The Insulin Receptor of Rat Brain Is Coupled to Tyrosine Kinase Activity*

Robert W. Rees-Jones‡, S. Anne Hendrick§, Merrit Quarum, and Jesse Roth

From the Diabetes Branch, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Insulin receptors from rat brain were studied for receptor-associated tyrosine kinase activity. In solubilized, lectin-purified receptor preparations, insulin stimulated the phosphorylation of the β subunit of its receptor as well as of exogenous substrates. Phosphoamino acid analysis of casein phosphorylated by these preparations revealed that 32P incorporation occurred predominantly on tyrosine residues. Receptor and casein phosphorylations were specific for insulin and analogues that also bind to the insulin receptor. The insulin dose response for phosphorylation of brain receptor resembled that reported for the purified insulin receptor from human placenta (Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., and Kahn, C. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2137–2141), suggesting similar insulin sensitivity and coupling of the brain receptor kinase. Four polyclonal antisera to the insulin receptor were able to bind and immunoprecipitate the brain receptor; however, only two antisera activated the receptor-associated kinase. Thus, the brain insulin receptor, like the well studied non-neural receptor, is coupled to tyrosine kinase activity, making regulation of cellular events by insulin in neural tissue possible.

The function of insulin in nervous tissue is still unclear. The search for insulin’s actions there has consisted mainly of experiments using intact animals or whole cell preparations to measure final metabolic events typical of insulin’s action in non-neural tissues (for review, see Ref. 1). Such investigations have suggested that insulin has little influence on these classic metabolic pathways in nervous tissue, although there have been occasional reports to the contrary (2, 3). Other studies have suggested that insulin may influence growth and development of fetal nervous tissue (4). Overall, this approach, by pinpointing specific effects to be studied, may overlook a wide range of other possible functions of insulin in the central nervous system.

A second approach, which does not require a knowledge of end events in nervous tissue, has been to look for early events in insulin action. The presence of insulin receptors, which are widely distributed in the mammalian central nervous system (5), has strongly supported the view that insulin has a role in this system. As characterized by insulin-binding studies, the brain receptor appears identical with peripheral (non-neural) receptors (5–7). However, brain receptor number may not be regulated as the peripheral receptor is (6, 8), and there may be structural differences in the carbohydrate components (9). Despite these studies, the possibility remains that the brain insulin receptor is present constitutively, and is not coupled to cellular events.

No information is available at present on early events after insulin binding to its receptor in nervous tissue. Recent reports have identified activation of a receptor-associated tyrosine kinase as an early event after insulin binding in a variety of non-neural tissues (10–17). In this study, we show that the insulin receptor of rat brain is coupled to tyrosine kinase activity, which phosphorylates the receptor as well as artificial substrates. This demonstration may aid in the search for insulin’s functions in the nervous system.

**EXPERIMENTAL PROCEDURES**

Materials—Wheat germ agglutinin coupled to agarose was obtained from Miles Laboratories (Elkhart, IN). [γ-32P]ATP (specific activity, 2900 Ci/mmol) was from New England Nuclear (Boston, MA). Porcine insulin was purchased from Eli Lilly and Co. (Indianapolis, IN), and porcine proinsulin was the gift of R. E. Chance of Eli Lilly and Co. (Indianapolis, IN). Human IGF-I, purified by R. Humbel (Biochemisches Institut der Universitāt Zürich, CH-8026 Zürich, Switzerland) was kindly provided by M. M. Rechler (National Institutes of Health, Bethesda, MD). Other peptide hormones were obtained through the Research Resources program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health (Bethesda, MD). Casein and synthetic tyrosine-containing polypeptides were from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were reagent grade. Antireceptor antisera have been characterized in detail elsewhere (19).

Receptor Preparation—A membrane preparation from whole brain (or liver) of 200-g Sprague-Dawley rats was obtained by differential centrifugation (5). These membranes were solubilized in Triton X-100, and the insulin receptor was partially purified by affinity chromatography with wheat germ agglutinin coupled to agarose (20).

