Insulin uptake across the luminal membrane of the rat proximal tubule in vivo and in vitro

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Kolman P, Pica A, Carvou N, Boyde A, Cockcroft S, Loesch A, Pizze A, Simeoni M, Capasso G, Unwin RJ. Insulin uptake across the luminal membrane of the rat proximal tubule in vivo and in vitro. Am J Physiol Renal Physiol 296: F1227–F1237, 2009. First published March 4, 2009; doi:10.1152/ajprenal.90351.2008.—We visualized insulin uptake in vivo across the apical membrane of the rat proximal tubule (PT) by confocal microscopy; we compared it with in vitro findings in a PT cell line (WKPT) using fluorescence microscopy and flow cytometry. Surface tubules were observed in vivo with a 633-nm single laser-illuminated real-time video-rate confocal scanning microscope in upright configuration for optical sectioning below the renal capsule. Fields were selected containing proximal and distal tubules; Cy5-labeled insulin was injected twice (the second time after ~140 min) into the right jugular vein, and the fluorescence signal (at 650–670 nm) was recorded. Fluorescence was detected almost immediately at the brush-border membrane (BBM) of PT cells only, moving inside cells within 30–40 min. As a measure of insulin uptake, the ratio of the fluorescence signal after the second injection to the first doubled (ratio: 2.11 ± 0.26, mean ± SE, n = 10), indicating a “priming,” or stimulating, effect of insulin on its uptake mechanism at the BBM. This effect did not occur after pretreatment with intravenous lysine (ratio: 1.03 ± 0.07, n = 6; P < 0.01). Cy2- or Cy3-labeled insulin uptake in a PT cell line in vitro was monitored by 488-nm excitation fluorescence microscopy using an inverted microscope. Insulin localized toward the apical membrane of these cells. Semiquantitative analysis of insulin uptake by flow cytometry also demonstrated a priming effect (upregulation) on insulin internalization in the presence of increasing amounts of insulin, as was observed in vivo; moreover, this effect was not seen with, or affected by, the similarly endocytosed ligand β2-glycoprotein.

intravital confocal microscopy; fluorescence; kidney

RENAL HANDLING OF INSULIN has been described previously using radiolabeled insulin combined with histochemical studies after timed injections in vivo (2) or in isolated proximal tubule (PT) perfusion studies in vivo (10) and in vitro (21, 20, 17) and in cultured cells (24). Insulin uptake in rabbit PT has also been monitored by electron microscopy (EM) after perfusion of gold-labeled insulin and subsequent cross-linking to its receptor with disuccinimidylsuberate (18). These studies have shown that insulin binding and uptake occur predominantly at the brush-border membrane (BBM) of PT cells (2, 22), where it is then translocated to endocytic vesicles and vacuoles, and eventually degraded in lysosomes; a small portion (~0.5%) is directly transcytosed to the basolateral membrane (19). However, unlike many other filtered peptides (34), insulin seems not to be degraded by brush-border enzymes (11). The receptors for insulin binding are recycled to the plasma membrane via dense apical tubules, either from small vesicles or from larger endocytic vacuoles; although the early mechanisms involved in insulin binding and internalization in the PT are still unclear.

Higher levels of insulin receptors are expressed on the basolateral membrane of PT cells than on the apical membrane (9, 29), and it is the basolateral receptors, present at other nephron sites (4), that are believed to mediate insulin’s biological effects (32). Thus it was originally proposed that nonspecific charge interactions were responsible for the initial binding of insulin to the BBM (28). However, later studies reported a high-capacity, low-affinity receptor for insulin in the PT (15), which was eventually thought to be the endocytic receptor megalin. Orlando et al. (23), using chemical cross-linking of 125I-labeled insulin to a renal microvillar membrane preparation, identified megalin as the binding protein, which required a 1,000-fold excess of unlabeled insulin to compete for binding; it was also competed with by glucagon. In contrast, RAP (receptor-associated protein), which inhibits binding of other endocytosed ligands to megalin, such as retinol binding protein (RBP) (13), transthyretin (27), and β2-glycoprotein (β2gpI) (16), did not inhibit binding. In the same study (23), uptake of FITC-labeled insulin in L2 yolk sac-derived cells (which are rich in megalin) was inhibited by a 50-fold excess of unlabeled insulin and by anti-megalin IgG, but again not by RAP.

