Insulin Binding and Degradation by Luminal and Basolateral Tubular Membranes from Rabbit Kidney

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ABSTRACT Insulin influences certain metabolic and transport renal functions and is avidly degraded by the kidney, but the relative contribution of the luminal and basolateral tubular membranes to these events remains controversial. We studied ^{125}I -insulin degradation [TCA and immunoprecipitation (IP) methods] and the specific binding of the hormone by purified luminal (L) and basolateral (BL) tubular membranes. These were prepared from rabbit kidney cortical homogenates by differential and gradient centrifugation and ionic precipitation steps in sequence, which resulted in enrichment vs. homogenate of marker enzymes' activities (sodium-potassium-activated adenosine triphosphatase for BL and maltase for L) of 8- and 12-fold, respectively. Both fractions degraded insulin avidly and bound the hormone specifically without saturation even at pharmacologic concentrations ($10\text{ }\mu\text{M}$). At physiologic insulin concentrations (0.157 nM) BL membranes degraded substantial amounts of insulin (44.2 ± 2.6 and $40.7\pm 2.2\text{ pg/mg protein per min}$ by the TCA and IP methods, respectively), even though at lesser rates ($P < 0.001$) than the luminal fraction (67.2 ± 2.3 and $75\pm 6.2\text{ pg/mg protein per min}$, respectively); the rate of insulin catabolism by BL membranes was significantly higher ($P < 0.001$) than that which could be attributed to their contamination by luminal components [$12.2\pm 1.9\text{ pg/mg per min}$ (TCA method), or $13.7\pm 1.9\text{ pg/mg per min}$ (IP method)]. Competition experiments suggested that insulin-degrading activity in both fractions includes both specific and nonspecific components. In contrast to degradation, insulin binding by both membranes was highly specific for native insulin and was severalfold higher in BL than L membranes [17.5 ± 1.3 vs. $4.5\pm 0.4\text{ fmol/mg protein}$ ($P < 0.001$) at physiologic insulin concentrations]. Despite the marked difference in the binding capacity for insulin by the two membranes,

the patterns of labeled insulin displacement by increasing amounts of unlabeled hormone were superimposable (50% displacement required $\sim 3\text{ nM}$), suggesting that their receptors' affinity for insulin was similar. These observations provide direct evidence that interaction of insulin with the kidney involves binding and degradation of the hormone at the peritubular cell membrane.

INTRODUCTION

The kidney plays a major role in the degradation of insulin (1), but the mechanisms involved in this process are not agreed upon. A subject of continuing controversy is the route of insulin uptake and catabolism by the tubular cell, i.e., the relative contribution of the luminal and basolateral membranes to these events.

Because insulin influences tubular transport of ions and probably also modulates certain metabolic processes in the kidney (2-7), an interaction with the basolateral cell membrane is suggested by analogy with other peptide hormones, e.g., parathyroid hormone and vasopressin, which bind to specific receptors on the contraluminal aspect of target tubular cells as a first step in their action on the kidney (8, 9). Binding of insulin to receptors at this site, besides initiating the cellular action of the hormone, could be linked in some way to its degradation, the latter performing a regulatory function by terminating the hormone signal on the tubular cell. Measurements of renal organ clearance of insulin in humans (1, 10), dogs (11), and rats (12) (in vivo), studies with the isolated perfused kidney (13, 14), and injection of the hormone in the portal circulation of the chicken (15) all strongly suggest that insulin degradation by the kidney involves in addition to glomerular filtration and luminal uptake, also catabolism of the hormone by peritubular mechanisms. Further support for this view was provided by recent observations that tubule fragments bound specifically and degraded insulin avidly in vitro, despite the fact

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that their collapsed lumina were exposed only to a very limited extent to the hormone (14, 16, 17). In contrast, studies based on electron microscopic autoradiography and tubule micropfusion were interpreted to indicate only negligible participation of the contraluminal uptake route in the renal metabolism of insulin (18).

The aim of the present study was to evaluate directly the contribution of luminal and basolateral renal tubular membranes to insulin binding and degradation. The results indicate that specific binding of insulin is substantially higher to basolateral than to luminal membranes, and that both membrane preparations degrade insulin rapidly. These observations provide direct evidence that basolateral tubular cell membranes participate in the renal catabolism of insulin and suggest that the peritubular aspect of the cell is an important site in the mediation of the insulin signal on the kidney.