Insulin Receptor Phosphorylation—This was performed by modifications of previously described techniques (11). Receptor preparations in wheat germ column elution buffer (20) (40 μl containing about 10 μg of protein) were preincubated in the presence or absence of stimulants (insulin, hormones, antireceptor antibodies) in a final volume of 140 μl containing 50 mM HEPES (pH 7.0) and 1.5 mg % bovine serum albumin. After 1 h at 4°C or 30 min at 24°C (as noted), phosphorylation was initiated by addition of 40 μl of reaction mix to give final concentrations of 50 μM [γ-32P]ATP (specific activity ~3 Ci/mmol), 1 mM CTP, and 3 mM Mn acetate. After 10 min at 24°C, 75-μl samples were added to 25 μl of "stopping solution" (11) and 25

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† To whom reprint requests should be addressed.

‡ Pharmacology Research Associate of the National Institute of General Medical Sciences. Present address, Neurochemistry Section, Waisman Center, University of Wisconsin, Madison, WI 53796.
μl of 5X electrophoresis sample buffer (11), and heated to 97 °C for 10 min.

Phosphorylated receptor preparations were analyzed on NaDodSO4-polyacrylamide gels (4% stacking, 7.5% resolving) (21). The gels were stained (50% trichloroacetic acid, 0.1% Coomassie blue), destained (7% acetic acid), dried, and autoradiographed using Kodak X-Omat-AR film and DuPont Lightning Plus intensifying screens. The major insulin-stimulated phosphoprotein (Mr, ~95,000) was cut from the gel and the 32P incorporated was quantitated by liquid scintillation. In some experiments, equal areas of the gel at Mr, ~66,000 (no phosphoproteins present) were cut, counted, and subtracted as background. Molecular weight determinations were by comparison to Bio-Rad (Richmond, CA) high molecular weight standards.

Artificial Substrate Phosphorylation—An identical protocol was followed for preincubation and phosphorylation of artificial substrates, with 400 μg of substrate present in the preincubation solution. The phosphorylation reaction was terminated by spotting 75 μl samples onto Whatman 3MM filter paper followed by extensive washing in 10% trichloroacetic acid containing 10 mM Na pyrophosphate. Filter papers were washed twice with ethanol and dried, and 32P incorporation was determined by liquid scintillation. In some experiments, equal areas of the gel at Mr, ~200,000-500,000 were cut and the 32P incorporated was quantitated by liquid scintillation. In 10 experiments, the mean Mr, ~85,000, and 105,000; these were not further characterized in this study.

**RESULTS**

**Insulin-stimulated Brain Receptor Phosphorylation**—In solubilized, lectin-purified preparations of insulin receptors from rat brain, insulin stimulated the phosphorylation of one major protein with a Mr, ~95,000 (Fig. 1). This phosphoprotein was identified as the β subunit of the insulin receptor by immunoprecipitation with four different antireceptor antibodies (B2, B3, B10, B-d) but not with control sera. Phosphorylation of the α subunit (Mr, ~135,000) of the brain insulin receptor was not observed in this cell-free system, in contrast to that seen with solubilized liver receptor (11).

Recent studies have demonstrated a different electrophoretic mobility of the insulin receptor subunits in brain compared to those in peripheral tissues as visualized by photoaffinity labeling or 125I-cross-linking (9), with brain subunits showing lower Mr, values. Interestingly, the present studies of

32P-labeled β subunit suggest a similar mobility difference, with the brain β subunit migrating at Mr, ~6,000 less than that from liver analyzed in a similar manner. Several minor insulin-stimulated phosphoproteins were also frequently noted, at Mr, ~85,000 and 105,000; these were not further characterized in this study.

**TABLE I**

| Substrate | Kinase activity with [insulin] at 100 nM | Stimulation |
|-----------|----------------------------------------|-------------|
| Casein    | 2.9                                    | 2.3         |
| Poly(Glu, Tyr), 1:1 | 6.6                                    | 2.3         |
| Poly(Glu, Tyr), 4:1 | 1.1                                    | 2.3         |
| Poly(Glu, Ala, Tyr), 1:1:1 | 2.4                                    | 1.8         |
| Poly(Glu, Ala, Tyr), 6:3:1 | 2.4                                    | 1.8         |
| Poly(Ala, Glu, Lys, Tyr), 6:2:5:1 | 7.6                                    | 2.2         |

