were solubilized by Triton X-100 (0.5% v/v), ultracentrifuged and the glycoproteins purified from the supernatant by WGA-agarose chromatography. Partially purified receptors were incubated 30 min at 20°C with buffer (**A** and **C**), IGF I (**B** and **D**) at a concentration of 0.1 μmol/l and phosphorylated with [γ-^{21}P]ATP as described in Materials and methods. The phosphorylated proteins were incubated for 6 h at 4°C with either normal mouse serum (1:400) alone (**A** and **B**), or normal mouse serum (1:400) plus monoclonal anti-IGF I receptor antibody αIR-3 (1:200) (**C** – **D**) followed by immunoprecipitation with anti-mouse immunoglobulin IgG for 12 h at 4°C. The immunoprecipitates were solubilized in 3% SDS plus mercaptoethanol and analyzed by gel electrophoresis and autoradiography.

1984b, 1985). It is possible that IGF I and insulin receptors in CNS undergo different post-translational glycosylation or proteolytic cleavage. This is an example of receptor heterogeneity among mammalian tissues as reviewed for insulin receptors (Gammeltoft, 1984).

The presence of two types of IGF receptor in the murine and human CNS suggests a physiological role of IGFs in the regulation of nerve and glial cell functions. Recently, immunoreactive IGF II and its putative precursors have been identified in extracts of human brain and in the cerebrospinal fluid, whereas IGF I could not be detected (Haselbacher and Humbel, 1982; Haselbacher *et al.*, 1985). IGF II immunoreactivity was found in several brain regions including the cerebral cortex, forebrain nuclei, thalamus, hippocampus, cerebellum and pituitary gland, and was concentrated 8 – 10 times in the synaptosomes, which also contain the IGF receptors. It seems likely that endogenous brain IGF II interacts with the IGF receptors on nerve cell membranes.

Observations suggest that IGF II might stimulate nerve cell growth. First, IGF II levels in the frontal cortex of a 3-month-old child with megaencephaly were ~10 times higher than in a normal control, whereas the numbers of receptors for IGF I and IGF II, as well as for insulin, were unchanged (Schoenle *et al.*, 1985). Second, IGFs stimulate DNA synthesis in fetal rat brain cell cultures, IGF I being more potent than IGF II and insulin, although the dose-response relationship of IGF II was not studied in detail (Lenoir and Honegger, 1982). Previously, immunoreactive insulin has been detected in brain-tissue extracts from several animal species (Havrankova *et al.*, 1978b), and insulin stimulates cell growth in fetal brain (Raizada *et al.*, 1980; Roger and Fellows, 1980) *via* binding to specific receptors (Havrankova *et al.*, 1978a; Gammeltoft *et al.*, 1984a).

The presence of receptors for IGFs and insulin together with the occurrence of IGF II and insulin, but not IGF I in the mammalian CNS leads us to propose the following hypothesis for the