In the present study, we describe a method we have developed for quantitative assessment of the disposition of fluorescently labeled substances filtered at the kidney glomerulus using conventional confocal, rather than multiphoton (8), microscopy in vivo. We chose to investigate insulin because of the available literature for comparison and as a prototypical filtered peptide hormone. We found that insulin’s visible uptake in vivo and in real time is confined to the PT and that it occurs apically, and we made the novel observation that insulin uptake is self-priming and upregulated. This finding was also confirmed in vitro in cultured PT cells and contrasted with the similarly endocytosed ligand β2gpI.
Innovative Methodology

METHODS

Insulin Labeling

Insulin was purchased from Sigma, and β-gpI was provided by Scipac (Sittingbourne, UK). For the in vitro cell culture experiments, insulin was prepared and labeled with Cy2 or Cy3, as previously described (6). For the in vivo study, Cy5 was chosen as a suitable fluorophor, because we were using a 633-nm HeNe laser to curtail mitochondrial autofluorescence (3) (Cy5 is a far red fluorescent dye) and to improve reflection mode imaging. Insulin was labeled with Cy5 according to the manufacturer’s instructions as follows: 1 mg of insulin was mixed with 75 μg of Cy5, and the labeled insulin was separated by size exclusion using a Sephadex G-15 column and then eluted with PBS. Labeling efficiency was estimated to be ~30%.

Confocal Microscopy In Vivo

Male Sprague-Dawley rats (n = 4) weighing ~200 g were maintained on a standard laboratory diet and handled using approved protocols in accordance with institutional and Home Office license regulations. They were anesthetized with sodium thiopentone (100 mg/kg ip; Link Pharmaceuticals, Horsham, UK) and prepared surgically as for micropuncture of the left kidney (5). Rats were infused intravenously via a right jugular venous catheter with a 0.154 M NaCl solution (“normal saline”) at a rate of 2 ml/h. During each experiment, the rat was placed beneath the objective of a confocal microscope (Noran Odyssey video-rate laser scanning confocal unit with a 633-nm laser on a Nikon Optiphot upright microscope) on a specially adapted and thermoregulated stage. We used a 40/1.0 oil-immersion objective lens with glass coverslips sandwiched to a thickness of 425 μm to fill the space between the kidney surface and the objective lens; this also stabilized the kidney surface and minimized transmitted movement from respiration and arterial pulsation. The confocal slit for the fluorescence signal was set at 100 μm. In those experiments in which the rats were preinfused with lysine, a 10% solution in 0.154 M NaCl was started ~30 min before the first insulin injection and continued throughout the experiment at dose of 200 mg/kg body wt, according to the protocol of Tucker et al. (33).

Image recording and analysis. Insulin uptake was rapid and suitable for imaging renal tubules at video rate. The Noran Odyssey video-rate laser scanning confocal unit has standard video outputs, one for reflection mode, another for fluorescence, and one for the mixed signal (not used in this study). We were not able to record both channels simultaneously. The signal was recorded using a SONY digital camcorder. We used a microswitch controlled by the left knee of the operator to select the reflection or fluorescence imaging modes. The time delay between the captured reflection and fluorescence images was 1 or 2/25 of a second. The image proved stable enough to treat the two frames as coming from the same place.

