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Research Article

Insulin binds specifically to basolateral renal cortical membranes and modifies tubular electrolyte transport, but the target sites of this hormone in the nephron have not been identified. Using a microassay that permits measurement of hormone binding in discrete tubule segments we have determined the binding sites of ^{125}I -insulin along the rabbit nephron. Assays were performed under conditions that minimize insulin degradation, and specific binding was measured as the difference between ^{125}I -insulin bound in the presence or absence of excess (10^{-5} M) unlabeled hormone. Insulin monoiodinated in position A14 was used in all assays. Specific insulin binding (attomol . cm⁻² +/- SE) was highest in the distal convoluted tubule (180.5 ± 15.0) and medullary thick ascending limb of Henle's loop (132.9 ± 14.6), followed by the proximal convoluted and straight tubule. When expressed per milligram protein, insulin binding capacity was highest along the entire thick ascending limb (medullary and cortical portions) and the distal convoluted tubule, i.e., the "diluting segment" (congruent to $10(-13)$ mol . mg protein⁻¹), and was lower (congruent to $4 \times 10(-14)$ mol . mg protein⁻¹), and remarkably similar, in all other nephron segments. Binding specificity was verified in competition studies with unlabeled insulin, insulin analogues (proinsulin and desoctapeptide insulin), and unrelated hormones (glucagon, 1-34 parathyroid hormone, prolactin, follicle-stimulating hormone). In addition, serum containing antiinsulin receptor antibody from [...]

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Insulin Binding Sites in Various Segments of the Rabbit Nephron

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ABSTRACT Insulin binds specifically to basolateral renal cortical membranes and modifies tubular electrolyte transport, but the target sites of this hormone in the nephron have not been identified. Using a microassay that permits measurement of hormone binding in discrete tubule segments we have determined the binding sites of ^{125}I -insulin along the rabbit nephron. Assays were performed under conditions that minimize insulin degradation, and specific binding was measured as the difference between ^{125}I -insulin bound in the presence or absence of excess (10^{-5} M) unlabeled hormone. Insulin monoiodinated in position A14 was used in all assays. Specific insulin binding (attomol $\cdot \text{cm}^{-1} \pm \text{SE}$) was highest in the distal convoluted tubule (180.5 ± 15.0) and medullary thick ascending limb of Henle's loop (132.9 ± 14.6), followed by the proximal convoluted and straight tubule. When expressed per milligram protein, insulin binding capacity was highest along the entire thick ascending limb (medullary and cortical portions) and the distal convoluted tubule, i.e., the "diluting segment" ($\approx 10^{-13} \text{ mol} \cdot \text{mg protein}^{-1}$), and was lower ($\approx 4 \times 10^{-14} \text{ mol} \cdot \text{mg protein}^{-1}$), and remarkably similar, in all other nephron segments. Binding specificity was verified in competition studies with unlabeled insulin, insulin analogues (proinsulin and desoctapeptide insulin), and unrelated hormones (glucagon, 1-34 parathyroid hormone, prolactin, follicle-stimulating hormone). In addition, serum containing antiinsulin receptor antibody from two patients with type B insulin resistance syndrome markedly inhibited insulin binding to isolated tubules.

Whether calculated per unit tubule length or protein

content, insulin binding is highest in the thick ascending limb and the distal convoluted tubule, the same nephron sites where a regulatory role in sodium transport has been postulated for this hormone.

INTRODUCTION

Insulin influences renal tubular transport functions, notably sodium reabsorption, in both man and animals (1-3). Because these effects are elicited by physiologic concentrations of the hormone, it has been postulated that insulin participates in the regulation of normal sodium balance and in the pathogenesis of certain disorders of sodium metabolism in humans (4).

Insulin decreases sodium excretion by the isolated perfused kidney, which excludes extrarenal factors as an explanation (1), and in both the isolated dog kidney (1) and the human kidney *in situ* (2) sodium retention occurs in the absence of changes in renal hemodynamics, indicating a direct effect on the renal tubule. Further evidence supporting a direct tubular effect of this hormone is provided by the demonstration of specific insulin binding to renal cortical tubules (5). Clearance and micropuncture studies suggest that the sodium-retaining effect of insulin takes place in the "diluting segment" (i.e., the thick ascending limb of the loop of Henle and the early distal convoluted tubule) and is independent of plasma glucose or aldosterone concentrations (2, 3).

