INSULIN RECEPTORS IN LACTATING MOUSE MAMMARY GLAND

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The binding of insulin to the crude membrane fraction of lactating mammary gland of white mouse was investigated. It has been demonstrated that insulin specifically binds to the tissue investigated and that the binding of [^{125}I]-insulin is inhibited by physiological concentrations of unlabeled insulin. Under the experimental conditions chosen an equilibrium of the binding is established after 30 min of incubation at 4°C. The specific binding increases with the increasing concentration of the membrane protein and becomes saturated as the concentration of the labelled hormone increases. Ten % of the labelled hormone is specifically bound at $0{\cdot}26 \cdot 10^{-9}$M concentration of [^{125}I]-insulin and at a concentration of the membrane protein equal 1·34 mg/ml. Scatchard analysis was used to demonstrate that the binding capacity is $3 \cdot 10^{-14}$ mol of insulin/mg protein and that the affinity constant is $3{\cdot}5 \cdot 10^{9}\text{M}^{-1}$. The pH-optimum of binding of insulin to the membrane fraction lies between 6·5 and 8·5.

An increasing number of studies carried out during the past few years have demonstrated that the first step of the effect of insulin and other polypeptide hormones is the binding to specific sites on the cell membrane, to the so-called receptors[1-3]. The presence of receptors for insulin has been shown in isolated lipid cells[4-7], erythrocytes and lymphocytes[8-11], thymocytes[12], mammary gland cells[13], liver membranes[14-20], lipid tissue membranes[21-22], placenta[23-25], soluble membrane fraction of liver and adipose tissue[26-27], and of lymphocytes[28,29].

The mammary gland is one of the organs sensitive to the action of insulin. This is reflected by the increase of α-aminoisobutyric acid[30,31], RNA (ref.[32]), and DNA (ref.[30]) synthesis and of mitosis in pregnant animals. By contrast, these effects have not been observed with the mammary gland of normal, non pregnant animals[30,31,33,34]. It has been postulated that prolactin stimulates the development of a sensitivity to insulin during gravidity and insulin thus becomes a growth factor of the mammary gland[35]. It has been admitted that prolactin affects the synthesis of membrane receptors for insulin or that it makes exposed receptors unaccessible to insulin action under normal conditions[13].

Oka and Topper[31,34] have been able to demonstrate that insulin covalently coupled to agarose stimulates amino acid transport to mammary gland cells of virgin females even though free insulin is lacking such an ability. This points to the presence of receptors for insulin in mammary gland cells of these animals. Hence, the existence of insulin receptors has been shown in mammary gland cells of not only pregnant and lactating but also of virgin animals even though no response has been obtained from the latter. It has been assumed that this difference is not due to an altered binding ability for insulin but to some other change which has occurred in the membrane[13]. The degree

of the binding of the hormone to cells of the lactating gland is 3—4 times higher compared to the binding to mammary gland cells of gravid or virgin females[13].

This study has been undertaken in an effort to confirm the existence of receptors for insulin in the membrane fraction of lactating mammary gland of mouse and to determine the characteristic features of the binding of insulin to receptors.

EXPERIMENTAL

Material. Crystalline porcine Zn-insulin (27 IU/mg) was purchased from Novo (Denmark), Na[^{125}I] was from Isotop (Hungary), bovine serum albumin from Boehring (FRG), Whatman CC-31 Cellulose from Whatman (England), and Sephadex G-100 from Pharmacia (Sweden). Unless stated otherwise the remaining chemicals were of analytical purity.

Lactating strain H white female mice, weighing 30—40 g, 10—14 after the delivery, were used in the experiments.

The iodination of insulin was effected by the method of Hunter and Greenwood[36]. Insulin (12·5 μg), dissolved in 50 μl of 0·4M sodium phosphate buffer, pH 7·4, and 25 μl of chloramine T solution (4 mg/ml) was added to an ampule containing 2mCi of Na[^{125}I] (volume 15—25 μl). Twenty seconds thereafter 100 μl of $Na_2S_2O_5$ solution (2·4 mg/ml) and 100 μl of KI solution (10 mg/ml) were added. [^{125}I]-Insulin was separated from free [^{125}I] as follows: *a*) the reaction mixture was chromatographed on a 1 × 33 cm column of Sephadex G-100 (ref.[33,37]), equilibrated with 0·04M sodium phosphate buffer, pH 7·4 containing 20% of albumin. The column was eluted with 0·25% albumin solution in phosphate buffer or *b*) the resulting solution was applied onto a Whatman CC-31 column. The subsequent procedure was identical with the method of Landon and coworkers[38]; β-mercaptoethanol was omitted.