METHODS

Preparation of basolateral and luminal membranes. Membranes were prepared from cortical homogenates of kidneys from male New Zealand White rabbits (3–4 kg body wt) that had free access to food and water until study. Animals were exsanguinated under light pentobarbital anesthesia and the kidneys were rapidly removed and placed in ice-cold isotonic saline. The cortex from both kidneys was homogenized at 4°C in Tris buffer (pH 7.0) containing 8% (wt/vol) sucrose in a glass-homogenizer with 10 strokes of a tight-fitting pestle. Basolateral (BL)¹ and luminal (L) membranes were prepared from this homogenate by the sequence of differential and gradient centrifugation combined with ionic precipitation described by Kinsella et al. (19), with minor modifications. After centrifugation at 1,000 *g* (10 min) the pellet was rehomogenized and centrifuged for a second time (1,000 *g*; 10 min) (model RC2-B, DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Newton, CT.). Supernates were combined and spun at 9,500 *g* (10 min), and the resulting supernate and lighter pellet portion were centrifuged at 47,000 *g* for 20 min (model L ultracentrifuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, CA.). The lighter pellet portions obtained after this step were resuspended in Tris Hepes Mannitol (THM) buffer (19) containing 2 mM CaCl₂ and 1 mM each MgCl₂ and MnCl₂ and incubated on ice for 60 min. After ionic precipitation supernates contained the luminal membranes and the pellet consisted of crude basolateral fragments. The latter was washed twice (12 min at 1,400 *g*), and both fractions were then dialyzed overnight against 50 vol of THM buffer. Luminal membranes were not processed further; basolateral membranes were further purified by centrifugation (90,000 *g*; 60 min.) in a discontinuous sucrose gradient (8/33.5%, wt/wt). BL membranes were harvested at the interphase,

diluted to isotonicity with distilled water, and spun at 75,000 *g* for 60 min. The pellet was resuspended in THM buffer and stored in aliquots at –90°C until used. Both membrane preparations consisted of intact vesicles of similar size, 100–300 nm in diameter, as revealed by electron microscopy (courtesy Dr. B. H. Spargo, Department of Pathology, The University of Chicago). In addition, the vesicles of both luminal and basolateral fractions have a similar orientation, i.e., over 75% are right-side out (19).

Activities of marker enzymes were determined in the crude homogenate and in each membrane batch prepared from individual animals. All marker enzyme assays were performed on deoxycholate-treated (0.1% final concentration) membrane fragments or homogenates. Sodium-potassium-activated adenosine triphosphatase (Na-K-ATPase), taken as the enzyme marker for basolateral membranes, was measured by methods described from this laboratory (20). Maltase, measured by Lloyd and Whelan's modification of the method of Dahlqvist (21), was considered the marker for luminal membranes. Activities of acid phosphatase and glucose-6-phosphatase (marker enzymes for lysosomes and microsomes, respectively) were determined by the method of Hübscher and West (22). Succinate dehydrogenase activity (mitochondrial marker) was measured by the method of Tisdale (23). Phosphate was determined by the method of Fiske and Subba row (24) and protein concentration by that of Lowry et al. (25).

Insulin degradation. Degradation of ¹²⁵I-labeled insulin was studied during incubation of the hormone with whole homogenate, and with luminal and basolateral membranes suspended in THM buffer containing 0.1% deoxycholate and stored at –90°C until use (final deoxycholate concentration in the assay media was 0.01%). In pilot studies this treatment was found to augment (1.4–3-fold), as well as to partially solubilize the insulin-degrading activity. Insulin degradation was determined as described in detail below by both TCA precipitation and immunoprecipitation methods. In preliminary experiments we determined the optimal pH, membrane protein concentration, and duration of incubation using physiologic ¹²⁵I-insulin concentrations (0.1 nM). pH optimum for insulin-degrading activity of whole homogenate was ~5.4 (consistent with that of acid hydrolases), while that residing in the luminal and basolateral membrane fragments displayed a pH optimum between 6.4 and 7.4. In subsequent experiments insulin degradation by these fractions was therefore assessed at physiologic pH. We then ascertained that insulin degradation was linearly dependent on membrane protein content up to 0.6 mg/ml and proceeded in linear fashion for at least 10 min. Accordingly, degradation experiments were performed at 37°C, pH 7.4, in 0.5 ml Krebs-Ringer-Tris (KRT) buffer (17) containing 0.5% bovine serum albumin (BSA) and ~0.2 mg membrane protein, during 5-min incubation. All incubations were carried out in duplicate, and the total insulin concentration ranged between 0.05 nM and 10 μM. The reaction was stopped by the addition of 0.25 ml ice-cold *N*-ethylmaleimide (NEM) (final concentration 1 mM), followed by rapid separation of the membranes from the incubation mixture by centrifugation at 35,000 *g* for 5 min at 4°C. At this temperature 1 mM NEM inhibited insulin degradation completely, and the supernatant prepared in this fashion had no further insulin-degrading activity.

