Evidence for the Presence of Insulin Binding Sites in Isolated Rat Intestinal Epithelial Cells*

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Summary. Insulin receptors have been demonstrated in isolated rat intestinal epithelial cells. The specific binding of ^{125}I -insulin was time – and temperature – dependent, the optimal temperature of study being 15° . Dissociation of bound ^{125}I -insulin by an excess of unlabelled hormone was rapid and attained $66 \pm 2\%$ in 2 h. When initiated by dilution, the dissociation attained $35 \pm 4\%$ in 2 h, and $72 \pm 1\%$ in 2 h when 10^{-7} mol/l unlabelled insulin was added. The pH optimum for the binding process was between 7.5 and 8, and the binding increased proportionally to cell protein concentration up to 1.5 mg/ml. Under standard conditions (2 h at 15°) the degradation of the labelled hormone in the medium accounted for 20–50% of total tracer, depending on the concentration of cells. At apparent equilibrium (2 h at 15°), unlabelled insulin in the range of 10^{-10} to 10^{-7} mol/l inhibited competitively the binding of $4.3\text{--}7 \times 10^{-11}$ mol/l ^{125}I -insulin; fifty per cent inhibition was obtained with 3×10^{-9} mol/l native insulin. Scatchard analysis, after correction for degradation, gave curvilinear plots, that may be explained by two orders of binding sites, with $2,000 \pm 200$ sites/cell of high affinity ($K_a = 2.2 \pm 0.2 \times 10^9$ l/mol) and $39,000 \pm 3,000$ sites/cell of low affinity ($K_a = 5.6 \pm 1.6 \times 10^7$ l/mol). The potency of proinsulin to compete with ^{125}I -insulin for the binding site was 3% that of insulin, unrelated peptides were inactive. Such results give a molecular basis to different reports suggesting that the intestine could be a target-tissue for insulin.

Key words: Insulin receptors, insulin degradation, enterocytes, epithelial cells, intestine.

Several lines of evidence indicate that intestinal epithelial cells, as other cells in the organism, exhibit specific binding sites for peptide hormones that might intervene in their regulation. Thus, binding sites for VIP [37] that are related to a cyclic AMP system [23] have been characterised. However, no studies were performed so far on the binding of insulin in the intestinal epithelium. The aims of this paper were to investigate insulin binding sites in rat isolated intestinal epithelial cells and if present, to define the characteristics of these binding sites as compared to those found in known target-tissues for the hormone [1, 10, 12, 14, 27, 33, 35, 43].

Material and Methods

Chemicals

Pork monocomponent insulin was iodinated by the chloramine T method to a specific activity of $150\text{--}180 \mu\text{Ci}/\mu\text{g}$ (0.4–0.5 atom of iodine per molecule, purified on Sephadex G 50 [30], and tested prior to use for its ability to bind to rat liver plasma membranes [12]. Pork monocomponent insulin, crystalline beef insulin, pork proinsulin were obtained from Novo, Novoallé, DK-2880 Bagsvaerd (Denmark). Human growth hormone was obtained from Prof. Dray, Institut Pasteur, Paris. Bovine serum albumin (BSA) fraction V, was obtained from Miles Laboratories, Elkhart, IN 46514, USA. Fatty acid free BSA was obtained from Sigma, P. O. Box 14508, St. Louis, M. O. 63178, USA. Epidermal growth factor was a generous gift of Dr. Moody, Novo Research Institute, 215 Nordre Fasanvej, DK-2200 Copenhagen (Denmark). All chemicals were of reagent grade.

Methods

Preparation of Cells. Intestinal epithelial cells were isolated from female Sprague-Dawley rats (160–200 g), fed ad libitum, according to a modification [22, 37] of the method of Mitjavila et al. [32]. Briefly, the jejunum-ileum was excised, flushed with an ice-cold solution of 0.24 mol/l NaCl pH 7.4, everted over a glass rod, filled

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with 0.34 mol/l NaCl pH 7.4, and washed with a dispersing solution containing 2.5 mmol/l EDTA and 0.24 mol/l NaCl pH 7.4. The cells were obtained by mild hand-shaking, sedimented at 200 g for 2 min, and washed 5 times in 100 ml of 0.15 mol/l NaCl. The preparations contained almost exclusively villous cells as attested by the presence of brush borders (microvilli) in 90–95% of the cells, and were free of non epithelial cells and parasites. Cells that excluded trypan blue were more than 90% after 4 h at 15°, and 95% after 1 h 30 at 30°. The cell preparation oxidised glucose and synthesized proteins in a linear fashion during 2 h at 15° (Laburthe, personal communication). Protein concentration was measured by the method of Lowry et al. [28]; 10^6 cells contained 0.24 mg of protein [37].