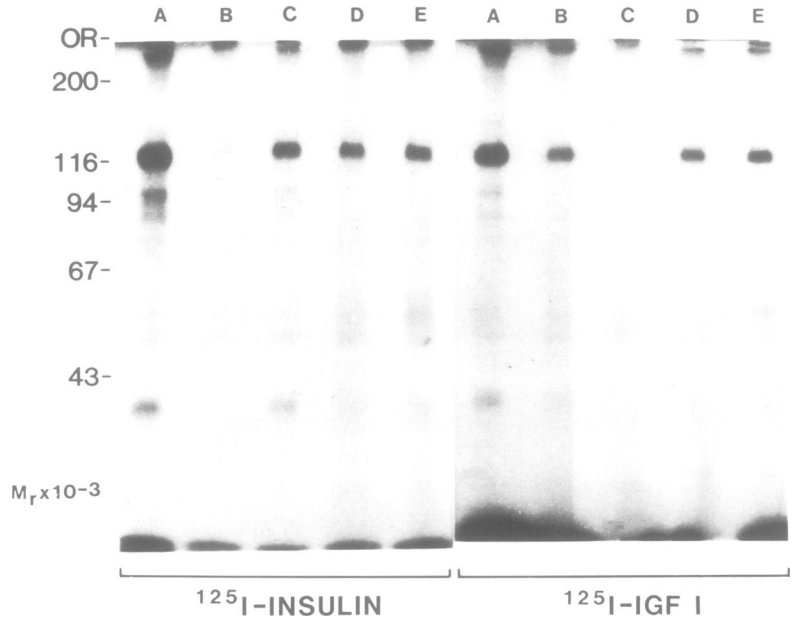


Fig. 4. Interaction of antibodies to insulin receptor with type I IGF and insulin receptors. Rat brain cortical membranes were pre-incubated for 90 min at 20°C with buffer (**A**), insulin (1 μmol/l) (**B**), IGF I (1 μmol/l) (**C**), antiserum to insulin receptor B_2 (1:100) (**D**), or B_9 (1:100) (**E**) followed by incubation for 90 min at 20°C with either [^{125}I]insulin (left) or [^{125}I]IGF I (right) at concentrations of 10 nmol/l. The samples were cross-linked with disuccinimidyl substrate for 15 min at 4°C, boiled in 3% SDS with mercaptoethanol, and analyzed by gel electrophoresis and autoradiography.

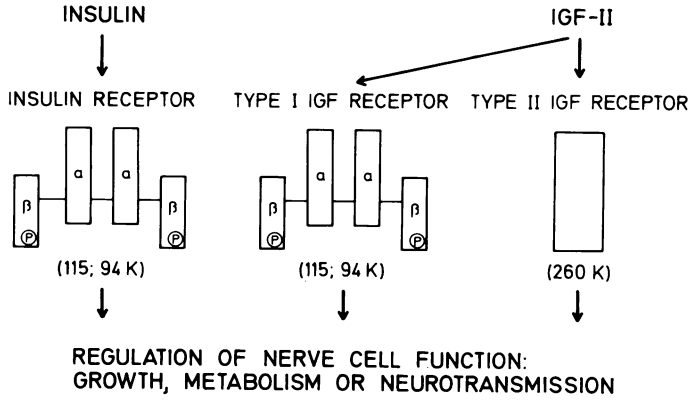


Fig. 5. Model of the mechanism of action of insulin and IGF II in mammalian brain. Three types of receptor are found in the murine and human CNS. Insulin in brain binds to its own receptor leading to stimulation of cell growth and metabolism, IGF II, but not IGF I, is present in human CNS, and interacts with both types of IGF receptors mediating actions on cell growth, metabolism and neurotransmission. The precise nature of these effects is currently unknown.

mechanism of action of the insulin family of peptides in brain (Figure 5). Insulin induces its actions on cellular growth and metabolism *via* binding to its own receptor. IGF II interacts with both types of IGF receptor mediating effects on brain cell growth, metabolism and neurotransmission. The nature of these actions is as yet unknown, but it is tempting to speculate that the type I IGF receptor with its tyrosine kinase activity is linked to stimulation of cell growth, in analogy with the tyrosine kinases of receptors for insulin (Kasuga *et al.*, 1982), for epidermal growth factor (Cohen *et al.*, 1980) and for platelet-derived growth factor (Heldin *et al.*, 1983) and of several cellular and retroviral oncogene proteins (reviewed by Bishop, 1983). Recently, amplification, enhanced expression and possible rearrangement of the epidermal growth factor receptor gene in primary human brain tumours of glial origin was reported (Libermann *et al.*, 1985), suggesting a role of receptor kinases in neoplasia. At present, the levels of IGF receptors in brain tumours are unknown, but studies are in progress.

The physiological role of the type II IGF receptor seems less clear. Studies in rat adipocytes have shown that insulin increases the apparent affinity of the IGF II receptor (King *et al.*, 1982) and causes a redistribution of the receptor between plasma membranes and low density microsomes (Oppenheimer *et al.*, 1983). This effect is similar to the insulin-induced redistribution of glucose transporters on fat cell plasma membranes (Cushman and Wardzala, 1980), and possibly, therefore, the IGF II receptor may have a role in cellular uptake of IGFs. In conclusion, the presence of IGF II and the two types of IGF receptor in mammalian CNS provides strong evidence for a physiological role of IGF II in the regulation of brain function, possibly related to cellular growth.

Materials and methods

Isotopes

[A14-^{125}I]Monoiodoinsulin was a gift from Novo Research Institute, Copenhagen, Denmark. [B26-^{125}I]Monoiodoinsulin was purchased from the Radiochemical Center, Amersham, UK. [^{125}I]IGF I and [^{125}I]IGF II were prepared by the chloramine T method. [γ-^{32}P]ATP was from Radiochemical Centre, Amersham, UK.

