Insulin receptors in rat brain: insulin stimulates phosphorylation of its receptor β-subunit

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In rat brain cortex synaptosomes insulin stimulated the phosphorylation of its own receptor β-subunit (94 kDa) as identified by immunoprecipitation with anti-insulin or anti-receptor antiserum. The receptor α-subunit (115 kDa) was characterized by specific labeling with ^{125}I-labeled photoreactive insulin. These observations indicate that: (i) insulin receptors in brain are composed of α-subunits which bind insulin, and β-subunits, the phosphorylation of which can be stimulated by insulin; (ii) the size of α-subunits in brain is significantly smaller than in other tissues (115 *vs* 130 kDa), whereas β-subunits (94 kDa) are identical. We suggest that brain insulin receptors represent a subtype regarding their binding function, whereas their enzyme function is more conserved.

Hormone receptor *Receptor subunit* *Phosphorylation* *Rat brain cortex synaptosome*

1. INTRODUCTION

Insulin receptors in all cell types studied so far are oligomeric glycoproteins composed of α-subunits (130 kDa) and β-subunits (94 kDa) linked by disulphide bonds [1]. This particular subunit composition provides the insulin receptor with a functional domain distribution. Thus, binding of insulin to the receptor α-subunit activates the protein kinase of the β-subunit, which is autophosphorylated in both intact cells and cell-free systems [2–4]. Insulin receptors are widespread in the rat central nervous system [5,6]. Recent studies of receptor binding have demonstrated differences in kinetics compared with other tissues [6], as well as a smaller molecular mass of the α-subunit (115 kDa) [7,8], suggesting that the brain insulin receptor might be a subtype. To evaluate this possibility, we have here studied the subunit structure of insulin receptors on purified rat brain cortex synaptosomes and searched for putative functional β-subunits using a cell-free phosphorylation assay.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Na^{125}I and [γ-^{32}P]ATP were purchased from Amersham; protein A (Pansorbin) from Calbiochem; reagents for SDS-polyacrylamide gel electrophoresis (SDS–PAGE) from Bio-Rad. Porcine insulin and anti-insulin guinea pig antiserum were gifts from L.G. Heding (Novo Research Institute, Copenhagen). Photoreactive B2-(2-nitro-4-azidophenylacetyl)des-PheB1-insulin (B2-Napa-insulin) was a gift from D. Brandenburg (Deutsche Wollforschungsinstitut, Aachen). Serum from patient B9 with autoantibodies to insulin receptors was a gift from C.R. Kahn (Joslin Research Laboratory, Boston, MA).

2.2. *Tissue preparation*

Purified synaptosomes were prepared from brain cortex of female Wistar rats (200 g) as in [9] as described in [10].

2.3. *Phosphorylation*

Synaptosomes were solubilized in 0.5% Triton

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X-100 and phosphorylated by incubation with [γ-^{32}P]ATP in the absence or presence of insulin (10 nM) as in [3,4].

2.4. *Photoaffinity labeling*

Intact synaptosomes were incubated with ^{125}I-labeled B2-Napa-insulin in the dark followed by UV-irradiation as in [11].

2.5. *Immunoprecipitation*

Solubilized receptors were incubated with insulin (10 nM) for 30 min at 20°C, conditions in which steady state is obtained [6], followed by incubation with anti-insulin or anti-receptor antiserum for 16 h at 4°C. Non-immune serum was used as a control. Antibody–insulin–receptor complexes were precipitated by protein A and