Binding Studies. Isolated cells (0.11–3.75 mg/ml of cell protein) were incubated in duplicate or triplicate samples with fixed concentrations of ^{125}I -insulin (5×10^{-11} to 7.8×10^{-11} mol/l) in 0.5 ml of Krebs Ringer phosphate buffer (KRP) containing 1 g per 100 ml of BSA, pH 7.8 unless stated otherwise. Fatty acid-free BSA has been used in all experiments (except for Fig. 4). Kinetics studies were performed at 15 and 30°. Equilibrium studies were performed during 2 h at 15°, in the presence of 0 to 10^{-7} mol/l unlabelled porcine insulin. After incubation, cell-bound insulin was separated by rapid centrifugation as described elsewhere [12] and the radioactivity of the washed pellets determined by gamma spectrometry. Data are reported as specific binding: this is obtained by subtracting from the total the non-specific binding, i. e. the amount of labelled insulin that is not displaced by an excess (1.6×10^{-5} mol/l) of unlabelled insulin. For dissociation by an excess of unlabelled hormone, the incubation was conducted at 15° for 2 h (time 0 of dissociation), in conditions previously described; then 10^{-7} mol/l unlabelled insulin was added in a small volume and the cell-bound radioactivity separated after various times of incubation as described above. Non-specific binding was subtracted at each point. For dissociation by dilution, the incubation

Fig. 1. Left: Time curves of ^{125}I -insulin binding to enterocytes at 30° (open circles) and 15° (closed circles). Cells (0.82–1.44 mg/ml of cell protein) were incubated with 5×10^{-11} mol/l ^{125}I -insulin for the times indicated on the abscissa. Specific binding was determined as described in Methods, each point is the mean $\pm$ SEM of 3 separate experiments, each performed in duplicate. The values are normalised to 1 mg/ml of cell protein. Right: Dissociation of bound ^{125}I -insulin to enterocytes at 15° by an excess of unlabelled insulin. Cells (0.67 mg/ml of cell protein) were incubated for 2 h at 15° with 7.5×10^{-11} mol/l ^{125}I -insulin and the specific binding determined. This corresponds to 100% on the ordinate, time 0 of dissociation. Then 10^{-7} mol/l unlabelled insulin was added in a small volume, and the incubation continued for various lengths of time as indicated on the abscissa, and the specific binding determined. Each point is the mean $\pm$ SEM of triplicate determinations in a single experiment

was conducted at 15° for 2 h (time 0 of dissociation); then 10 ml of KRP containing 0.1 g/100 ml of BSA and, when indicated, 10^{-7} mol/l unlabelled insulin were added and the cell-bound radioactivity separated by filtration on glass fibre filters (Whatman, GFC, Whatman-Ltd, Maidstone, England) after various times of incubation. The filters were then rinsed three times with 5 ml of ice-cold buffer (KRP containing 0.1 g/100 ml of BSA) and counted in a gamma spectrometer. Non-specific binding was subtracted at each point.

Degradation of ^{125}I -insulin. The integrity of insulin in the incubation medium was tested by its ability to bind to liver plasma membranes as previously described [13]. The degradation is expressed as % of unaltered insulin present in control simultaneously incubated without cells.

Degradation of Binding Sites. The degradation of binding sites was tested by preincubation of the cells at the temperature considered, followed by an incubation with ^{125}I -insulin for 2 h at 15°. The binding to these cells was compared to the binding to control cells (not preincubated).

Scatchard Analysis. The results obtained from competition experiments were analysed by the method of Scatchard [39]. In order to avoid interference between degradation of insulin and quantitative data, we corrected Scatchard analysis for degradation according to the method of Kahn et al. [20]. The following equation was applied to each point:

$$\frac{B \text{ (corrected)}}{F} = \frac{B - NS}{F \times (1 - DF)}$$

where B = bound hormone,
F = free hormone,
NS = non specific binding,
DF = fraction of hormone degraded.