[^{125}I]-Insulin monomer is obtained after additional electrophoretic separation in polyacrylamide gel by the method of Sirakov and Ditzov[39].

Preparation of subcellular insulin-binding fraction. White mice of H-line, 3—5 months old and lactating 10—14 days were used. The animals were decapitated, the mammary glands were isolated and immediately placed in cold Krebs–Ringer phosphate buffer, pH 7·4, containing 0·25M sucrose per 1 g of tissue in 10 ml of buffer. The tissue was cut to small pieces and homogenized in a Potter–Elvehjem homogenizer for 30 s. The homogenate was filtered through gauze and centrifuged 20 min at 3000 *g* (ref.[40]). The sediment was suspended in the same buffer and centrifuged again 10 min at 3000 *g*. This procedure was repeated three times. The sediment obtained from 1 g of tissue was suspended in 2 ml of buffer and immediately used in the experiment.

The concentration of proteins was determined by the method of Lowry[41].

Determination of binding of [^{125}I]-*insulin.* The incubation mixture contained [^{125}I]-insulin at $0{\cdot}77 \cdot 10^{-9}$M concentration, 0·83 mg/ml of the membrane protein in Krebs–Ringer phosphate buffer, pH7·4, with bovine serum albumin (final concentration 10 mg/ml). The total volume of the sample was 300 μl. Experiments with unlabelled insulin (0·167 mg per ml of incubation mixture) were carried out simultaneously. All experiments were performed in plastic tubes at 4°C. The experiments were terminated after 30 min by the addition of 1 ml of ice-cold Krebs–Ringer buffer containing 0·1% of albumin. The incubation mixture was immediately filtered by suction through Whatman GF/C glass filter; the filter was washed with 5 ml of the same buffer. The radioactivity of the filter was measured in a scintillation spectrometer (type Vakutronik, GDR). All measurements were repeated until stable values were obtained.

The radioactivity of the filter in the presence of the unlabelled hormone was considered as repre-

senting the total binding. Nonspecific binding corresponds to the radioactivity of the sample in the absence of an excess of unlabelled hormone. The difference between total and nonspecific binding is taken for specific binding[4].

RESULTS

Time profile of formation of hormone–receptor complex. The binding of [^{125}I]--insulin to the receptor in the mammary gland is a process depending on temperature and time. Our experiments were carried out at 4°C where the degradation of the hormone and of the receptor is considerably decreased[17,20,21]. The binding rapidly increases during 15 min and an equilibrium state is established in 30 min (Fig. 1).

Binding as function of [^{125}I]*-insulin concentration.* The specific binding of insulin to the crude membrane fraction of the lactating mammary gland is a process in which the concentration of the labelled hormone increases until a state of saturation is attained (Fig. 2). Specific binding can be observed even at low insulin concentrations

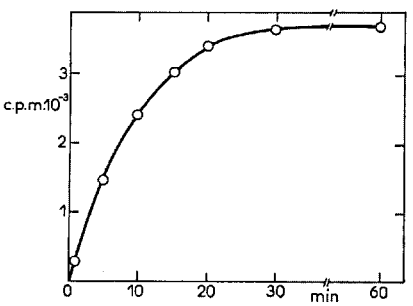


FIG. 1

Time Profile of Binding of [^{125}I]-Insulin to Membrane Fraction of Mouse Mammary Gland

The protein concentration of the membrane fraction was 0·83 mg/ml, the concentration of [^{125}I]-insulin was 0·77 . 10^{-9}M. The results were corrected for nonspecific binding. The specific binding was expressed as the difference between the total quantity of labelled hormone bound and the quantity of labelled hormone remaining bound to proteins in the presence of an excess of unlabelled insulin (0·167 mg/ml). Ordinate--bound [^{125}I]-insulin.

