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RECEPTORS FOR INSULIN-LIKE GROWTH FACTORS AND INSULIN ON MURINE FETAL CORTICAL BRAIN CELLS

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Fetal murine neuronal cells bear somatomedin receptors which can be classified according to their affinities for IGF-I, IGF-II and insulin. Binding of ^{125}I-IGF-I is half-maximally displaced by 7 ng/ml IGF-I while 15- and 700-fold higher concentrations are required for, respectively, IGF-II and insulin. Linear Scatchard plots of competitive-binding data with IGF-I suggest one single class of type I IGF receptors (K_a = 2.6 x 10^9 M^{-1}; R_o = 4500 sites per cell). The occurrence of IGF-II receptors appears from the specific binding of ^{125}I-IGF-II and competition by unlabeled IGF-II; the IGF-II binding sites display a low affinity for IGF-II and no affinity for insulin. IGF-II also interacts with insulin receptors although 50- to 100-fold less potent than insulin in competing for ^{125}I-insulin binding. The presence of distinct receptors for IGF-I, IGF-II and insulin on fetal neuronal cells is consistent with a role of these peptides in neuronal development, although our data also indicate that IGF-I receptors could mediate the growth promoting effects of insulin.

Somatomedins or insulin-like growth factors (Sm/IGF) are polypeptides which are structurally related to insulin and functionally characterized by growth promoting effects in various tissues(1). They include somatomedin-C/ insulin-like growth factor I (further denoted as IGF-I) and IGF-II which have been structurally characterized by Rinderknecht and Humbel (2,3) and Klapper *et al.* (4). In various cell types, biological effects of the somatomedins are mediated by specific cell surface receptors which have been clearly distinguished from insulin receptors (5,6). However, insulin and somatomedins have been found to interact with each others receptors (7,8,9,10,11,12). The latter characteristic may have functional consequences in the nervous system where not only insulin (13) and somatomedin (14,15) but also insulin

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receptors (16) and somatomedin receptors (17,18) have been demonstrated. Furthermore, insulin (19) as well as somatomedin (18,20) have been described to promote survival, differentiation and/or replication of fetal neuronal cells in vitro. In the light of these observations we investigated the occurrence of somatomedin receptors in freshly isolated murine fetal cortical brain cells and compared their interactions with IGF-I, IGF-II and insulin with those of the earlier described insulin receptors (21).

MATERIALS AND METHODS

Peptides.

The somatomedin preparations were isolated from Cohn fraction IV according to a modification of a previously reported procedure (22) or as described by Svoboda et al. (23). Isolation was monitored with an IGF-I radioreceptorassay (24). In brief, Cohn IV was suspended in 8 to 12 volumes 2M acetic acid and 75 mM NaCl and ultrafiltered through an Amicon cartridge filter with nominal cut-off of 30,000 daltons (PM 30). The ultrafiltrate was concentrated 10- to 40-fold on a cartridge with a cut-off of 2,000 daltons (H5P2). The concentrate was sequentially chromatographed twice on SP-Sephadex C25 using a combination of a salt and pH-gradient. The tubes were arbitrarily pooled into larger fractions based on pH and salt changes. They were dialysed, lyophilized and passed over Sephadex G50. The active fractions were pooled, lyophilized and chromatographed on CM Sepharose using a pH gradient with 0.1 M acetic acid (pH 5.0) as starting buffer and chromatographed for a second time on Sephadex G50. The active fractions were pooled. This preparation had a specific activity of 40 U/mg as determined by IGF-I radioreceptor assay (n° 685) and was employed in excess (4 U/ml) to determine non-specific binding in the receptor binding assay. Further purification consisted of preparative isoelectric focussing followed by gel filtration on Sephadex G50. The basic fraction of P_I 8.85 - corresponding to Sm-C/IGF-I (prep.717 I, spec. act : 1230 U/mg) and the neutral fraction with P_I 6.80, containing IGF-II (prep.761, spec. act : 925 U/mg) were used in competition experiments in IGF-binding. They were also further purified by HPLC (25,26) on a Vydac 218 TPcolumn (0.1% TFA H_2O/CH_3CN), in order to prepare Sm-C/IGF-I with a specific activity of 13000 U/mg (prep.960 II/18) and IGF-II with a specific activity of 1000 U/mg (prep.943). The HPLC-purified Sm-C/IGF-I was used for competitive-binding assays with ^{125}I-IGF-I and subsequent determination of receptor affinity and binding site concentration. The highly purified preparations were also employed for iodionation by the lactoperoxidase method (27). The labeled peptides displayed a specific activity in the range of 60 to 150 μCi/μg. Bovine insulin was purchased from Novo (Copenhagen, Denmark). The ^{125}I-insulin preparation was prepared by Chloramin-T iodination and submitted to HPLC (28) in order to isolate the ^{125}I-TyrA14-insulin isomer for binding studies (29).