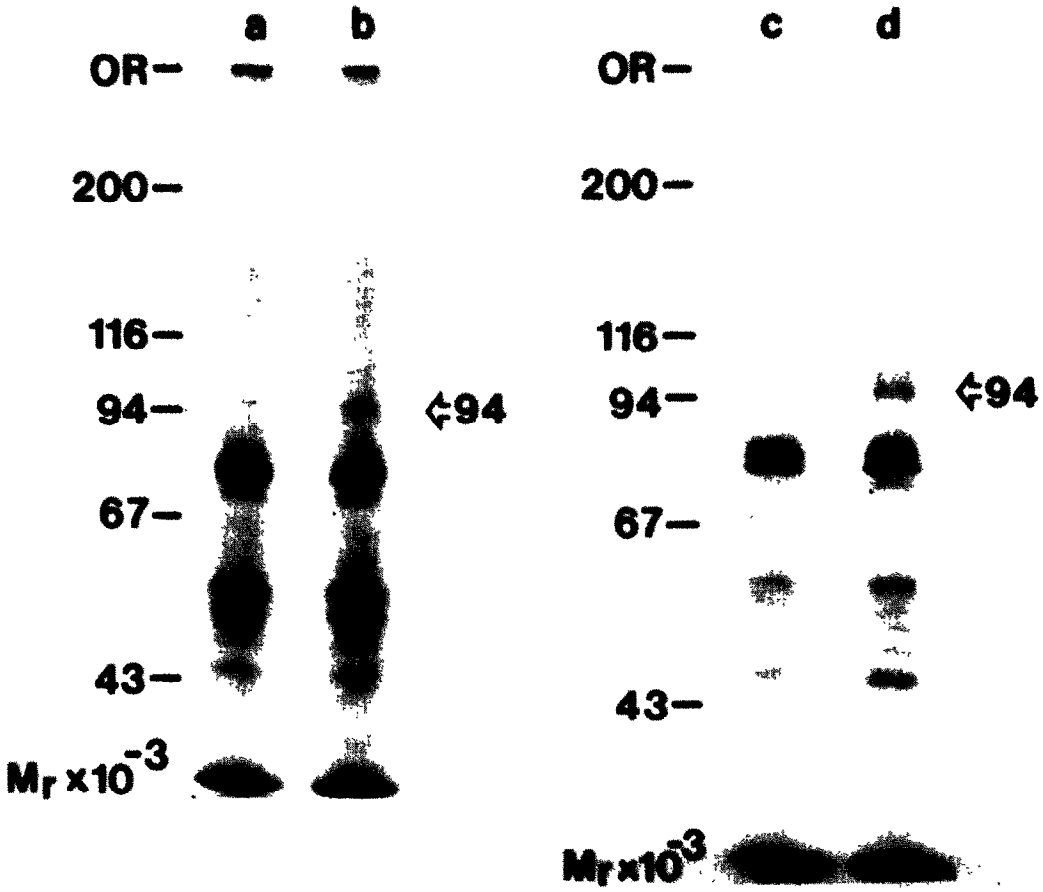

Fig.1. Phosphorylation of insulin receptors. Purified rat brain cortex synaptosomes (20 mg protein/ml) in Hepes buffer (50 mM, pH 7.6) containing NaCl (150 mM), bacitracin (1 mM), aprotinin (1000 trypsin-inhibitor units/ml) and PMSF (2 mM) were solubilized by Triton X-100 (0.5%, v/v) for 90 min at 4°C, and ultracentrifuged (100000 × *g*, 90 min). The supernatants were diluted with 4 vols of buffer with bovine serum albumin (2 mg/ml) and incubated for 30 min at 20°C in the absence (a), or presence (b–d) of insulin (10 nM). Phosphorylation was measured in a reaction mixture containing (final conc.) $MnCl_2$ (4 mM), $MgCl_2$ (8 mM), and [γ-^{32}P]ATP (15 μM) for 15 min at 20°C, and terminated by addition of EDTA (25 mM) and NaF (50 mM). All samples (a–d) were then incubated with insulin (10 nM) for 30 min at 20°C, followed by incubation with serum (final dilutions) for 16 h at 4°C: anti-insulin antiserum (1:100) (a,b), normal serum (1:100) (c), or anti-receptor antiserum (1:200) (d). After precipitation by protein A, the pellets were boiled for 5 min in a solution containing SDS (30 mg/ml), sodium phosphate (10 mM), glycerol (10%, v/v), 2-mercaptoethanol (2%, v/v) and bromophenol blue (0.01%, v/v), centrifuged (10000 × *g*, 5 min) and the supernatants were analyzed by one-dimensional SDS–PAGE (7.5% polyacrylamide) using standards as in [4]. The gels were stained, dried and autoradiographed by exposure to Kodak X-Omat film.

analyzed on SDS–PAGE under reducing conditions, as in [12] and autoradiographed as in [3,4].