For the calculations concerning the high affinity sites (Fig. 5, right, slope C) the contribution of the low affinity sites has been subtracted from each point: $C = A - B$.

Statistical Methods. Numerical results are expressed as means $\pm$ SEM of several experiments, or as means of duplicate determinations or means $\pm$ SEM of triplicate determinations in a single experiment. For Scatchard analysis [39], in each individual experiment, the regression lines were obtained from the experimental points using the method of least squares.

Results

Kinetics of Binding

Association. The binding of ^{125}I -insulin to rat enterocytes was time – and temperature – dependent. (Fig. 1, left panel). At 30°, binding was unstable, reached a peak ($1.08 \pm 0.04\%$ of total radioactivity) in 30 min and then decreased rapidly. At 15°, binding increased with time, reached a plateau ($2.6 \pm 0.3\%$ of total) in 2 h, and then remained constant until at least 4 h. Non specific binding varied with time and temperature, attaining $49 \pm 4\%$ of total binding in 30 min at 30° and $25 \pm 3\%$ in 2 h at 15°. The absence of steady state of binding at 30° was presumably due

to the degradation of ^{125}I -insulin (Table 1), the rise of non specific binding ($49 \pm 4\%$ of total binding after 30 min, and $74 \pm 5\%$ after 90 min) and the degradation of binding sites (see forward). No amelioration could be obtained by the use of insulin degradation inhibitors [13, 25] like bacitracin (1.5 mg/ml), N-Ethyl maleimide (1–10 mmol/l), aprotinin (1500 U/ml) or iodoacetamide (54 mmol/l). At the concentrations cited above, previously shown to protect insulin from degradation [13, 25], these substances were toxic to the cells and lowered total binding. Lowering temperature (Table 1) and adding unlabelled hormone (see forward) resulted in a clear decrease in the degradation of insulin. For all these reasons, all subsequent studies were performed at 15° , without inhibitor.

Table 1. Time course of degradation of ^{125}I -insulin at 15° and 30°

15°		30°	
Time (h)	% of degradation	Time (min)	% of degradation
0.5	18 ± 1	15	22 ± 1
1	30 ± 8	30	40 ± 7
2	42 ± 7	60	64 ± 11
4	55 ± 9	90	71 ± 10

The supernatants of experiments shown in Figure 1 were tested for insulin integrity as described in Methods. Each value is the mean $\pm$ SEM of three separate experiments

Fig. 2. Dissociation of bound ^{125}I -insulin to enterocytes at 15° , initiated by dilution (closed circles) and dilution plus 10^{-7} mol/l unlabelled insulin (open circles). Enterocytes (protein concentration 0.82 mg/ml) were incubated with 7.8×10^{-11} mol/l ^{125}I -insulin for 2 h at 15° . This corresponds to 100% on the ordinate, time 0 of dissociation. After dilution of the incubation medium ($\times 20$), the specific binding was determined by filtration (as indicated under Methods) at the times indicated on the abscissa. Each value is the mean $\pm$ SEM of triplicate determinations in a single experiment

Dissociation. Dissociation, at 15° , of bound ^{125}I -insulin by 10^{-7} mol/l unlabelled insulin is shown in Figure 1, right panel. Dissociation was rapid, with 50% of bound ^{125}I -insulin dissociated in 20–30 min; after 2 h, $34 \pm 2\%$ of labelled hormone remained associated with the cells. Figure 2 shows the dissociation, at 15° , of ^{125}I -insulin bound to enterocytes by dilution ($\times 20$) of the incubation medium. By dilution alone, the dissociation was weak, attaining $22 \pm 1\%$ and $35 \pm 4\%$ of initial binding in 30 and 120 min, respectively. The maximal dissociation was obtained by addition of 10^{-7} mol/l unlabelled insulin to the medium of dilution. In these conditions, $72 \pm 1\%$ of labelled hormone was dissociated after 120 min. The addition of a higher concentration of unlabelled insulin (1.6×10^{-5} mol/l) resulted in a