Cortical cells.

Brain cells were dissected from the cortex of fetal C57/BL mice as previously described (30, 31). Briefly, cortices were removed from 14½ - 15 day-old mice embryos and cleaned from adhering tissue. Excised

tissue was cut into pieces and then mechanically dissociated in Ca^{2+}-free Krebs medium (pH 7.5). All experiments were carried out on freshly dissociated cells, suspended at approximately 10^7 cells/ml; these cell preparations are highly enriched in neurons, as appears from their positive stain (95%) for neuron-specific enolase; only few cells (5%) stain for glial fibrillary acid proteins (32). During the experiments, the cells remained structurally uncoupled and viable.

Binding assay.

Binding experiments were carried out in Ca^{2+}-free Krebs-Ringer medium buffered with Hepes and bicarbonate, containing 1 mM EGTA and glucose (0.1 g/dl) (pH 7.5) and supplemented with 1% (w/v) bovine serum albumin (fraction V, RIA-grade; Sigma), bacitracin (0.8 mg/ml; Sigma) and Trasylol (1000 kallikrein-inhibition units/ml Bayer, Leverkussen, Germany) (assay buffer). The binding assay started after addition of 400 µl cell suspension to siliconized polystyrene tubes containing 50 µl labeled peptide and 50 µl assay buffer with or without unlabeled peptide. After 120 min incubation at 15°C, duplicate 200 µl samples were withdrawn from the assay tubes, mixed with 200 µl ice-cold assay buffer and centrifuged at 10,000 g for 45 sec in a Beckman Microfuge B. After aspirating the supernatant, tube tips were cut and counted. Specific binding was calculated as the difference in binding between conditions containing only ^{125}I-insulin or ^{125}I-IGF-I,II and those containing an excess of unlabeled insulin (10 µg/ml) or insulin-like growth factor (SM 685; 4 Units/ml).

RESULTS

Binding of ^{125}I-IGF-I and effects of somatomedin and insulin.

Specific binding of ^{125}I-IGF-I to brain-cortical cells increased with time, being half-maximal at min 20 and in steady state at min 120 (Fig. 1). Under these conditions, specific binding was reached with 5 to 9% of labeled IGF-I, while non-specific binding represented 8 to 10% of total binding. At the end of a 120 min incubation at 15°C, the TCA-soluble radioactivity was inferior to 15%. Brain-cortical cells were incubated with ^{125}I-IGF-I in the absence or presence of increasing concentrations of unlabeled IGF-I, IGF-II or insulin (Fig.2). IGF-I was a more potent competitor for ^{125}I-IGF-I binding than IGF-II, provoking half-maximal displacement at 15-fold lower concentrations IGF-I (7 ng/ml) than IGF-II (100 ng/ml). A competition was also observed with insulin, but this effect only occurred at concentrations higher than 1000 ng/ml, whereas half-maximal displacement required 5000 ng/ml (8×10^{-7} M). When binding of IGF-I to brain-cortical cells was expressed as a function of free IGF-I, saturation of IGF-I binding sites appeared achieved at 6 nM IGF-I (Fig. 3). The same competitive-binding data analysed according to Scatchard (33) resulted in linear plots (Fig.3, inset) with an affinity constant (K_a) of 2.56 ($\pm$ 0.23) $\times 10^9$ M^{-1} (SEM;