**Insulin-stimulated phosphorylation of artificial substrates by brain receptor**

Insulin receptor preparations (~10 μg of protein) from rat brain were preincubated in 50 mM HEPES (pH 7.6) at 4 °C for 1 h with 400 μg of artificial substrate in the presence or absence of 10−7 M porc insulin. Phosphorylation and quantitation of 32P incorporation were performed as described under "Experimental Procedures." Results are expressed in kinase activity units, where 1 unit equals 1 pmol of phosphate incorporated into 1 mg of artificial substrate in 10 min. The tyrosine-containing synthetic polypeptides are random copolymers of amino acids at the indicated molar ratios, with Mr = 20,000−50,000.

**FIG. 1.** Insulin-stimulated phosphorylation of brain receptor. Microsomes were obtained from rat brain by differential centrifugation, and were solubilized in Triton X-100. The insulin receptor was partially purified by affinity chromatography on wheat germ agglutinin coupled to agarose. Receptor preparations (~30 μg of protein) were incubated without (lanes a and b) and with (lanes c and d) 10−7 M porc insulin for 30 min at 24 °C. Phosphorylation was performed for 10 min at 24 °C with [γ-32P]ATP as described under "Experimental Procedures." Phosphorylated receptor preparations were then immunoprecipitated with control sera (lanes a and c) or antireceptor antisera (lanes b and d) and analyzed by NaDodSO4-polyacrylamide gel electrophoresis. In 10 experiments, the mean Mr, ~85,000 and 105,000; these were not further characterized in this study.
several synthetic tyrosine-containing polypeptides, the most efficient of these being poly(Glu-Tyr) (4:1). Other tyrosine-containing peptides did not serve as substrates, suggesting specific recognition sites for substrate acceptance. The magnitude of artificial substrate phosphorylation by brain receptor was comparable to that obtained using liver receptor (data not shown). Phosphoamino acid analysis of phosphorylated casein showed that the insulin-stimulated $^{32}$P incorporation occurred predominantly on tyrosine residues, also reflecting the presence of tyrosine kinase activity (Table II).

**Specificity of Insulin-Stimulated Phosphorylation**—Casein and receptor phosphorylation by these brain receptor preparations was stimulated specifically by insulin and related peptides that are capable of binding to the insulin receptor. Neither casein nor receptor phosphorylation was enhanced after preincubation with a variety of unrelated peptides (nerve growth factor, ACTH, epidermal growth factor, luteinizing hormone-releasing hormone) or cAMP, at concentrations of $10^{-7}$ M (data not shown). Of note, epidermal growth factor, which is known to stimulate tyrosine kinase activity mediated by its receptor (25), failed to stimulate casein phosphorylation or phosphorylation of a $M_c \approx 170,000$ protein on NaDodSO$_4$-polyacrylamide gels. This suggests the absence of epidermal growth factor receptors in our preparation.

In contrast, insulin-like growth factor I ($10^{-7}$ M) was about 50% as effective as pork insulin ($10^{-7}$ M) in stimulating casein and receptor phosphorylation (data not shown), compatible with IGF-1's lower affinity for the insulin receptor (26). Both pork insulin and IGF-I stimulated the phosphorylation of what appeared to be the same 95K band, suggesting the absence in this preparation of IGF-I receptor, which may also contain associated tyrosine kinase activity (27) and migrates on NaDodSO$_4$-polyacrylamide gels with a slightly higher $M_c$, than the insulin receptor (28). Thus, the action of IGF-I was most likely mediated via the insulin receptor.

Guinea pig insulin and pork proinsulin, which have less than 10% of pork insulin's affinity for the insulin receptor (29), showed appropriate shifts to the right in a dose-response curve of 95K phosphorylation (Fig. 2). The dose-response curve obtained with pork insulin is comparable to that obtained for the purified insulin receptor from human placenta (15), suggesting a similar sensitivity to insulin and coupling of binding to kinase activity by the brain receptor. Indistinguishable dose-response curves were obtained for casein phosphorylation by these brain receptor preparations (data not shown).