In a previous report we have shown that insulin binds preferentially to basolateral membranes from rabbit kidney cortex and have defined the characteristics of this process (6). The present study was designed to map the distribution of insulin-binding sites along the rabbit nephron, using microtechniques that

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allow measurement of hormone binding in individual nephron segments (7).

METHODS

Tubule microdissection. Kidneys were obtained from New Zealand White rabbits (Thompson Research Foundation, Monee, IL) weighing 1.5–2.5 kg and fed ad lib. until study. Procedures for tissue preparation and tubule microdissection have been described in detail (8) and will be presented only briefly here. Under pentobarbital anesthesia the left kidney was removed and perfused with ice-cold Krebs-Ringer-Tris-HCl (KRT)¹ solution (NaCl, 114 mM; KCl, 5 mM; NaHCO₃, 17 mM; KH₂PO₄, 1.2 mM; MgSO₄, 1.2 mM; CaCl₂, 1 mM; Tris-HCl, 16 mM, pH 7.4) containing 200 U/ml collagenase, type I (Sigma Chemical Co., St. Louis, MO), and 0.2% bovine serum albumin (BSA). Thin pyramids were cut along the corticopapillary axis and incubated for 25 min in the same medium at 35°C, after which they were rinsed with cold microdissection solution (identical to the above except that collagenase and BSA were omitted) and stored on ice until use. Tubule segments were dissected freehand under stereomicroscopic observation, transferred to the concavity of a sunken bacteriological slide, and photographed to determine their length.

¹²⁵I-Insulin binding. Several tubules from the same nephron segment (average total length 4–8 mm) were pooled in each sample and placed on small (5 × 5 mm) squares of aluminum foil. Tubules were incubated for 90 min at 22°C in 5 µl of KRT buffer (pH 7.4) containing porcine ¹²⁵I-insulin (2.5 nM) moniodinated in position A14 (mean sp act 336 µCi/µg); BSA, 1%; and N-ethylmaleimide 1 mM. A14-¹²⁵I-insulin was chosen because it is stable, has a high specific activity, and its binding characteristics and biologic properties are similar to native insulin (9). These experimental conditions were found by us to be optimal for insulin binding to tubular cell membranes and to minimize insulin degradation during incubation (6). The reaction was stopped by cooling on ice, and 2 µl of the assay medium was removed to assess the amount of intact insulin remaining after incubation; this volume was added to 50 µl of 20% trichloroacetic acid (TCA) (wt/vol) and the radioactivity in the TCA-soluble and -insoluble fractions was counted. The remaining incubation medium was aspirated, the tubules were washed three times in cold KRT, and the bound radioactivity counted for 20 min in a Packard gamma spectrometer² (Packard Instrument Co., Downers Grove, IL). Nonspecific binding was determined in parallel experiments in the presence of excess (10^{-5} M) unlabeled insulin, and the amount of specifically bound hormone was derived from the differ-

ence between these two determinations. Blanks incubated in identical conditions were run in each experiment. A14-¹²⁵I-insulin was >97% TCA-precipitable, and incubation without tubules did not alter this appreciably; adherence of the labeled hormone to aluminum foil was also negligible (<0.1%). Binding was corrected for the small amount of degradation during the assay (0.8–5.1%) and expressed as percentage of intact insulin present. Assays were done in triplicate, average values of all tubules from a single animal representing a single data point in figures and table.

To determine the specificity of insulin binding we performed two sets of experiments. In the first we incubated medullary thick ascending limbs of Henle's loop (MAL) with 2.5 nM ¹²⁵I-insulin in the presence of increasing concentrations of unlabeled insulin, insulin analogues (proinsulin and desoctapeptide insulin), or unrelated peptide hormones (prolactin, glucagon, follicle-stimulating hormone [FSH], and synthetic 1–34 N-terminal parathyroid hormone [PTH]). In the second set of experiments we tested the effect on ¹²⁵I-insulin binding to MAL of serum from two patients with type B insulin resistance due to autoantibodies to the insulin receptor. Serum was obtained from patients VQ (University of Chicago Medical Center) and B₁₀ (courtesy Dr. Simeon I. Taylor, Diabetes Branch, National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD), whose clinical histories have been reported elsewhere (10, 11). Tubules were incubated for 60 min at 22°C in 2 µl of undiluted serum from the patients and from a normal control. After this preincubation, serum was aspirated and the insulin binding assay was carried out as outlined above. Care was taken to remove serum completely before adding the incubation medium containing radiolabeled insulin. MAL were chosen for these studies because of their high insulin-binding capacity and relative facility of microdissection.