Fig. 3. Binding of ^{125}I -insulin as a function of the concentration of cell protein. Enterocytes (0.11 to 3.75 mg/ml of cell protein) were incubated at the different concentrations indicated on the abscissa with 7.2×10^{-11} mol/l ^{125}I -insulin for 2 h at 15° . Each value is the mean of duplicate determinations in a single experiment

Fig. 4. pH dependence of insulin binding. Enterocytes (0.54 mg/ml of cell protein) were incubated with ^{125}I -insulin (6×10^{-11} mol/l) at the pH values indicated for 2 h at 15° . Each value is the mean $\pm$ SEM of triplicate determinations in a single experiment

lower dissociation than that obtained with 10^{-7} mol/l (data not shown), in agreement with the previous observations by De Meyts et al. [9].

Effect of Cell Protein Concentration and pH

Results (Fig. 3) indicated a linear relationship between ^{125}I -insulin binding and the amount of cell protein concentration in the range of 0.1 to 1.5 mg/ml. For higher concentrations, the binding increased more slowly.

Figure 4 shows the pH dependence of ^{125}I -insulin binding to rat enterocytes at 15° . The pH value optimum was between 7.5 and 8. All other experiments were performed at pH 7.8 and with concentrations of cell protein below 1.5 mg/ml.

Degradation of Insulin and Binding Sites

The degradation of ^{125}I -insulin in the medium increased with temperature (Table 1) and with the concentration of cell protein (Table 2). The amount of tracer degraded in the medium after 1 h accounted for 30 ± 8 and $64 \pm 11\%$ at 15 and 30° , respectively (Table 1). Under the conditions of incubation used (0.5 to 1.5 mg/ml of cell protein, 2 h at 15°), the degradation was also dependent on the concentration of insulin (labelled plus unlabelled) added to the medium: for example, with 1.2 mg/ml of cell protein, the degradation of insulin was 43, 29, 21 and 18% in the presence of 4×10^{-11} , 1.4×10^{-10} , 5.4×10^{-10} and 10^{-9} mol/l insulin, respectively.

No degradation of the binding sites could be detected after 2 h at 15° . At 30° , 25% of the binding sites were degraded after 1 h (data not shown).

Concentration Dependence of Insulin Binding

The concentration dependence of insulin binding was determined by adding increasing concentrations of unlabelled insulin (10^{-10} to 10^{-7} mol/l) to a fixed concentration of ^{125}I -insulin in the incubation mixture

Table 2. Degradation of ^{125}I -insulin (7×10^{-11} mol/l) as a function of cell protein

Concentration of cell protein	0.44	0.60	1.10	2.24	3.75
% of ^{125}I -insulin degraded after 2 h at 15°	13	27	40	63	77

The supernatants from experiment shown in Figure 3 were assayed for insulin integrity as described in Methods. Each value is the mean of duplicate determinations

under the conditions of apparent equilibrium. Native insulin reduced tracer binding in a dose-dependent fashion, half-maximum binding being obtained at about 3×10^{-9} mol/l unlabelled insulin (Fig. 5, left). Scatchard analysis of the binding data (Fig. 5, right), gave curvilinear plots similar to those obtained for insulin in various tissues [21]. That may be explained by two independent binding sites and/or negative cooperativity [9]. In order to compare the data with those of others on a quantitative basis, we performed the calculations following the Scatchard method [39]. As calculated from the curves obtained from individual experiments, the numbers of binding sites per cell were $2,000 \pm 200$ and $39,00 \pm 3,000$ of high and low affinity, the corresponding constants being $2.2 \pm 0.2 \times 10^9$ l/mol and $5.6 \pm 1.6 \times 10^7$ l/mol, respectively (mean $\pm$ SEM of 6 separate experiments).

Specificity of Binding

The specificity of binding was tested in competition experiments between ^{125}I -insulin (5×10^{-11} mol/l), unlabelled insulin (5×10^{-10} to 10^{-7} mol/l), proinsulin (10^{-9} to 10^{-6} mol/l) and unrelated peptides. Fifty per cent of the labelled hormone was displaced by 10^{-7} mol/l proinsulin, indicating that the ability of proinsulin to bind to enterocytes was 3% that of native insulin. Human growth hormone (10^{-6} mol/l), glucagon (10^{-6} mol/l) and epidermal growth factor (10^{-6} mol/l) did not compete with ^{125}I -insulin for the binding site (data not shown).