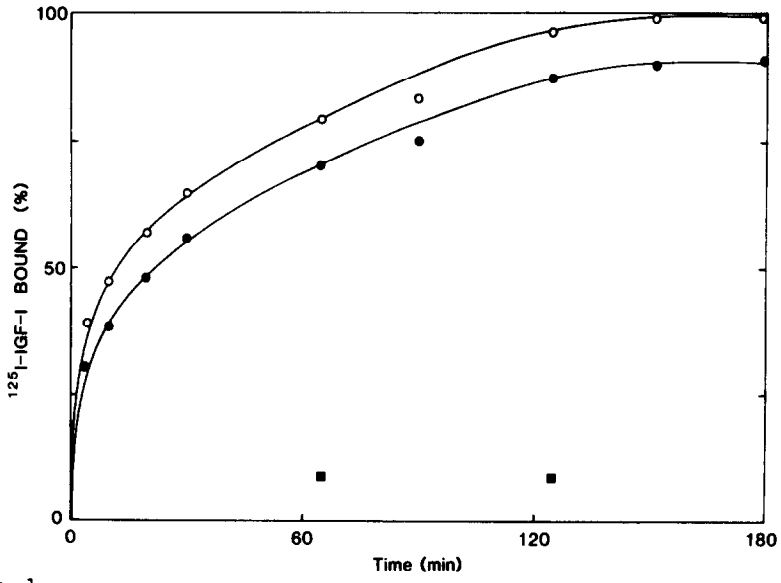

Fig. 1.
Association kinetics of ^{125}I-IGF-I to brain-cortical cells. Brain cortical cells (5.8 x 10^6/ml) were incubated with ^{125}I-IGF-I (380 pM). At the time indicated, cell-bound radioactivity was measured and expressed as a percent of total radioactivity bound at 180 min (O). Non-specific binding was determined at min 65 and 125 (■); specific binding (●) was obtained as described under Methods.

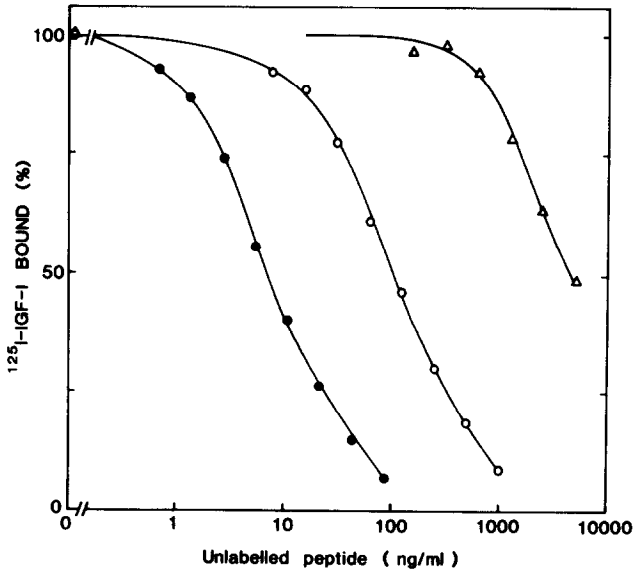

Fig. 2.
Competition for ^{125}I-IGF-I binding by unlabeled IGF-I, IGF-II and insulin. Brain-cortical cells (9 x 10^6/ml) were incubated with ^{125}I-IGF-I (550 pM) in the presence of the indicated concentrations of unlabeled IGF-I (●), IGF-II (O), or insulin (Δ). Binding of ^{125}I-IGF-I was expressed as a percent of binding in the absence of any other peptide. Correction was made for non-specific binding.