Cy5-insulin was injected into the jugular vein catheter (0.1 mg/kg body wt) in a volume of 0.1 ml (although we also did some experiments using a femoral artery cannula in the aorta and placed just above the left renal artery). Two consecutive injections were given at an interval of ~140 min. The reflection and fluorescence images (Fig. 1, A and C, respectively) were “grabbed” from the short video sequences using Pinnacle Studio software (Pinnacle Systems; Avid Technology, Daly City, CA). The average time interval between repeated recording sequences was ~10 min; the format of the grabbed image frames was 640 × 480 pixels at 8 bits/pixel. Recordings were made from several tubules (in different fields) during each experiment. Once we had captured the images, we used JASC Paint Shop Pro 5 (Corel, Ottawa, ON, Canada), with its freehand smart edge selection tool, to select from the reflection image (Fig. 1A) the tubule segment to be analyzed, filling in the cells, including their white reflective (negative black in Fig. 1, A–D) brush border, to create a binary mask. (The images shown in Fig. 1, A–D, are cropped free of artifacts.) For further analysis, we wrote a procedure in MATLAB software (The MathWorks, Natick, MA). The fluorescence intensity image (Fig. 1C) was transformed to an insulin pseudoconcentration map with the use of a calibration function determined from calibration measurements. The mask was then divided into equal-width strips of ~0.85 μm (Fig. 1B); it was applied to monitor the spatial distribution of insulin in the tubular cells of the selected PT segment by calculating the averaged concentration in each strip separately. The concentration...
values were corrected by using another calibration function to compensate for the effect of photobleaching.

Figure 2 illustrates the output of the MATLAB program in a representative control and lysine experiment. Figure 2, Ai and Bi, shows the temporospatial concentration of insulin inside proximal tubular cells. The origin of the spatial axis corresponds to the inner border of the tubule (the apical brush border). Figure 2, Aii–Aiii and Bii–Biii, shows the linear uptake and rate of uptake, respectively, after the first and second injections of labeled insulin; insulin values are in pseudoconcentration units (see calibration below).

**Calibration of the fluorescence signal.** We found that the intensity distribution in fluorescence mode across the field of view was not constant and that a calibration procedure had to be performed to correct for this, again using MATLAB. Calibration of Cy5-labeled insulin concentration was performed in vitro with Cy5. We used a special bacteriological chamber with a depth of 10 μm (Weber Scientific, Trenton, NJ). A series of solutions with different Cy5-insulin concentrations was then measured. The image data taken as a short video sequence were averaged using MATLAB software, and the resulting image was smoothed in Paint Shop Pro 5 using a Gaussian blur filter (radius 3). However, we found that the intensity of fluorescence for calibration measurements of high concentrations (~0.26 mg/ml) of Cy5-insulin varied by as much as 50%, probably because of significant evaporation from a small sample volume on a relatively large surface area. Therefore, it was not possible to calibrate and transform the intensity values recorded into real concentration values for insulin. The intensity values were in the range of 0 to 255, with a value of 16 for the blank (water). Thus pseudoconcentrations were derived by assuming a linear relationship between a given mean fluorescence intensity in the image (I) and Cy5-insulin concentration (C) equal to 1/255 × (I – 16).

**Correction for bleaching.** After each Cy5-insulin injection, tubules were imaged approximately every 10 min, and the duration of irradiation in every measurement was ~10 s. This gave a total of ~5 min of irradiation in any one region so that the effect of photobleaching could not be ignored. We determined experimentally how the fluorescence intensity dropped on bleaching the labeled insulin in renal tubular cells. The concentration values calculated from the fluorescence images were corrected using these data (see Fig. 3).

**Insulin Localization by EM in Kidney Tissue at ~1 and ~45 Min After Insulin Injection**

**Tissue preparation.** For ultrastructural study, kidneys were perfused through the renal artery at 1 min and 30–45 min after the insulin injection with 5 ml of normal saline, followed by 10 ml of fixative, consisting of 4% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The kidneys were then dissected out and immersed in the same fixative for 5 h (at 4°C), followed by washing with phosphate buffer and 0.1 M Tris-buffered saline (TBS; pH 7.6). Sections (60–70 μm thick) were cut through the kidney on a vibratome and collected in TBS. These were then processed with insulin antibody by using a preembedding immunogold-silver labeling technique for EM (see below).