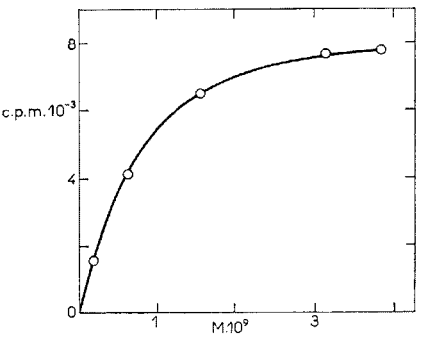


FIG. 2

Specific Binding of [^{125}I]-Insulin to Membrane Fraction of Mouse Mammary Gland; Dependence on Concentration of [^{125}I]--Insulin

The membrane fraction (1·34 mg of protein/ml) was incubated with labelled insulin under standard conditions, *cf.* Experimental. Ordinate quantity of [^{125}I]--insulin bound to the protein fraction (c.p.m.); abscissa quantity of [^{125}I]-insulin in the reaction mixture [M].

(0·19 . 10^{-9}M). The saturation is almost complete at insulin concentrations higher than 3 . 10^{-9}M. At 0·64 . 10^{-9}M concentration of insulin 50% of the maximal saturation is attained.

The results obtained were treated according to Scatchard[42]. The binding capacity of the crude membrane fraction examined and the equilibrium constants were determined. The results point to the presence of one kind of receptor with K_{aff} equal approximately to 3·5 . 10^{9}M^{-1} (Fig. 3) and to a binding capacity of crude membrane fraction for insulin of 3 . 10^{-4} mol/mg protein.

Binding as function of concentration of membrane proteins. The relation between the specific binding and the concentration of membrane proteins is shown in Fig. 4. It is obvious that the binding proportionally increases with the increasing concentration of proteins. This dependence is approximately linear. The results obtained permit us to choose the optimal concentration of membrane proteins for our binding experiments.

Displacement of [^{125}I]*-insulin by unlabelled insulin.* Low concentrations of unlabelled insulin compete with [^{125}I]-insulin for the binding to the membrane protein

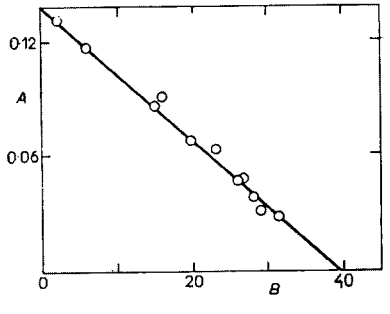


FIG. 3

Scatchard Plot of Binding of [^{125}I]-Insulin to Membrane Fraction of Mouse Mammary Gland

A Ratio of bound to free [^{125}I]-Insulin, B-bound [^{125}I]-insulin, fmol/ml.

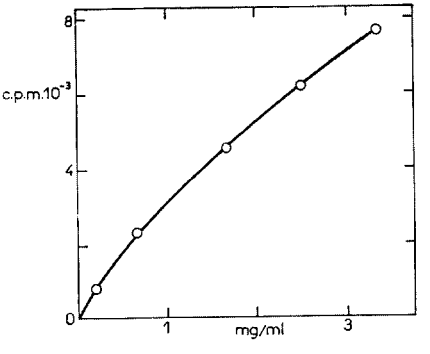


FIG. 4

Specific Binding of [^{125}I]-Insulin to Membrane Fraction of Mouse Mammary Gland; Dependence on Protein Concentration of the Membrane Fraction

[^{125}I]-Insulin (0·64 . 10^{-9}M was incubated with the protein fraction under standard conditions, see Experimental for details. The specific binding of [^{125}I]-insulin is expressed as described in the legend to Fig. 1. Ordinate quantity of [^{125}I]-insulin (c.p.m.); abscissa conc. of membrane proteins in reaction mixture (mg/ml).

(Fig. 5). The binding of the labelled hormone is decreased by 30% in the presence of the unlabelled hormone at a concentration of 1 ng/ml and a decrease of 70% is observed at a concentration of 10 ng/ml. This shows that the binding characteristics of [^{125}I]-insulin and of unlabelled insulin share many features in common.