Incubation media were assayed for change in intact insulin content both by TCA precipitation and by immunoprecipitation. 2 150-μl aliquots were diluted with borate buffer (pH 8.0) containing 0.5% BSA (to a volume of 0.5 ml each) mixed with an equal volume of 20% ice-cold TCA, vortexed,

¹ *Abbreviations used in this paper:* BL, basolateral; BSA, bovine serum albumin; IP, immunoprecipitation; KRT, Krebs-Ringer-Tris; L, luminal; Na-K-ATPase, sodium-potassium-activated adenosine triphosphatase; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; THM, Tris Hepes Mannitol; TLCK, *N*-α-*p*-tosyl-L-lysine-chloromethyl ketone hydrochloride; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone.

and centrifuged at 3,000 rpm for 30 min at 4°C. The radioactivity of the resulting pellet and supernatant were determined in a Packard auto gamma spectrometer (Packard Instrument Co., Downers Grove, IL.). The fraction of insulin degraded was calculated from the difference between the radioactivity present in the supernatant and that of control tubes incubated without membranes and treated similarly. Two other 150- μ l aliquots of incubation medium were diluted to a volume of 0.3 ml each with borate buffer (pH 8.0) containing 0.5% BSA and excess antiinsulin guinea pig antibody in a final dilution of 1:10,000. After 24 h of incubation at 4°C bovine γ -globulin (50 μ l of a 1.6 g/dl solution) and polyethylene glycol (final concentration 14%) were added, and the tubes were incubated for 30 min at 4°C. After centrifugation the percentage of insulin degraded was calculated from the decrement in the radioactivity of the pellet in comparison to that from control tubes incubated without membranes.

Possible differences in insulin-degrading activity between luminal and basolateral membranes and the specificity of this process were examined in separate experiments. Excess amounts (10 μ M) of unlabeled insulin, insulin analogues (proinsulin and desoctapeptide insulin), or peptide hormones structurally unrelated to insulin (bovine glucagon, rat prolactin, rat FSH, and synthetic 1-34 N-terminal PTH) were added to the incubation medium. In other experiments we examined the effects of various enzyme inhibitors on the insulin-degrading activity of the two membranes: these incubations were carried out in the presence of either chloroquine (0.1, 1, and 100 mM), alloxan (1 and 10 mg/ml), *p*-chloromercuribenzoate (PCMB; 1 mM), NEM (1 mM), bacitracin (8 and 0.8 mg/ml), aprotinin (4,000 U/ml), ammonium chloride (100 mM), and 50 μ g/ml each of soybean-trypsin inhibitor, *N*-alpha-*p*-tosyl-L-lysine-chloromethyl ketone hydrochloride (TLCK), and L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK). Degradation rates of 125 I-insulin were measured in these studies by the TCA method and compared with those of control incubations performed in parallel in the absence of enzyme inhibitors or excess amounts of unlabeled hormones. Results from these experiments are therefore expressed as insulin degradation in percent of that measured in control incubations.

Insulin binding. For these studies we used basolateral and luminal membranes without deoxycholate treatment, because this detergent interfered with binding by the assay method selected. Experiments were performed with concentrations of 125 I-insulin ranging from 3 pM to 2 nM; incubations were carried out at 22°C in 0.5 ml KRT buffer (pH 7.4), containing 1% BSA and 1 mM *N*-ethylmaleimide. In preliminary experiments we ascertained that at this temperature the addition of NEM (1 mM) inhibited insulin degradation by >97%, thus allowing measurement of binding after prolonged incubation. Because insulin binding increased linearly with increasing membrane protein concentrations up to 0.8 mg/ml, experiments were performed using 0.4 mg/ml. Under these conditions insulin binding reached equilibrium between 60 and 90 min. Incubation was terminated after 90 min by the addition of ice-cold KRT buffer (1.0 ml) and immediate separation of the membranes from the incubation mixture by centrifugation in the cold (35,000 g; 5 min). An equal volume of 20% ice-cold TCA was added to 500 μ l of the supernate, and the mixture was centrifuged at 3,000 rpm for 30 min to determine the amount of insulin degraded during incubation. Radioactivity was also counted in the rest of the original supernate and in the original pellet

(washed in 1 ml cold KRT and reprecipitated at 35,000 g), which represented total bound insulin. Nonspecific binding of the hormone (average 0.35%) was determined in parallel experiments in the presence of a large excess (10 μ M) of unlabeled insulin, and the amount of specifically bound insulin was derived from the difference between these two determinations. In each experiment, control tubes (without membranes) were treated identically to experimental tubes (including the addition of equal amounts of iodinated and unlabeled hormone) and the percent "binding" to these tubes (average $\leq$ 1%) was subtracted from the values obtained in experimental tubes. All determinations were conducted in duplicate. The TCA-precipitable insulin present in the supernatant at the end of the incubation is referred to as intact hormone and results are expressed as percentage of intact insulin bound.

In other experiments, variable amounts (final concentration of 0.1 nM to 10 μ M) of unlabeled insulin, insulin analogues (proinsulin and desoctapeptide insulin), or unrelated peptide hormones (bovine glucagon, rat FSH, synthetic 1-34 N-terminal PTH) were added to the medium to examine the specificity of the insulin binding. These results were also corrected for binding to blank tubes and for insulin degradation during the incubation.