Finally, the pork insulin dose-response for receptor phosphorylation (Fig. 2) closely paralleled insulin binding to this receptor preparation (Fig. 3). This suggests that brain receptor

| Phosphoamino Acid | Insulin | Phosphoserine | Phosphotyrosine |
|------------------|---------|--------------|----------------|
| $M_c$            | cpm     | cpm          |
| $0$              | 203     | 194          |
| $10^{-7}$        | 211     | 418          |

phosphorylation is an intramolecular event, i.e. only occupied receptors are phosphorylated.

**Recogntion of Brain Receptor by Antireceptor Antibodies**—Recent work in our laboratory has demonstrated that while some antireceptor antibodies are capable of stimulating phosphorylation of insulin receptors from rat liver, others bind to the receptor but fail to activate the kinase. To address possible antigenic, and thus structural, differences between the brain and hepatic insulin receptor, we tested the ability of several polyclonal antisera to the insulin receptor to stimulate brain receptor and casein phosphorylation.

Sera B-2 and B-10 failed to stimulate receptor phosphorylation compared to control sera (Fig. 4). They also did not enhance casein phosphorylation, suggesting a failure of kinase activation, rather than alterations of receptor as substrate. Both antibodies were capable of binding to the brain insulin receptor, as judged by their ability to immunoprecipitate the

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phosphoreceptor (data not shown). In contrast, sera B-d and B-S enhanced receptor phosphorylation, and to a lesser extent, serum B-d increased casein phosphorylation (Fig. 4). Results with all four sera are similar to those found using the rat liver insulin receptor, suggesting similar recognition of both insulin receptors by these four antisera.

DISCUSSION

In insulin receptor preparations from rat brain, insulin stimulated the phosphorylation of the β subunit of its receptor as well as of exogenous substrates. In every manner studied, the brain insulin receptor functioned as a tyrosine kinase similar to the well studied peripheral (non-neural) receptor. Activation of the kinase was specific for insulin and lower affinity analogues that also bind to the insulin receptor. The efficiency of artificial substrate phosphorylation by brain receptor was comparable to that of peripheral receptor. The dose response of receptor phosphorylation for pork insulin closely paralleled insulin binding and resembled that seen with purified placental insulin receptor (15), suggesting similar insulin sensitivity and coupling of the brain receptor kinase. In contrast to a recent report suggesting differential insulin sensitivity and coupling of the brain receptor (13, 16), our studies have failed to confirm these findings. The reported structural differences in the brain receptor (9), perhaps in carbohydrate residues, appear not to influence the coupling of insulin binding to kinase activity.

The mechanism of transmission of insulin's signal to the cell interior, probably via the β subunit, is currently unclear. It may involve phosphorylation of cellular proteins by the tyrosine kinase, generation of soluble mediators (34, 35), and/or other as of yet unsuspected mechanisms. Clearly, the presence of tyrosine kinase activity associated with the brain insulin receptor provides a mechanism for regulation of cellular events by insulin in nervous tissue. When studied in intact cells, insulin stimulates receptor phosphorylation on both serine and tyrosine residues (12); one possible explanation for this phenomenon is phosphorylation and activation of a serine kinase by the receptor-associated tyrosine kinase. Likewise, in nervous tissue, the receptor kinase may well phosphorylate intracellular proteins to regulate cellular events. This mechanism would be compatible with actions of insulin in nervous tissue distinct from those known in peripheral tissues, and would require only the presence of different biochemical pathways after receptor kinase activation in nervous tissue. An analogy can be drawn to hormone-activated adenylate cyclase. The common resulting signal, cAMP, is translated into different responses in different tissues by the presence of different substrates for cAMP-dependent protein kinases (36). Alternatively, the insulin receptor kinase may not be involved in insulin's actions, but may subserve some other cellular function common to both peripheral and nervous tissue.

In conclusion, insulin binding and tyrosine kinase activation can be studied in a cell-free preparation of brain insulin receptors. Further investigation of this phenomenon may provide insight into the functions of insulin in nervous tissue.

Acknowledgments—We thank S. I. Taylor and V. Moncada for performing phosphoamino acid analyses, G. Grunberger and Y. Zick for data on the synthetic artificial substrates, and J. L. Young for secretarial assistance. S. I. Taylor and V. Moncada gratefully acknowledge support by the American Diabetes Association (Rogel Staubach Research Feasibility grant award).

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