Fig. 5. Left: concentration dependence of insulin binding. Enterocytes (0.99–1.44 mg/ml of cell protein) were incubated for 2 h at 15° with ^{125}I -insulin (4.9 – 7×10^{-11} mol/l) in the absence (100% on the ordinate) and presence of increasing concentrations of unlabelled insulin (10^{-10} to 10^{-7} mol/l). Each value is the mean $\pm$ SEM of 6 separate experiments, each performed in duplicate. Right: individual Scatchard plots obtained from the data (see Methods). The same symbols indicate measurements performed in a single experiment. The continuous lines A and B represent the mean of the individual lines derived from the experimental points of each individual Scatchard plot. In dotted lines is C = A - B (see Methods)

Discussion

Binding Assay of Insulin

The main characteristics of the insulin binding sites on rat enterocytes are similar to those described in other systems: The high affinity constant of the insulin-receptor interaction is of the same order of magnitude than that obtained for rat liver membranes [12]. The numbers of sites/cell are comparable with those given for isolated human adipocytes [1] and cancer colon cells [11]. The temperature and pH dependence of binding is also in accordance with the results obtained in other systems for the binding of insulin to its receptors [14, 16, 32, 35, 43, 44]. The specificity of binding of ^{125}I -insulin to enterocytes, as tested with proinsulin and unrelated peptides is the same that in liver membranes [12]. The studies of dissociation of ^{125}I -insulin from rat enterocytes (Fig. 2) indicate that the insulin receptors of rat enterocytes exhibit a property which may be negative cooperativity [9] or other causes of non linear Scatchard plots [36, 41]. This property is described for many insulin receptors [2, 9, 21, 35, 36, 44], and seems to be a common feature for all systems used to measure insulin binding [21].

Degradation of Insulin

The degradation of ^{125}I -insulin was reduced by performing the experiments at 15° as was described for the binding of insulin to other isolated cells [2, 16, 34, 35, 44, 45]. Degradation increased with the concentration of cells (Table 2), but was reduced in the presence of small quantities of unlabelled hormone. Terris and Steiner [42] have postulated that the degradation of insulin by isolated hepatocytes requires its prior association with receptors. However, in the case of enterocytes, it would be difficult to distinguish between a "specific" degradation of the hormone, and a "non-specific" degradation due to the enzymes present in the brush border membrane, which is not in contact with insulin under physiological conditions. In favour of the latter hypothesis, no degradation of insulin was detected at 37° by attached cancer colon cells HT 29 [11], and only weak degradation (10% after 2 h at 15°) by detached cancer duodenum cells HUTU 80 (Cezard et al., in preparation). Similarly, the fact that the dissociation of bound ^{125}I -insulin is incomplete can be explained, at least for a part, by an internalisation of intact or degraded hormone [6, 17]. As it was shown for human monocytes [3] and rat hepatocytes [42] the labelled compounds released from the cells may be degraded ^{125}I -insulin.

Insulin Action

The presence of insulin receptors in rat enterocytes gives a molecular basis to observations reported about the physiological role of insulin in the intestine. Until recently, the biological activity of insulin on the digestive tube has been doubtful and conflicting [4, 7, 8, 15, 19, 26]. These discrepancies may be due to the fact that most studies were performed in vivo and that insulin does not seem to stimulate, in the intestine, the uptake of glucose and amino-acids [4, 7, 8, 15, 19, 26], which is one of the specific actions of insulin on target-tissues [19, 24,]. However, when injected in vivo to diabetic rats, insulin restores to normal the depressed Ca^{2+} transport [40]. In addition, insulin injected to control rats stimulates the brush border enzymes activities such as lactase and sucrase [29]. When added in vitro, insulin increases the cGMP content of scraped rabbit ileal mucosa [5] and stimulates slightly the guanylate cyclase activity of isolated brush border and basolateral membranes of the rat duodenum [46]. The hormone also intervenes in the ontogenesis of mouse intestinal sucrase and so seems to play a role in intestinal maturation [31]. Furthermore, insulin stimulates growth and macromolecules synthesis in two human intestinal cell lines, HT 29 and HUTU 80 (Cezard et al., in preparation).

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