Materials. Porcine monocomponent insulin, was iodinated by the method of Freychet et al. (26) as modified by Terris and Steiner (27). The iodinated hormone was purified by column chromatography on a Biogel P-30 column (Bio-Rad Laboratories, Richmond, CA) and subsequently adsorbed on a cellulose acetate column from which it was eluted by 30 mM phosphate buffer (pH 7.4) containing 10% BSA. 125 I-insulin thus prepared contained $\sim$ 0.2 atoms of iodine per mole of insulin, was 97–99% TCA-precipitable, and 88–92% immunoprecipitable. After each purification the specific radioactivity of the iodinated hormone was determined by the method of self displacement as modified by Morris (28); the specific radioactivity of the preparations used ranged between 80 and 120 μ Ci/ μ g. Incubation of iodinated insulin without membranes under the conditions prevailing in both the binding and degradation experiments described above did not increase the TCA-soluble fraction of the labeled hormone.

Porcine monocomponent insulin, proinsulin, desoctapeptide insulin, and bovine glucagon were generously supplied by Dr. R. Chance and Dr. W. Bromer of the Eli Lilly Company (Indianapolis, IN). Insulin iodinations were performed by the laboratory of Dr. A. H. Rubenstein, which also provided the antiinsulin guinea pig antibody. Rat prolactin [National Institute of Arthritis, Metabolism, 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 parathyroid hormone was purchased from Beckman Instrument Co., Palo Alto, CA. Glucose oxidase and horseradish peroxidase were purchased from Boehringer Mannheim Biochemicals, (Indianapolis, IN), and bovine- γ -globulin from Miles Laboratories Inc., (Elkhart IN). Aprotinin was obtained from FBA pharmaceuticals (New York). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO), and all chemicals were reagent grade.

Statistical methods. Results are presented as means $\pm$ SE, unless otherwise specified. Regression lines were calculated by the method of least squares. Statistical significance ($P < 0.05$) was assessed by the Student's *t* test for paired or unpaired samples, as appropriate.

RESULTS

Characterization of basolateral and luminal membranes. The relative enrichment of marker enzymes for BL and L membranes compared with the starting homogenate is depicted in Fig. 1. Activity of Na-K-ATPase (the BL membrane marker) increased eight-fold vs. homogenate in this fraction; similarly, maltase (the L membrane marker) was enriched 12-fold in the luminal membrane preparation. Cross contamination between these fractions was small: the basolateral preparation included at most 18% luminal fragments, while only 9% of the brushborder preparation consisted of BL fraction components². There was little contamination of either membrane fraction with mitochondria, lysosomes, or microsomes as indicated by the lack of enrichment of succinate dehydrogenase, acid phosphatase, and glucose-6-phosphatase, respectively.

Insulin degradation. The change in the intact insulin content of the supernate, as assessed by both TCA precipitation and immunoprecipitation (IP), was taken to represent degraded insulin. Although the two methods corresponded satisfactorily, we confirmed the observation of Kurokawa and Lerner (17) that the TCA method tends to slightly underestimate insulin degradation when the fraction of degraded hormone increases, presumably due to precipitation of nonimmunoreactive fragments by TCA. Insulin degradation velocity of luminal and basolateral membrane fractions at a physiologic concentration of the hormone (0.157 nM) is presented in Fig. 2. Basolateral membranes degraded substantial amounts of insulin (44.2 ± 2.6 and 40.7 ± 2.2 pg/mg protein per min by the TCA and IP methods, respectively; $n = 8$) even though at lesser rates ($P < 0.001$) than the luminal fraction [67.2 ± 2.3 and 75 ± 6.2 pg/mg protein per min, respectively ($n = 8$)]. The rate of insulin catabolism by the basolateral fraction was significantly higher than that which could be attributed to contamination of this preparation by luminal membrane fragments [44.2 ± 2.6 vs. 12.2 ± 1.9 pg/mg per min (TCA method) or 40.7 ± 2.2 vs. 13.7 ± 1.9 pg/mg per min (IP method); both $P < 0.001$]. Insulin-degrading activity of luminal and basolateral membranes averaged, respectively, 1.6 and 0.4% of the total degrading activity present in the original homogenate and parallels the protein yield of these two fractions (2.4 and 1.0%, respectively, of the total protein content of the starting homogenate).

² Cross-contamination was calculated as follows: Percent contamination of membrane A by membrane B = activity of marker enzyme b in A $\times$ 100/activity of marker b in B; where A and B are the respective membranes, and a and b the corresponding marker enzyme activities.

FIGURE 1 Relative enrichment (compared with the starting homogenate) of marker enzymes from luminal and basolateral membranes prepared from rabbit kidney cortex. Activity of Na-K-ATPase in the basolateral fraction, increased 8-fold while that of maltase was enriched in the luminal membrane 12-fold vs. the original homogenate. There was little contamination of either fraction with mitochondria (SDH), lysosomes (acid phosphatase) or microsomes (G-6-phosphatase), or by each other. □, luminal; ■, basolateral.