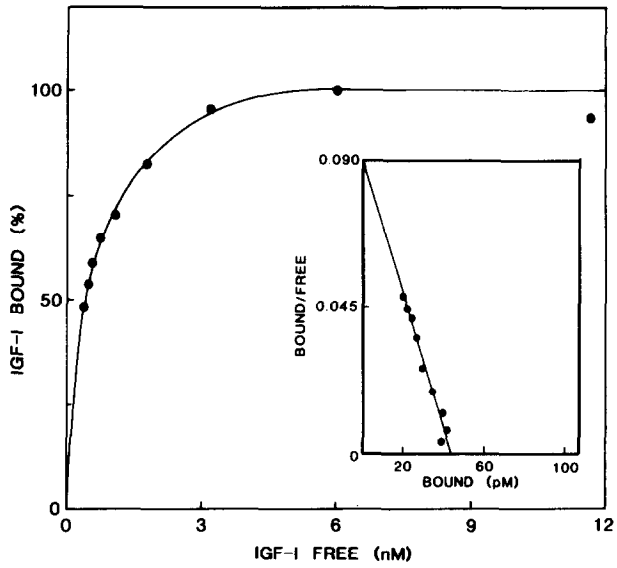

Fig. 3.
Saturation of IGF-I binding sites by IGF-I. Brain-cortical cells (7 x 10^6/ml) were incubated with ^{125}I-IGF-I (435 pM) in the presence of various concentrations of unlabeled IGF-I. Specific binding of IGF-I to the brain cells was expressed as a percent of maximum binding and plotted as a function of free IGF-I.
Inset : Scatchard plot of the same binding data. Bound/free ^{125}I-IGF-I was plotted as a function of specific IGF-I binding to the brain cortical cells. Data were corrected for non-specific binding and submitted to linear regression (correlation coefficient higher than -0.97).

n=5). The total number of binding sites (R_o) was estimated at 4483 (± 567) (SEM; n=5) per cell.

Binding of ^{125}I-IGF-II and effects of IGF-II and insulin.

^{125}I-IGF-II was also found to bind to brain-cortical cells. Under the present conditions, specific binding occurred for 2 percent of the labeled peptide while non-specific binding represented 27 to 30 percent of total binding. In the presence of 10 ng/ml IGF-II, binding of labeled IGF-II was 30 percent higher than in the absence of IGF-II (Fig. 4). At 100 ng/ml IGF-II, binding of ^{125}I-IGF-II was half-maximally displaced (Fig. 4), while a similar concentration of IGF-I induced only a slight decrease in maximal binding (data not shown). Insulin - even at 5000 ng/ml - exerted no competition (Fig. 4).

Binding of ^{125}I-insulin and effects of somatomedins and insulin.

Brain-cortical cells were incubated with ^{125}I-insulin in the presence or absence of increasing concentrations IGF-I, IGF-II or insulin. Up to 100 ng/ml, IGF-I did not compete with insulin binding (data not shown). Both IGF-II and insulin competed with ^{125}I-insulin binding, but

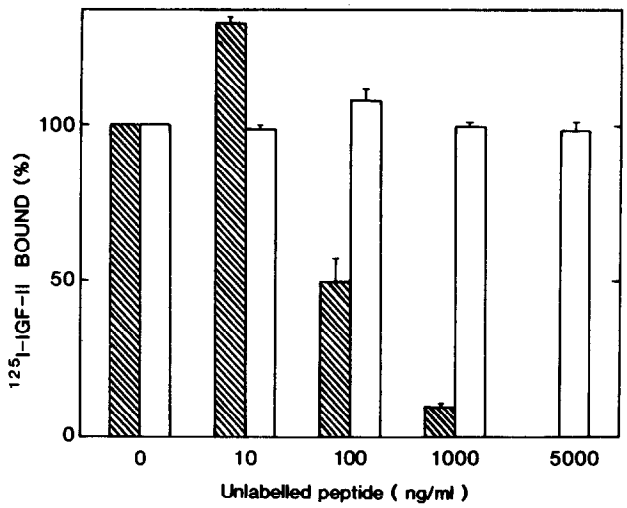

Fig. 4.
Competition for ^{125}I-IGF-II binding by unlabeled IGF-II and insulin. Brain-cortical cells (10^7/ml) were incubated with ^{125}I-IGF-II (300 pM) and the indicated concentrations of unlabeled IGF-II (▨) or insulin (□). Binding of ^{125}I-IGF-II was expressed as a percent of binding in the absence of unlabeled peptide after correction for non-specific binding.