Materials. A14-¹²⁵I-insulin was a kind gift from Dr. B. Frank, and porcine monocomponent insulin, proinsulin, desoctapeptide insulin, and bovine glucagon from Dr. R. Chance and Dr. W. Bromer, Eli Lilly & Co., Indianapolis, IN. Rat prolactin (National Institute of Arthritis, Metabolic, and Digestive Diseases [NIAMDD]-rat PRL-B-1) and rat FSH (NIAMDD rat FSH-B-1) were provided by Dr. A. F. Parlow (NIAMDD-Rat Pituitary Hormone Distribution Program, University of California, Los Angeles, CA). 1–34 PTH was purchased from Beckman Instruments, Inc., Palo Alto, CA, and all other chemicals (reagent grade) from Sigma Chemical Co.

RESULTS

Preliminary experiments using several nephron segments revealed that binding was similar in fed or fasted (48 h) rabbits, and consequently animals were allowed food and water ad lib. We also verified that pretreatment with collagenase did not alter significantly insulin binding, which was comparable in tubules obtained from the left and the right kidney of the same animal perfused with or without collagenase, respectively. The profile of specific ¹²⁵I-insulin binding along the rabbit nephron is shown in Fig. 1. Highest binding capacity (per unit tubule length, lower panel) was found in the distal convoluted tubule and medullary thick ascending limb (180.5±15.0 and

¹ Abbreviations used in this paper: FSH, follicle-stimulating hormone; KRT, Krebs-Ringer-Tris-HCl solution; MAL, medullary thick ascending limb(s) of Henle's loop; NIAMDD, National Institute of Arthritis, Metabolic, and Digestive Diseases; PTH, parathyroid hormone.

² Counts per minute of total binding ranged between approximately four times the background counts in segments rich in binding sites (e.g., MAL) and two to three times the background counts in tubules with lower binding capacity (e.g., cortical collecting tubules). Examples of actual counts per 20 min for total and nonspecific binding, respectively, were 4,200 and 2,900 in MAL and 2,600 and 1,800 in cortical collecting tubules; background counts averaged 1,100/20 min.

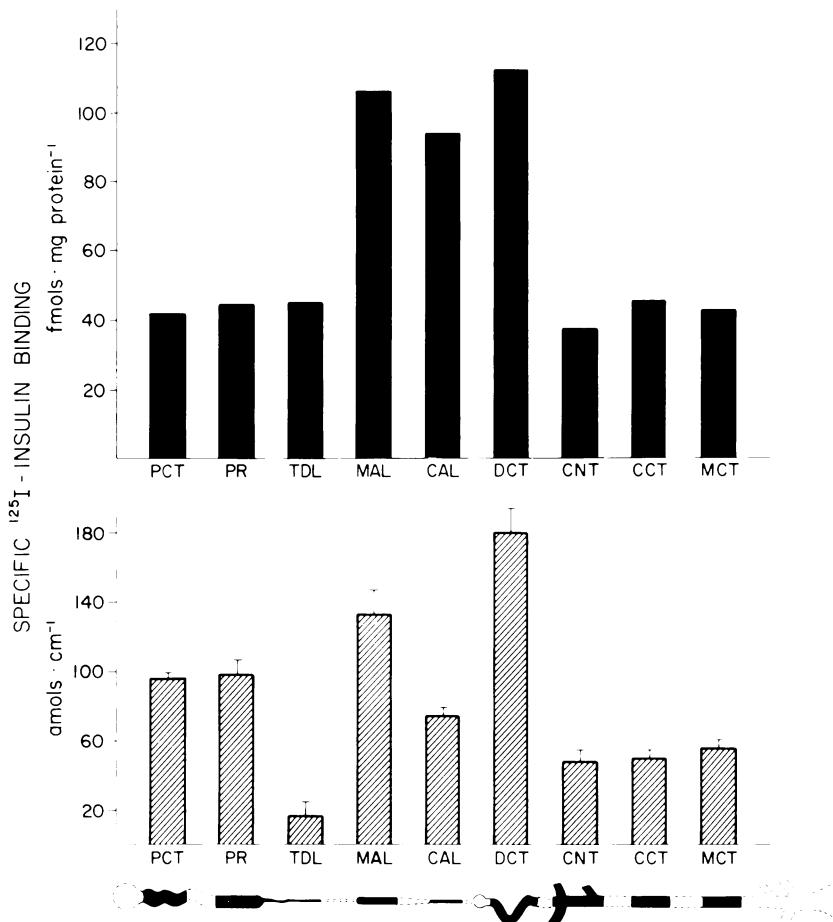


FIGURE 1. Specific binding of A14-¹²⁵I-insulin along the rabbit nephron, expressed per milligram protein (*upper panel*) and tubule length (*lower panel*). Binding capacity was highest in the thick ascending limb of Henle's loop and the distal convoluted tubule. $n = 6$.