**Immunogold-silver labeling.** To improve immunoreactivity, the vibratome sections were infiltrated for 45 min with cryoprotectant, consisting of 25% sucrose and 10% glycerol in phosphate buffer, before being immersed in liquid nitrogen-cooled isopentane and then...
in liquid nitrogen for ~10 s and were then thawed in cryoprotectant before being washed in TBS and placed for 1 h at room temperature in 10% nonimmune normal goat serum (British BioCell International, Cardiff, UK) diluted in TBS containing 0.1% sodium azide. Sections were then 1) exposed for 20 h at 4°C to a mouse monoclonal insulin antibody (see below for details) at a concentration of 0.3 μg/ml TBS with 0.1% sodium azide, 2) washed 3 times for 10 min each in TBS, 3) incubated for 20 h at 4°C with a goat anti-mouse IgG:1-nm gold conjugate (GAM1; British BioCell International) diluted 1:100 in TBS with sodium azide, 4) washed in TBS and then in distilled water, 5) fixed for 10 min with 1% glutaraldehyde, 6) washed 10 times for 5 min with deionized distilled water, and 7) subjected to augmented gold labeling for 10 min with a silver enhancement kit (British BioCell International). The specimens were then washed with distilled water and sodium cacodylate buffer (0.1 M), treated with 1% osmium tetroxide for 10 min, and dehydrated in progressively higher concentrations of ethanol followed by propylene oxide. The specimens were embedded flat in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and subsequently examined with a JEM-1010 transmission electron microscope (TEM).

**Immunocytochemical controls.** The insulin antibody (2D11-H5) used in this study was a mouse monoclonal IgG1 antibody corresponding to amino acids 1-84, representing full-length porcine insulin (Santa Cruz Biotechnology, Santa Cruz, CA). According to the supplier, this antibody reacts with insulin of human, porcine, and bovine origin by immunohistochemistry, immunoprecipitation, and ELISA. In the present study, the specificity of immunolabeling was investigated routinely by omission of the insulin antibody and IgG steps, as well as by replacement of primary antibody with nonimmune normal goat serum.

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Fig. 3. Derivation of the bleaching correction. The curve shows the pseudoconcentration of Cy5-insulin as a function of time under constant conditions of laser irradiation (see text for details). ΔC, change in concentration (increment); ΔT, duration of irradiation.

Fig. 4. Electron micrograph of insulin binding and uptake into cells of the PT detected by an immunogold-silver labeling method at 1–2 min (A) and 30–45 min (B) after insulin injection and under control conditions (C). A: at 1–2 min, insulin immunogold-silver grains (arrows) localize to the brush border. N, cell nucleus; Mv, brush-border microvilli; Cap, a peritubular capillary. B: insulin immunogold-silver grains are localized in the cytoplasm (arrows) and in an apical vacuole/multivesicular body (mvb) (at ~45 min postinjection). C: no insulin immunogold-silver grains were seen when the primary antibody step was omitted or in control tissue (not shown). Bar, 1 μm.
Cell Culture

The WKPT 9302 Cl.2 cell line was a gift from Prof. F. Thévenod (Witten, Germany) and cultured as described previously (31). This epithelial cell line is derived from the early segment (S1) of the PT of normotensive Wistar-Kyoto rats and transduced with the onco-gene SV40 large T-antigen (35); it forms confluent, electrically resistive polarized monolayers expressing apical microvilli, tight junctions, and convolutions of the basolateral plasma membrane. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s nutrient mixture F-12 (1:1; Sigma) supplemented with 10% bovine calf serum, 1.2 mg/ml NaHCO3, 5 μg/ml insulin, 4 μg/ml apotransferrin, 10 ng/ml EGF, and 100 U/ml penicillin and streptomycin. Cells were grown in 75-cm2 flasks at 37°C with 5.0% CO2 and split 1/10 or 1/20 three times a week.