Peptides

Porcine insulin and proinsulin were purchased from Novo Research Institute, Copenhagen, Denmark. Coypu insulin was a gift from T.L.Blundell, Department of Crystallography, Birkbeck College, London, UK. IGF I and IGF II were purified from human serum as described (Rinderknecht and Humbel, 1976).

Antisera

Anti-insulin receptor antisera, B_2 and B_9 were gifts from C.R.Kahn, Joslin Research Laboratory, Boston, MA, USA. Monoclonal antibody (mouse) to the human IGF I receptor, αIR-3 was a gift from S.Jacobs, Wellcome Research Labs., Triangle Park, NC, USA. Anti-mouse immunoglobulin antiserum was purchased from Dako, Copenhagen, Denmark.

Chemicals

The materials for SDS-polyacrylamide gel electrophoresis were from Bio-Rad. Pansorbin (*Staphylococcus* protein A) was from Calbiochem and wheat germ agglutinin (WGA)-agarose from Sigma.

Tissue preparation

The brains of adult female Wistar rats (200 g), and human brains (12 – 24 h post mortum) were dissected and the tissue homogenized at 0°C in sucrose 0.32 nmol/l. Plasma membranes were prepared by differential centrifugation followed by centrifugation on a discontinuous sucrose density gradient as described (Gammeltoft *et al.*, 1984a).

Membrane-binding assay

Plasma membranes (0.5 mg protein/ml) in Krebs-Ringer solution containing Hepes (25 mmol/l, pH 8.0), albumin (10 mg/ml) and bacitracin (1 mg/ml) were incubated for 2 h at 15°C with ^{125}I-labelled IGF I, IGF II or insulin (100 pmol/l) and unlabelled peptides added as indicated. The receptor-bound ^{125}I-activity was determined as described (Gammeltoft *et al.*, 1984a).

Affinity labelling

Plasma membranes (3 mg protein/ml) in NaCl (150 mmol/l) containing Hepes (50 nmol/l, pH 7.8), albumin (10 mg/ml) and bacitracin (1.8 mg/ml) were in incubated for 2 h at 20°C with ^{125}I-labelled IGFs or insulin (10 nmol/l). After centrifugation and resuspension in NaCl-Hepes buffer (without albumin and bacitracin), disuccinimidyl suberate (0.1 mmol/l) dissolved in dimethyl sulphoxide was added. The reaction was stopped after 15 min at 4°C by addition of Tris (10 mmol/l, pH 7.4) and EDTA (1 mmol/l) as described (Gammeltoft *et al.*, 1984b).

Phosphorylation

Plasma membranes (30 mg protein/ml) were solubilized in NaCl-Hepes buffer with bacitracin (1.8 mg/ml), trasylol (100 KIU) and PMSF (0.17 mg/ml) and Triton X-100 (0.5% v/v). Glycoproteins were purified by WGA-agarose chromatography (see below) and incubated in the absence or presence of insulin or IGFs (100 nmol/l) for 30 min at 20°C followed by phopshorylation with [γ-^{32}P]-ATP (15 μmol/l) for 15 min at 20°C after addition of $MgCl_2$ (8 mmol/l) and $MnCl_2$ (4 mmol/l). The reaction was stopped by addition of EDTA (25 mmol/l) and NaF (50 mmol/l) (Van Obberghen and Kowalski, 1982).

Immunoprecipitation

Solubilized receptors were incubated overnight at 4°C with antisera or monoclonal antibodies followed by precipitation with protein A or anti-mouse IgG (Kasuga *et al.*, 1983; Jacobs *et al.*, 1983).

WGA-agarose chromatography

Solublized receptors were applied to column, washed with buffer and eluted with N-acetylglycosamine (Van Obberghen *et al.*, 1983).

SDS-polyacrylamide gel electrophoresis

Membrane pellets or immunoprecipitates were solubilized in SDS (3%) with mercaptoethanol and bromophenol and boiled for 5 min. Electrophoresis was performed on 7.5% polyacrylamide gel with mol. wt. standards (mol. wt. ~40 000 – 200 000) (Laemmli, 1970). The gel was autoradiographed on Kodak X-omat film.

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