To test the relation between substrate concentration and degradation insulin concentrations were varied between 0.05 nM (simulating low fasting levels) and 10 μ M, which is many times higher than levels obtainable even after pharmacologic doses of the hormone. Results are depicted in Fig. 3, where the variables are presented in logarithmic scales because of the very wide concentration range used (left panel). It is apparent that there was no saturation of the insulin-degrading activity of either membrane, even at extremely high insulin concentrations. However, in the physiologically relevant concentration range insulin degradation showed a curvilinear relationship to insulin concentration when drawn on linear scale (Fig. 3, right panel). At all insulin concentration studied luminal membranes degraded more of the hormone than the basolateral fraction, and the rate of insulin catabolism by the latter preparation could not be accounted for by cross-contamination with luminal membrane fragments.

The specificity of the insulin-degrading activity of the two membranes was evaluated in experiments in which excess amounts (final concentration 1 μ M) of unlabeled insulin, insulin analogues, or peptide hormones structurally unrelated to insulin were added to the incubation mixture (Table I). The results demonstrate that the velocity of ¹²⁵I-insulin degradation by basolateral and luminal membranes was slowed by 55 and 62%, respectively, by the addition of a 10,000-fold excess of unlabeled insulin (1 μ M). Excess amounts of desoctapeptide insulin were more effective in this

FIGURE 2 Insulin degradation velocity by luminal and basolateral membranes (protein content ~ 0.4 mg/ml) at physiologic concentrations of the hormone (0.157 nM), as determined by TCA and IP techniques. BL degraded substantial amounts of insulin, even though at lesser rates than the luminal fraction. The rate of insulin catabolism by BL was significantly higher than that which could be attributed to its contamination by luminal fragments (solid bars). Experiments were performed with membranes purified from eight animals. ▨, TCA; □, IP.

respect than equimolar concentrations of the native hormone, whereas the influence of proinsulin was less pronounced. The effects of synthetic 1–34 PTH and rFSH were intermediate, while rat prolactin and porcine glucagon interfered less with insulin degradation. Despite the higher degrading capacity of the luminal side, the behavior of the two membrane preparations

TABLE I
Inhibition of ^{125}I -Insulin Degradation by Unlabeled Insulin, Insulin Analogues, and Unrelated Peptide Hormones (all $1 \mu\text{M}$)

	Basolateral	Luminal
Degradation, % control*		
Insulin	45.5 $\pm$ 1.1	38.3 $\pm$ 3.7
Desoctapeptide insulin	33.5 $\pm$ 1.5	24.4 $\pm$ 3.0
Proinsulin	62.9 $\pm$ 2.4	60.0 $\pm$ 3.5
1–34 PTH	69.2 $\pm$ 2.1	74.8 $\pm$ 3.1
rFSH	68.0 $\pm$ 1.8	76.5 $\pm$ 4.6
rPRL	82.1 $\pm$ 8.3	86.7 $\pm$ 4.1
Glucagon	84.6 $\pm$ 3.1	91.9 $\pm$ 2.1

* Data are expressed in percentage of control values ($9.0 \pm 0.1\%$ /mg protein per min for basolateral and $14.1 \pm 1.3\%$ /mg protein/min for luminal membranes), measured in incubations with 0.17 nM ^{125}I -insulin in the absence of unlabeled insulin or other hormones. Results are expressed as the mean of two separate estimations $\pm$ one half the range.

TABLE II
Effect of Enzyme Inhibitors on ^{125}I -Insulin Degradation

	Basolateral	Luminal
Degradation, % control*		
Chloroquine, 100 mM	0	0
Chloroquine, 1 mM	102.4	101.2
Chloroquine, 0.1 mM	97.2	100.6
PCMB, 1 mM	1.7	6.8
Alloxan, 10 mg/ml	4.1	10.8
Alloxan, 1 mg/ml	46.1	69.2
Bacitracin, 8 mg/ml	9.7	8.0
Bacitracin, 0.8 mg/ml	61.4	77.1
NEM, 1 mM	24.1	27.4
Aprotinin, 4000 U/ml	82.4	85.6
Soybean-trypsin, inhibitor 50 $\mu\text{g}/\text{ml}$	114.1	111.0
TLCK, 50 $\mu\text{g}/\text{ml}$	116.7	112.0
TPCK, 50 $\mu\text{g}/\text{ml}$	118.1	117.0
NH_4Cl , 100 mM	110.2	102.4

* Data are expressed in percentage of control values ($8.0 \pm 2.1\%$ /mg protein per min for basolateral and $12.2 \pm 1.2\%$ /mg protein per min for luminal membranes), measured in incubations with 0.11 – 0.18 nM ^{125}I -insulin in the absence of inhibitors.

regarding the inhibition of insulin degradation was qualitatively similar.

To further evaluate possible differences in the insulin-degrading activity of the two membranes we examined the effects of various enzyme inhibitors on insulin degradation velocity (Table II). The pattern of enzyme inhibitor potency regarding insulin degradation was also strikingly similar in the two membrane preparations: low concentrations of chloroquine (0.1 and 1 mM) were without effect, while high concentrations (100 mM) inhibited both luminal and basolateral membrane insulin-degrading activity completely. PCMB, high concentrations of alloxan, bacitracin, and NEM³, in that order, were somewhat less potent. High levels of aprotinin, another peptidase inhibitor, had a small but demonstrable effect, but inhibitors of trypsinlike and chymotrypsinlike peptidases were ineffective.