3. RESULTS

3.1. *Phosphorylation of insulin receptors*

To search for insulin receptor β-subunits whose phosphorylation is stimulated by insulin, solubilized synaptosomes were first incubated without or with insulin (10 nM) and then with ^{32}P-labeled ATP. The phosphorylation reaction was stopped by EDTA. Thereafter, to allow for immunoprecipitation with anti-insulin antibodies, insulin was added to both incubations at 10 nM. After precipitation with protein A, the labeled proteins were reduced and analyzed by SDS–PAGE. Several proteins (40–200 kDa) of solubilized synaptosomes were phosphorylated in the absence of insulin (fig.1a). Only one additional labeled protein of 94 kDa was observed after stimulation with insulin (fig.1b). This phosphoprotein represents the β-subunit of the insulin receptor, since it was immunoprecipitable by anti-insulin antiserum (fig.1b), but not normal serum (fig.1c), indicating its association with insulin. This was further confirmed, since anti-receptor antiserum immunoprecipitated the same 94-kDa phosphoprotein (fig.1d). The insulin-induced phosphorylation of its own receptor β-subunit was specific for insulin, since vasoactive intestinal polypeptide, a peptidergic neurotransmitter [10], had no effect on the receptor labeling up to 1 μM (not shown). Similar results were obtained with solubilized rat liver plasma membranes, when the phosphorylated insulin receptor β-subunit (94 kDa) was immunoprecipitated with anti-insulin or anti-receptor antiserum (not shown). Thus, the molecular size of the receptor β-subunit of brain cortex is identical to that of the liver.

3.2. *Photoaffinity labeling of insulin receptors*

To characterize insulin receptor α-subunits, purified synaptosomes were incubated with ^{125}I-labeled B_2-Napa-insulin in the dark followed by UV-irradiation. One band (115 kDa) was heavily labeled and several smaller proteins (20–40 kDa) showed weaker labeling (fig.2a). The 115-kDa protein represents the insulin receptor α-subunit for the following reasons. Firstly, photoaffinity labeling was specific, since addition of unlabeled insulin

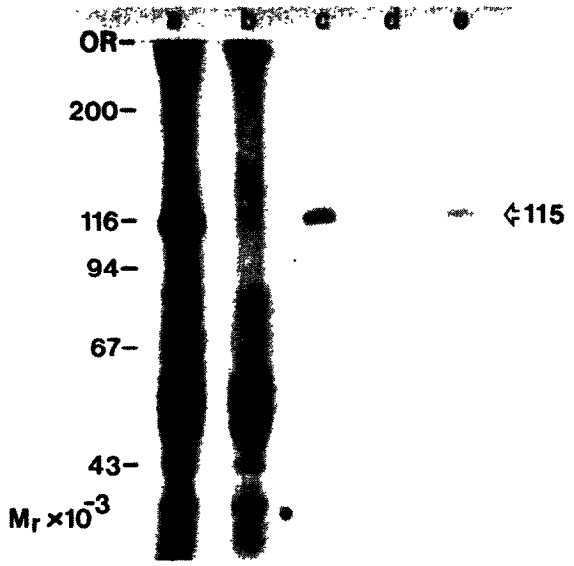

Fig.2. Photoaffinity labeling of insulin receptors. Purified synaptosomes (3 mg protein/ml) in Hepes buffer (50 mM, pH 7.6) containing NaCl (150 mM), bovine serum albumin (10 mg/ml) and bacitracin (1 mM) were incubated for 2 h at 20°C in the dark with ^{125}I-labeled B_2-Napa-insulin (10 nM) either in the absence (a) or presence (b) of unlabeled insulin (1 μM). The incubation was terminated by UV-irradiation for 5 min at 4°C followed by centrifugation (10000 × g, 5 min), and the pellets were boiled in SDS solution (see legend to fig.1). Alternatively, the pellets were solubilized in 0.5% Triton X-100 (see legend to fig.1) for 90 min at 4°C (c–e), and ultracentrifuged (100000 × g, 90 min). The supernatants were diluted with 1 vol. buffer, and incubated for 16 h at 4°C with serum (see legend to fig.1): anti-insulin antiserum (c), normal serum (d), or anti-receptor antiserum (e). After precipitation by protein A the samples were analyzed by SDS–PAGE and autoradiography.