132.9 ± 14.6 attomol · $\text{cm}^{-1} \pm \text{SE}$, respectively), substantial binding (≤ 100 attomol · cm^{-1}) being found in the proximal tubule as well. When expressed per milligram protein (*upper panel*), calculated from the relationship between tubule length and protein content in the rabbit nephron (12), binding capacity was high along the entire thick ascending limb (medullary and cortical portions) and the distal convoluted tubule ($\approx 10^{-13}$ mol · mg protein $^{-1}$) and was lower ($\approx 4 \times 10^{-14}$ mol · mg protein $^{-1}$), and remarkably similar, in all other nephron segments.

Experiments aimed at determining the specificity of the observed insulin binding are presented in Fig. 2 and Table I. In competition studies (Fig. 2) insulin competed most effectively for binding sites with the labeled hormone, which it displaced in a dose-dependent manner. In agreement with results previously reported with isolated tubular cell membranes (6), proinsulin and desoctapeptide insulin produced only

partial displacement, proportional to the biologic activity of these analogues (13). Hormones structurally unrelated to insulin (prolactin, glucagon, FSH, and 1-34 PTH) were without effect even at the pharmacologic concentration of 10^{-6} M, but at 10^{-5} M some of these hormones did show partial binding competition. Because of the very high concentrations where this effect occurred, its significance is obscure.

Specificity of insulin binding was also examined after preincubation of tubules with sera from two patients with antiinsulin receptor antibodies. These studies reveal (Table I) that both sera inhibited specific insulin binding to MAL by $\sim 55\%$.

DISCUSSION

Insulin binds specifically to high-affinity receptors in cortical tubules and in plasma membranes from mammalian kidney cortex (5, 14). We have recently shown