In Vitro Endocytosis Assay

WKPT cells were plated onto 13-mm-diameter glass coverslips placed in 16-mm-diameter wells (24-well plate) and washed twice in warm HEPES buffer (20 mM HEPES, 137 mM NaCl, 3.0 mM KCl, 2.0 mM MgCl2, 1.0 mM CaCl2, and 1 mg/ml glucose, 37°C). The Cy-labeled ligand (1–5 μM) was added to the cells (250 μl/well) and incubated for 2 h at 37°C. The cells were incubated in 0.2 M acetic acid and 0.5 M NaCl for 5 min at room temperature to remove any residual extracellular fluorescence, washed four times with PBS, and either fixed in 4.0% formaldehyde for 10 min at room temperature for analysis by confocal microscopy or detached in trypsin-EDTA for analysis by flow cytometry. In competition experiments, the cells were preincubated with unlabeled insulin or β2gpI for 10 min at 37°C before the Cy2-labeled ligand was added. In priming experiments, the cells were incubated at 37°C for 60 min with unlabeled insulin or unlabeled β2gpI and washed four times with HEPES buffer before incubation with labeled ligand.

Confocal Microscopy In Vitro

After the fixed cells were washed three times in PBS, the coverslips were mounted onto a 10-μl drop of Mowiol overnight at 4°C. Cell fluorescence was recorded by excitation at 488 nm with an Omnichrome series 43 (PerkinElmer, Milan, Italy) argon ion laser system using an Olympus IX70 microscope fitted with a ×100 oil-immersion objective, unless stated otherwise. Images were acquired with a charge-coupled device camera (PerkinElmer) cooled to ~35°C and controlled with the Ultraview 4.0 software (PerkinElmer).

Analysis by Flow Cytometry

After 2 h of incubation with fluorescent ligand, the WKPT cells were washed four times with ice-cold HEPES buffer supplemented with 1 mM EDTA. Cells were then incubated on ice with HEPES buffer plus 10 mM EDTA for 10 min and detached with trypsin-EDTA at 37°C for 5–15 min. The trypsin was then quenched by addition of WKPT culture medium, and the cells were pelleted by centrifugation at 1,300 rpm for 5 min. The cell pellets were resuspended in ice-cold HEPES buffer plus 1 mM EDTA and kept on ice until ready for analysis by flow cytometry. At least 10,000 cells per data point were analyzed for fluorescence on an EPICS Elite Flow cytometer (Beckman-Coulter, High Wycombe, UK). A gating strategy was employed that excluded cell aggregates and high-scatter cell events judged to be nonviable cells. The mean cell fluorescence was recorded for each data point. The data were then processed with Summit v3.1 software (Cytomation, Fort Collins, CO). Histogram charts were produced for each sample, and the mean fluorescence was resolved.

Statistics

All statistical comparisons were made using nonparametric Kruskal-Wallis one-way ANOVA and/or unpaired Mann-Whitney tests; P < 0.05 was considered significant.

Fig. 5. Absence of competition between insulin and β2-glycoprotein (β2gpI) uptake. WKPT cells were incubated with 1 μM Cy2-labeled β2gpI (A, a–c) or Cy2-labeled insulin (B, d–f) for 2 h at 37°C in the presence of either 10 μM unlabeled β2gpI (b and e) or 10 μM unlabeled insulin (c and f). For the control (a and d), no unlabeled ligand was added. Uptake of insulin and β2gpI was assessed by confocal microscopy.
RESULTS

In Vivo Confocal Microscopy

Descriptive analysis. As we (3) reported previously using the Noran Odyssey confocal microscope, the reflection mode image showed details of capillary blood flow in identifiable and distinguishable proximal and distal tubules: PTs were recognized by their intensely bright (light scattering) brush border, especially early PT segments (Fig. 1A; see Supplemental Video); erythrocytes were strongly reflective, and white cells were recognizable by their slower motion, adhering to capillary walls and some even marginating (3, 26). (Supplemental data for this article is available online at the American Journal of Physiology-Renal Physiology website.) After the injection of free Cy5, fluorescence was evident in the lumen of distal tubules within 1 min, but no uptake of fluorescence was seen in either the proximal or distal tubules (not shown).