half-maximal displacement occurred with markedly lower insulin concentrations (20 ng/ml) than IGF-II concentrations (1800 ng/ml) (Fig. 5). Thus IGF-II was only 1 to 2 percent as potent as insulin in

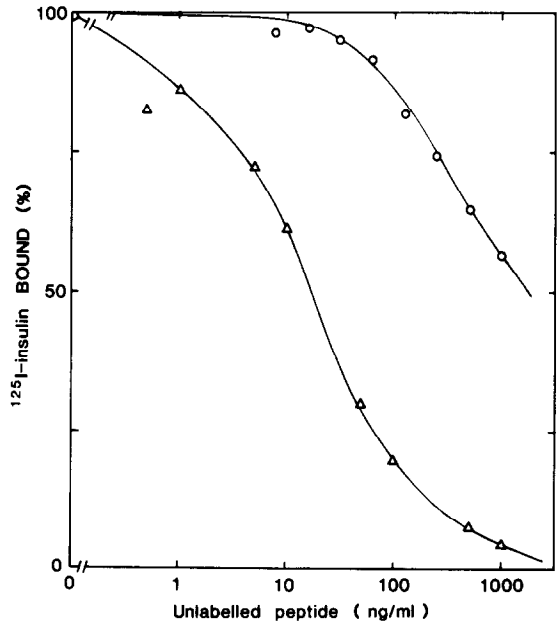

Fig. 5.
Competition for ^{125}I-insulin binding by unlabeled IGF-II and insulin. Brain-cortical cells were incubated with labeled insulin (150 pM) and the indicated concentrations of unlabeled IGF-II (O) or insulin (Δ). Binding of ^{125}I-insulin was expressed as a percent of binding in the absence of any other peptide. Correction was made for non-specific binding.

displacing ^{125}I-insulin binding on brain-cortical cells, while 20-fold lower concentrations of the peptide produced half-maximal displacement of ^{125}I-IGF-I binding.

DISCUSSION

Receptors for somatomedins have been identified in neural tissue from human and rat foetusses (17,18), but the relationship between somatomedin binding and insulin binding has not yet been clearly examined. The present study demonstrates the existence of somatomedin binding sites on freshly isolated intact neuronal cells from murine embryos and examines the possible interaction between binding of IGF-I, IGF-II and insulin. Competition experiments under steady state conditions indicated that IGF-I concentrations required for half-maximal displacement of ^{125}I-IGF-I were 7 ng/ml, while those of IGF-II and insulin were respectively 15- and 700-fold higher. This feature is a characteristic of type I IGF receptors which are considered to be structurally related to the insulin receptor (34). Analysis of the competitive-binding data with ^{125}I-IGF-I in the presence of unlabeled IGF-I indicated that the IGF-I binding sites are saturated by low concentrations of IGF-I; the linear plots in Scatchard analysis of these data suggest the presence of one class of non-interacting high affinity binding sites. Their binding affinity was calculated to be 2.6×10^9 M^{-1} whereas their concentration was estimated at 4500 sites per cell if they are homogeneously distributed over all cells. A similar affinity for IGF-I has been previously described in human fetal brain plasma membranes (17). Our experiments are less conclusive concerning the characteristics of a distinct IGF-II receptor. ^{125}I-labeled IGF-II bound to fetal neuronal cells, but specific binding was 3- to 5-fold lower than with a comparable concentration of ^{125}I-IGF-I; in addition, the concentration of unlabeled IGF-II required for half-maximal displacement of ^{125}I-IGF-II was close to that which exerted a similar effect upon ^{125}I-IGF-I binding. However, IGF-I and insulin were far less or totally ineffective in competing for ^{125}I-IGF-II binding at concentrations which markedly displaced ^{125}I-IGF-I from its binding sites. At 10-fold lower concentrations than those which reduced ^{125}I-IGF-II binding by 50 percent, unlabeled IGF-II enhanced binding of ^{125}I-IGF-II. Insulin did not induce such effect, although it was