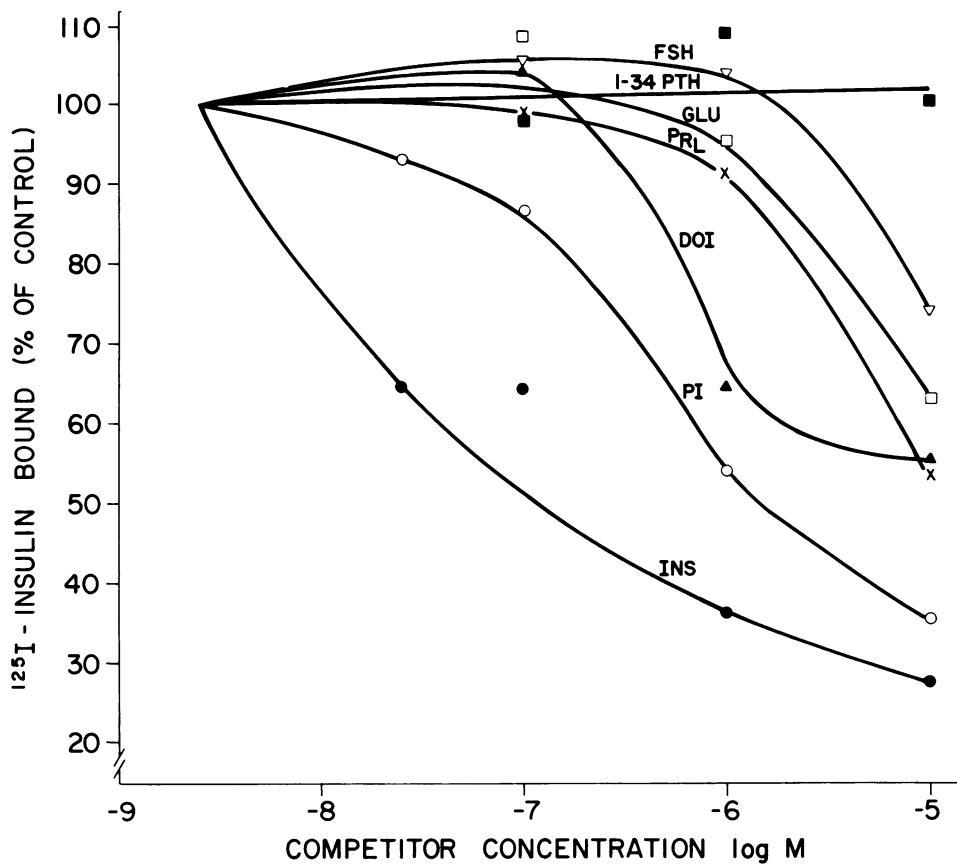


FIGURE 2 Displacement of A14-¹²⁵I-insulin bound to MAL by increasing amounts of unlabeled insulin (INS); insulin analogues (proinsulin, PI; desoctapeptide insulin, DOI); and unrelated hormones (prolactin, PRL; glucagon, GLU; 1-34 PTH; FSH). Labeled insulin was displaced most by the homologous unlabeled hormone itself followed by insulin analogues in rough proportion to their biologic activity, whereas unrelated hormones were not effective competitors in the relevant concentration range. Each point represents mean value of four to seven animals.

that insulin binds preferentially to the basolateral membrane (6), the postulated site of interaction between peptide hormones and their receptors in the kidney. The present study extends the latter obser-

vations and identifies the distribution of insulin-binding sites in the individual segments of the rabbit nephron: when related to unit tubule length, insulin binding was highest in the distal convoluted tubule and the MAL. Expressed per milligram protein, binding capacity was highest (and similar) in both the medullary and cortical portions of the thick ascending limb as well as in the distal convoluted tubule, i.e., along the entire length of the "distal nephron" before the beginning of the collecting tubule. Although fewer, binding sites were also present in all other nephron segments tested, where their concentration was remarkably similar when related to protein content. Besides determining insulin binding in individual nephron segments for the first time, these studies also demonstrated binding of the hormone to medullary structures, which were not evaluated before as possible binding sites. Tubule or membrane preparations used for this pur-

TABLE I
Inhibition of A14-¹²⁵I-Insulin Binding by Sera Containing Insulin Receptor Antibody

Serum	Specific binding $10^{-18} \text{ mol} \cdot \text{cm}^{-2}$	Percentage of control
Control	135±19	100
VQ	58±13	46±12
B ₁₀	58±16	45±12

Results are mean±SE of four separate experiments (four animals). Binding was measured in MAL.

pose were obtained in the past only from the renal cortex (5, 6, 14).

The experiments described in this study were performed in the same conditions found by us to be optimal for insulin binding to isolated basolateral membranes (6), with the sole exception of the hormone concentration used. Because of the limitations imposed by the minute amounts of tissue available (1–2 µg protein per assay), the minimal concentration of ligand required to measure binding reproducibly (2.5×10^{-9} M) was somewhat higher than the K_d of the high-affinity insulin receptor (6), and probably resulted in binding to more than one class of receptor sites. Nevertheless, because the kinetics of insulin binding to renal and other cell membranes indicate the presence of several "families" of receptors, and the concentration of insulin utilized here was still in the high physiologic range, we believe that the results reflect the distribution of insulin-binding sites in the nephron *in vivo*.

Binding of insulin to renal tubules raises the question of the role of this process in the intact animal. That it is of physiologic significance is indicated by the fact that binding is specific for this hormone, and that the kidney binds more insulin (per milligram protein) than the liver, which is an established site of insulin action (14). It is possible, but not proved, that receptor binding of the hormone is a prelude to its degradation on the basolateral membrane. Another possibility, suggested by the mode of action of other peptide hormones on the kidney and of insulin on its target tissues, is that binding of insulin to specific receptors on the basolateral membrane of renal tubular cells precedes its biologic effects on these cells. Results of the studies described above, indicating preferential binding in the thick ascending limb and distal convoluted tubule support this view: the best known effect of insulin on tubular electrolyte transport, increased sodium reabsorption, takes place in precisely the same regions of the nephron of man and experimental animals (2–4). The approximately equal binding of insulin to all other nephron segments could subserve other actions, of a more general character, of this hormone on the kidney, such as glucose uptake (15), as well as effects of insulin on the transport of other electrolytes. The nature of the events that follow the binding of insulin to its receptor in kidney cells, and especially how they translate into the antinatriuretic effect of the hormone, remain at present unknown; their elucidation will both depend on, and contribute to, an improved understanding of the molecular basis of insulin action.

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Ms. L. Karas prepared the manuscript.

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