Insulin binding. In preliminary experiments we determined the optimal conditions required to minimize degradation of the hormone during the pro-

³ Inhibition of insulin degradation by NEM was temperature dependent, averaging 75% at 37°C ; $>97\%$ at 22°C , and 100% at 4°C .

FIGURE 3 The relation between insulin concentration and degradation velocity by luminal and basolateral membranes (protein concentration: 0.4 mg/ml), as determined by the TCA method; results with immunoprecipitation (not shown) were essentially identical. Although saturation is not reached even at pharmacologic concentrations of the hormone (left panel, log scale), the relationship is not linear (right panel, linear scale).

longed incubation necessary for binding studies and to minimize its nonspecific adsorption to assay tubes. Specific binding of insulin by both membranes reached equilibrium between 60 and 90 min, and subsequent incubations were therefore terminated after 90 min. In contrast to degradation, the basolateral membranes bound considerably more insulin than the luminal preparations: results from all experiments (labeled insulin 0.12–0.21 nM) show binding of $20.5 \pm 1.0\%$ or 17.5 ± 1.3 fmol/mg to BL ($n = 11$) and only $4.8 \pm 0.3\%$ or 4.5 ± 0.4 fmol/mg for L ($n = 8$), both differences being significant at the <0.001 level. This could not be attributed to differences in insulin degradation velocity by the two membranes because degradation was minimal ($<3\%$), and binding results are expressed as percentage of intact insulin bound after correction for degradation.

To determine the specificity of insulin binding, increasing amounts (final concentrations of 0.1 nM to 10 μ M) of unlabeled insulin were added in competition experiments to the incubation medium that contained ~ 0.1 nM labeled hormone. Displacement patterns were similar (Fig. 4), and the concentration of unlabeled insulin necessary for 50% displacement of the labeled hormone bound was ~ 3 nM for the two preparations, despite the marked differences in their binding capacity. Furthermore, when excess unlabeled hormone (10 μ M) was added after reaching binding equilibrium, bound ^{125}I -insulin was displaced in a similar fashion in both membrane fractions (Fig. 5).

The specificity of insulin binding was also examined by adding increasing amounts (final concentration 0.1 nM–10 μ M) of insulin analogues (proinsulin and desoctapeptide insulin) to the incubation mixture. Results from these competition experiments, performed with basolateral membranes, are shown in Fig. 6. Displacement of labeled insulin (0.1 nM) was most effectively accomplished by the native hormone itself, while proinsulin and desoctapeptide insulin were substantially less effective. The ability of these peptides to compete for insulin binding sites paralleled their *in vitro* insulin-like biologic activity (29).

Table III summarizes data from competition experiments in which results of control incubations using 0.1 nM labeled insulin alone (expressed as 100% binding) were compared with those obtained in the presence of a large excess (1 or 10 μ M) of unlabeled insulin, insulin analogues, and peptides unrelated to insulin. Only insulin and proinsulin competed effectively with the labeled hormone for binding sites. Desoctapeptide insulin produced only a partial displacement, whereas other hormones were ineffective despite the supra-maximal concentrations used. It is noteworthy that the two membrane preparations behaved similarly in these competition experiments as well.

The kinetic analysis of insulin binding to basolateral membranes is shown in Fig. 7. The Scatchard plot including all data points reveals a curvilinear relationship between the ratio of bound/free insulin and the amount of hormone bound, as has been uniformly ob-

FIGURE 4 Displacement of labeled insulin (0.12–0.21 nM) bound to luminal and basolateral membranes by increasing amounts (0.1 nM–10 μ M) of unlabeled insulin. Displacement patterns for luminal (○) and basolateral (●) membranes are superimposable, suggesting similar receptor affinity for insulin. Each point represents the mean of two or three duplicate determinations performed with membranes from five animals.

FIGURE 5 Dissociation of bound ^{125}I -insulin by excess (10 μ M) unlabeled hormone. Pattern of dissociation was similar in luminal and basolateral membranes. Results are mean of duplicate determinations from two animals.

served for insulin binding to a variety of tissues by other investigators. The data are thus compatible with three or more classes of binding sites with different affinities and binding capacities for insulin, or with interactions between receptor sites consistent with negative cooperativity (30, 31). A linear estimate of “low affinity” sites was derived by fitting a straight line (A) to points corresponding to total insulin concentrations of 10 to 1,000 ng/ml (1.7 nM–0.17 μ M). For the “high affinity” line (B), bound insulin points below those used in the low affinity estimate were replotted against their respective B/F ratios, corrected by subtracting the B/F ratios contributed by the low affinity line at each point. The apparent dissociation constants for the “low” and “high affinity” sites were 35 and 2.4 nM, respectively. Using only data from experiments with labeled insulin, i.e., with the lowest concentrations of

FIGURE 6 Displacement of labeled insulin (0.12–0.18 nM) bound to basolateral membranes by increasing amounts (0.1 nM–10 μ M) of unlabeled insulin, proinsulin and desoctapeptide insulin. Labeled insulin is displaced most by the native hormone itself, while proinsulin and desoctapeptide insulin are substantially less effective. Each point represents the mean of two duplicate determinations performed with membranes from two animals.