previously found to do so in rat adipocytes (11,12,35). Whether the presence of 10 ng/ml IGF-II increases the number of IGF-II binding sites at the plasma membrane, or their affinity, remains to be elucidated. In contrast to the IGF-I receptors, the type II binding sites on fetal neuronal cells display a relatively low affinity for IGF-II, an even lower affinity for IGF-I and no affinity for insulin. It seems therefore not excluded that the endogenous ligand for the IGF-II binding sites is an IGF-II related substance (36) rather than IGF-II itself. Besides its low affinity binding to IGF receptors, IGF-II also interacts with insulin receptors on fetal brain cortical cells, but its potency to displace ^{125}I-insulin is 50- to 100-fold lower than that of insulin. A similar weak competition with insulin binding has also been observed in other cell types such as rat adipocytes (8) and IM-9 cells (37) which may indicate that the absence of negative cooperativity in insulin binding (21) is not necessarily associated with enhanced sensitivity for IGF-II (38).These and previously reported (21) data permit an assesment of the relationship between the binding of somatomedins and that of insulin to freshly isolated fetal neuronal cells. These cells have been shown to display specific receptors for IGF-I as well as for IGF-II and for insulin. Somatomedins and insulin bind with high affinity to their own receptor and with markedly lower affinity to each others' receptor. This cross reactivity is compatible with structural similarities between both the receptors and the peptides themselves (10). It is furthermore compatible with a heterogeneity of insulin binding on these cells, which could not be attributed to negative cooperativity (21). From 10^{-7} M on, insulin appears to interact with the type I IGF receptor, whereas it can be assumed that at lower concentrations this hormone binds solely to the insulin receptor. Consistent with this view is the fact that insulin binding to neuronal cells is represented by linear scatchard plots when non-specific binding is determined at 10^{-7} M instead of 10^{-6} M unlabeled insulin (data not shown). It seems therefore conceivable that some of the biological effects described for insulin concentrations of 10^{-7} M or higher are mediated through IGF-I receptors rather than through insulin receptors or IGF-II receptors (35,39). This possibility should be examined in the case of insulin-induced growth of fetal neuronal cells. The simultaneous expression of insulin receptors and type I IGF receptors

has not been observed on well known target cells for insulin such as adipocytes and hepatocytes (40) nor on H35 rat hepatoma cells (34) where the growth stimulatory effect of insulin (41) is mediated by insulin receptors which lack negative cooperativity (42). On the other hand, chick embryo fibroblasts present type I IGF receptors together with insulin receptors without negative cooperativity (37). However, the type I receptors on the latter cells exhibit a markedly higher affinity for IGF-II (9) than those on freshly isolated fetal-mouse brain-cortical cells, cultured rat brain cells (18) or human IM-9 lymphoblastoid cells (43). This apparent discrepancy may reflect the variation in affinity and in specificity of somatomedin binding which has been earlier described in fetal neuronal tissue (17). Regulation of biological effects at the receptor level may be a crucial mechanism during fetal development where the circulating IGF levels seem to lack the hormonal regulation by growth hormone (44,45). In conclusion, we have identified specific high affinity receptors for IGF-I in fetal murine brain-cortical cells. Type I IGF receptors differ from insulin receptors, but could well mediate the biological effects of high insulin concentrations upon fetal brain cells. Further studies are now required to further characterize the IGF-II binding sites and to define the respective biological effects of IGF-I, IGF-II and insulin upon neural development.

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