Effect of pH *of medium on binding of insulin.* The optimum of binding of [^{125}I]--insulin to membrane proteins of the mammary gland lies between 6·5 and 8·5 under our experimental conditions and reaches a maximum at 7·5.

DISCUSSION

The interaction of insulin with the crude membrane fraction of mammary gland, investigated in this study, results in the binding of the hormon to biologically important insulin receptors in the cell membrane of this organ. In parallel experiments the binding of insulin and the biological response were examined by a similar technique applied to intact cells. It has been shown[13] that the binding is the primary process leading to the biological response. The activity bound to intact cells quantitatively appears in the membrane fraction obtained by homogenization. Even though the biological response is lost as a result of the destruction of the cell it can be concluded that the specific binding to the crude membrane fraction reflects the interaction of insulin with receptors in a relatively unaltered form.

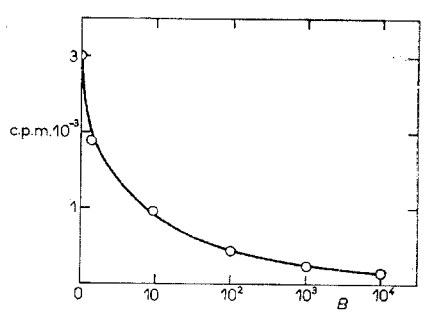
Similarly to intact cells[13], the interaction of insulin with the crude membrane fraction of the mammary gland is a reversible process depending on temperature and time. An equilibrium state of the hormone to receptor binding is attained approximately in 30 min at 4°C. The dissociation rate is decreased[21] and likewise the degradation of the hormone and of the receptor are minimal[20] at this temperature.

The optimum of binding of insulin to the receptor lies in the pH-range 6·5 – 8·5; similar values were observed with other tissues showing binding affinity for insulin[21].

FIG. 5

Displacement of [^{125}I]-Insulin from Binding to Membrane Fraction of Mouse Mammary Gland by Unlabelled Insulin

[^{125}I]-insulin ($0{\cdot}77 \cdot 10^{-9}$ M) was incubated with the membrane fraction (protein concn. 0·83 mg/ml), containing the quantity of unlabelled insulin required, under standard conditions. The specific binding of [^{125}I]--insulin was determined as described in the legend to Fig. 1. Ordinate-[^{125}I]-insulin bound. Abscissa: non-labelled insulin, ng/ml.



The binding increases in parallel with the increasing concentration of membrane proteins over the range examined. An increase of concentration of the bound hormone leads to a saturation of the binding thus indicating that the full number of receptor sites is occupied. Physiological concentrations of insulin leading to a complete binding are another piece of evidence in favour of the biological nature of the phenomenon observed.

The data obtained were treated according to Scatchard[42]. The binding capacity of the crude membrane fraction examined and the equilibrium constants were determined. Our data agree with those of O'Keefe and Cuatrecasas[14]. The values obtained by us are very similar to those obtained for insulin receptors in lipid tissue, liver, and lymphocytes[9,20-22]. This unambiguously demonstrates that molecular structures which recognize and specifically bind insulin to target tissues are similar and most likely identical. One kind of binding site was found in experiments with low concentrations of unlabelled insulin; a similar observation has been made by Cuatrecasas studying mammary gland cells[13]. Kahn and coworkers[20] found three kinds of binding sites when investigating liver membrane fractions, namely a site with high affinity and low capacity, a site with low affinity and high capacity, and a site nonspecifically binding insulin. The values of affinity constants K_{aff} as well as the values of binding capacity of sites showing high affinity and low capacity are identical with the values obtained by us. If we take into account the low insulin concentrations and the low temperature under which our experiments were carried out we arrive at the most probable conclusion that the receptor sites detected by us are those which the above authors describe as sites of high affinity and low capacity.

The found characteristics of insulin receptors of the mammary gland are similar to values found for prolactin[43]. A parallel investigation of the two kinds of receptors in virgin, pregnant, and lactating animals might contribute to the full elucidation of their role in the development of this organ.

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