TABLE III
Inhibition of ^{125}I -Insulin Binding by Unlabeled Insulin, Insulin Analogues, and Peptide Hormones Unrelated to Insulin

	Basolateral	Luminal
Residual binding, % control*		
Insulin, 1 μM	14.7 $\pm$ 1.4 (4)	10.6 $\pm$ 6.1 (4)
Insulin, 10 μM	8.7 $\pm$ 1.7 (10)	16.7 $\pm$ 3.5 (7)
Proinsulin, 1 μM	15.9 $\pm$ 1.1 (3)	21.3 $\pm$ 3.0 (4)
Proinsulin, 10 μM	7.8 $\pm$ 0.6 (3)	—
Desocta-insulin, 1 μM	59.9 $\pm$ 1.1 (3)	68.0
Desocta-insulin, 10 μM	28.3 $\pm$ 3.6 (3)	—
1-34 PTH, 1 μM	98.3	91.8
1-34 PTH, 10 μM	90.8	—
rFSH, 1 μM	102	85.2
rFSH, 10 μM	87.9	—
Glucagon, 1 μM	107.3	103.3
Glucagon, 10 μM	93.0	—

* Data are expressed in percentage of control values from incubations with 0.17 nM ^{125}I -insulin in the absence of unlabeled insulin, insulin analogues, or other hormones. Digits in parentheses show number of observations.

the hormone (Fig. 7, open circles and inset), one can demonstrate a class of binding sites with an even higher affinity for insulin (apparent K_D , 0.34 nM).

The relation between insulin concentration and the absolute amounts of insulin bound per milligram membrane protein is presented in Fig. 8. Insulin concentrations were varied between 3 pM and 10 μM . Even though the latter concentrations exceeded many times levels obtainable even after pharmacologic doses of the hormone, saturation of insulin binding could not be demonstrated for either membrane (left panel). However, analysis of results from experiments in which physiologic concentrations of the hormone were used (right panel) reveals a degree of saturation because this relationship is not linear. These findings are in agreement with those of other investigators who observed both saturable and nonsaturable components in the binding of insulin to a variety of tissues [reviewed by Terris and Steiner (27)].

DISCUSSION

Insulin uptake and inactivation by peritubular mechanisms has been hitherto controversial and evidence favoring its occurrence was largely indirect. Studies based on electron microscopic autoradiography and tubule microperfusion were interpreted to indicate negligible participation of the contraluminal uptake route in the renal metabolism of insulin (18). On the other hand, measurement of renal-organ clearance rates of this hormone in humans, dogs, and rats in vivo

FIGURE 7 Scatchard analysis of insulin binding to basolateral membranes. The plot including all data points reveals a curvilinear relation between bound/free hormone (ordinate) and hormone bound (abscissa), which is compatible with three or more classes of binding sites with different affinities and binding capacities for insulin. Open symbols and inset represent experiments with labeled hormone only, which illustrate the properties of the high affinity binding sites (see text). Experiments were performed with membranes purified from three animals.

FIGURE 8 The relation between insulin concentration and the absolute amount of insulin bound to basolateral and luminal membranes. Although saturation could not be demonstrated even at pharmacologic concentrations of the hormone (left panel, log scale), binding to basolateral membranes at low insulin concentrations is not linear (right panel, linear scale). Experiments were performed with membranes purified from three animals.

(1, 10–12), studies with isolated perfused kidneys (13, 14), and experiments in which insulin was injected into the renal portal circulation of the chicken (15) suggested that the renal handling of this hormone involves in addition to filtration and luminal uptake, also its catabolism by peritubular mechanisms. Duckworth (32) and Blanchard et al. (33) have demonstrated binding and degradation of insulin by kidney cell membranes, but no distinction was made between luminal and antiluminal fractions. In our study we evaluated the interaction of insulin with the two components of the renal tubular cell membrane, and demonstrated that both basolateral and luminal membranes purified from rabbit kidney cortex degrade insulin avidly and contain specific binding sites for this hormone. Our observations provide, therefore, direct evidence for the notion that the contraluminal membrane participates in the renal degradation of insulin, and suggest that this membrane plays an important role in the mediation of the insulin signal to the renal tubular cell.