(1 μM) prevented ^{125}I-B_2-Napa-insulin binding (fig.2b), whereas vasoactive intestinal polypeptide was without effect (not shown). Secondly, it was immunoprecipitable by anti-insulin antiserum (fig.2c), and not by normal serum (fig.2d) proving its association with insulin. Finally, anti-receptor antiserum immunoprecipitated the labeled 115-kDa protein (fig.2e). As expected from previous studies [1,11] labeling of liver plasma membranes with ^{125}I-labeled photoreactive insulin revealed the insulin receptor α-subunit of 130 kDa which was observed after immunoprecipitation with anti-in-

sulin or anti-receptor antiserum (not shown). The molecular size of the α-subunit of the brain insulin receptor (115 kDa) is thus significantly smaller than that of the hepatic insulin receptor α-subunit (130 kDa).

4. DISCUSSION

Our study presents the first demonstration that insulin stimulates phosphorylation of its own receptor β-subunit in rat brain cortex. Thus, insulin receptors in brain share with several other tissues the fundamental property of β-subunit phosphorylation induced by insulin [2–4]. Furthermore, the molecular size of the receptor β-subunit in brain cortex (94 kDa) is identical to that of the liver [3,4]. In contrast, the molecular mass of the receptor α-subunit in brain cortex (115 kDa) is smaller than that of other tissues (130 kDa) [1,11]. The latter is in agreement with previous studies of insulin receptor α-subunits identified on membranes of whole rat brain [7] and of olfactory tubercle, hippocampus and hypothalamus of rat brain [8].

Here, we have primarily used immunoprecipitation of insulin–receptor complexes with anti-insulin antiserum for purification of receptors and identification of α- and β-subunits after disulfide reduction, an approach which unambiguously establishes the insulin binding capacity of the precipitated receptors. In addition, we observed that anti-receptor antiserum, as in other rat tissues [1], interacted with insulin receptors from rat brain cortex, suggesting common antigenic determinants.

The physiological role of insulin in brain is still obscure, but the presence of receptors which consist of two subunits with separate functions, insulin binding and insulin-stimulated phosphorylation, like insulin receptors in well-defined insulin target tissues, strongly suggests a regulatory role of insulin in nerve cell function and growth. More importantly, intriguing differences were observed. Thus, the insulin binding α-subunit of the receptor in brain is significantly smaller than that of other cell types, and this is in accordance with the binding kinetics and insulin specificity of the brain receptors which are also different compared to other tissues [6]. In contrast, the size of the β-subunit of the brain insulin receptor is identical to that of other tissues. Taken together, it is tempting to suggest that the enzyme function of the insulin receptor has been more conserved than its binding function.

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REFERENCES

[1] Kahn, C.R., Baird, K.L., Flier, J.S., Grunfeld, C., Harmon, J.T., Harrison, L.C., Karlsson, F.A., Kasuga, M., King, G.L., Lang, U., Podskalny, J.M. and Van Obberghen, E. (1981) Recent Prog. Horm. Res. 37, 447–538.

[2] Kasuga, M., Karlsson, F.A. and Kahn, C.R. (1982) Science 215, 185–187.

[3] Van Obberghen, E. and Kowalski, A. (1982) FEBS Lett. 143, 179–182.

[4] Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H. and Ponzio, G. (1983) Proc. Natl. Acad. Sci. USA 80, 945–949.

[5] Havrankova, J., Roth, J. and Brownstein, M. (1978) Proc. Natl. Acad. Sci. USA 75, 5737–5741.

[6] Gammeltoft, S., Staun-Olsen, P., Ottesen, B. and Fahrenkrug, J. (1984) Peptides, in press.

[7] Yip, C.C., Moule, M.M. and Yeung, C.W.T. (1980) Biochem. Biophys. Res. Commun. 96, 1671–1678.

[8] Heidenreich, K.A., Zahnisser, N.R., Berhanu, P., Brandenburg, D. and Olefsky, J.M. (1983) J. Biol. Chem. 258, 8527–8530.

[9] Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 96, 79–96.

[10] Staun-Olsen, P., Ottesen, B., Bartels, P.D., Nielsen, M.H., Gammeltoft, S. and Fahrenkrug, J. (1982) J. Neurochem. 39, 1242–1251.

[11] Fehlmann, M., Carpentier, J.L., Van Obberghen, E., Freychet, P., Thamm, P., Saunders, D., Brandenburg, D. and Orci, L. (1982) Proc. Natl. Acad. Sci. USA 79, 5921–5925.

[12] Laemmli, U.K. (1970) Nature 227, 680–685.