The insulin-degrading activity of either tubular membrane could not be attributed to their contamination with other cell constituents because enzyme markers for mitochondria (succinate dehydrogenase), microsomes (glucose-6-phosphatase), and lysosomes (acid phosphatase) remained at or below their level in the original homogenate (Fig. 1). Additional evidence against the contribution of lysosomal peptidases to the insulin-degrading activity of the two membranes is provided by the observation that the pH optimum for

insulin degradation in purified luminal and basolateral membranes was several pH units higher than that of the original homogenate containing lysosomes.

Degradation of insulin by both membranes proceeded without evidence of saturation, consistent with *in vivo* and *in vitro* observations by others that insulin degradation by the renal parenchyma fails to reach saturation even at pharmacologic insulin concentrations (1, 10, 12, 14). Although brush border membranes were more active in this respect, substantial insulin-degrading activity was also demonstrated in the contraluminal membrane fraction (Figs. 2 and 3). Moreover, binding capacity for insulin was several-fold higher in basolateral than in luminal membranes, indicating that cross contamination could not account for our observations. These findings provide therefore direct evidence for degradation of insulin by the contraluminal membrane of renal tubular cells.

Results of competition experiments (Table I) suggest that the insulin-degrading activity consists of both insulin specific and nonspecific components because other hormones, structurally unrelated to insulin, were also capable of reducing insulin degradation. Further evidence for heterogeneity of insulin degradation is suggested by the hyperbolic relationship between insulin concentrations and degradation velocity (Fig. 3, right panel), which may reflect the summation of saturable and nonsaturable components in this catabolic process. It is noteworthy that the pattern of inhibition of insulin degradation by excess concentrations of an-

alogues or unrelated peptides (Table I), as well as the effect of various peptidase inhibitors (Table II), were remarkably similar in the two membrane preparations.

In contrast to degradation, basolateral membranes bound considerably more insulin per milligram protein than the luminal preparations. However, the patterns of insulin displacement by increasing amounts of unlabeled hormone (Fig. 4) or by addition of excess unlabeled insulin after binding equilibrium was reached (Fig. 5) were similar or superimposable, suggesting that the receptor affinity for insulin was comparable in the two-membrane fractions. To verify that insulin is bound to membrane rather than contained inside the vesicles, we measured binding to membranes pretreated with 5 mM acetic acid which "opens" the vesicles (34): electron micrographs revealed that vesicles treated with acetic acid were empty and binding to both basolateral and luminal membranes (22.4 and 4.9%/mg protein, respectively) was unaltered by this procedure.

The specificity of insulin binding was examined in competition experiments using increasing concentrations of unlabeled insulin or its analogues (Fig. 6). As expected, displacement of labeled insulin was most effectively accomplished by the native hormone itself, while proinsulin and desoctapeptide insulin were substantially less effective. The ability of these peptides to compete for insulin binding sites paralleled their *in vitro* insulin-like biologic activity (29). Further evidence suggesting that the binding of insulin to renal tubular cell membranes is specific was provided by experiments in which we assessed the effect of a large excess (1 and 10 μ M) of unlabeled insulin, insulin analogues, or unrelated peptide hormone on the binding of physiologic concentration (0.1 nM) of labeled insulin (Table III). Only insulin and proinsulin competed effectively with the labeled hormone for binding sites, while the other peptides were either ineffective or displaced insulin incompletely despite the very large concentrations used.

The demonstration of specific binding sites for insulin along the basolateral membrane of the renal tubular cell raises the obvious question about their function and biologic significance. Because the properties of these sites (Fig. 7) are similar to those of insulin receptors observed by others in a variety of insulin target tissues (reviewed in 35) it is probable that the insulin binding sites we describe are also true receptors. Insulin is thought to modulate tubular transport processes (2, 3, 5) and to alter renal metabolism (4, 5, 7), and it is likely that these processes require binding of insulin to specific renal tubular cell receptors, usually associated with the basolateral membrane; this membrane is known to contain specific receptors for other peptide hormones with renal action (e.g., PTH

and vasopressin), and recent evidence suggests that it participates in the catabolism of these peptides (36, 37). In contrast to PTH and vasopressin, however, the second messenger involved in the transduction of the insulin signal to the tubular cell is unknown, and it is not presently possible to monitor insulin-mediated postreceptor events in isolated membrane preparations.

It has been proposed that in a variety of tissues insulin binding may be linked to its degradation (27), but this notion remains controversial (38, 39). If such a link does indeed exist it is possible that at the basolateral membrane insulin binding and degradation are coupled, the latter performing a regulatory function by terminating the insulin signal on the renal cell. Degradation of insulin on the basolateral cell membrane would also explain the lack of label accumulation in tubular cells reported by Bourdeau et al. (18).

The aim of the present study was to define the interaction of insulin with different components of the renal tubular cell membrane, because the polar orientation of these cells imparts distinct functional properties to the luminal and basolateral aspects of their plasma membrane. The experiments reported herein provide direct evidence that insulin handling by the kidney involves binding and degradation of the hormone at the peritubular cell membrane. Additional studies using intact cells, ideally at the single nephron level, should add important information on the many facets of the relationship between insulin and the kidney.

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