After injection of Cy5-insulin, fluorescence appeared in peritubular capillaries within 0.5 min; soon afterward, fluorescence was seen at the PT brush border, although fluorescence intensity and distribution were variable (see Supplemental Video). By ~20–30 min, there was an increase in intracellular fluorescence with a punctate pattern distributed toward the basolateral membrane (Fig. 1, C and D; see Supplemental Video). No fluorescence was detectable in the distal tubules (Fig. 1, C and D; see Supplemental Video). In each recording in fluorescence mode, before injection of Cy5-insulin, there was no tubular autofluorescence; after Cy5-insulin, there were no changes in the reflection mode images.

Quantitative analysis. Quantitative analysis of the intracellular fluorescence signal after Cy5-insulin injection (Fig. 2) showed an increase in intracellular fluorescence intensity up to ~20 min, when it had almost reached a steady state. However, after the second injection of insulin, uptake of insulin seemed to be enhanced in magnitude (Fig. 2Aii) and rate (Fig. 2Aiii). The mean ratio for the change in concentration, as a measure of insulin uptake, after the second injection (second/first) was 2.11 ± 0.26 (mean ± SE). After pretreatment with lysine, this enhancement, or priming effect, was abolished with a ratio of 1.03 ± 0.07 (Fig. 2, Bii and Biii). These ratios were significantly different when compared using the nonparametric Mann-Whitney test (two-tailed exact test, P < 0.01) with median values of 2.03 vs. 0.98, respectively.

EM of insulin immunolabeling. EM examination of rat kidney tissue after injection of insulin showed localization of insulin at 1 and 45 min. Insulin immunolabeling was evident in PT cells: 1 min after insulin injection, label was detected predominantly along microvilli of the brush border of

Fig. 6. Analysis of insulin uptake by flow cytometry. WKPT cells were incubated with 5 μM Cy2-labeled insulin for 2 h at 37°C in the presence of 0, 20, 50, 100, or 200 μM unlabeled insulin. A: flow cytometry results. The shaded area shows the distribution for the no-ligand negative control (−ve ctrl), and the hatched area shows the distribution for the sample. B: mean fluorescence of the cells analyzed by flow cytometry. Values are means ± SE (n = 3).

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proximal tubular cells (Fig. 4A); after 45 min, there was mainly intracellular localization (Fig. 4B). Label was present throughout the cytoplasm and also in endocytic vesicles and/or multivesicular bodies (Fig. 4B). No immunolabeling was detected in control sections (Fig. 4C), when no insulin was injected or insulin was injected without incubation with anti-insulin antibody, or when the primary antibody step was omitted in sections from insulin-injected animals.

A: fluorescence-activated cell sorting (FACS) results. The shaded area shows the distribution for the no-ligand negative control, and the hatched area shows the distribution for the sample. B: mean fluorescence of the cells analyzed by FACS. Values are means ± SE (n = 3).

Fig. 8. L-Lysine does not affect insulin uptake in vitro. Confluent WKPT cells were incubated with 5 μM Cy2-insulin for 2 h at 37°C in presence of 40 μM unlabeled insulin, 40 μM β2gpI, or 40 μM L-lysine. Uptake of Cy2-insulin was assessed by confocal microscopy. A: confocal visualization of endocytosed Cy2-insulin (×40 objective). +ve ctrl, positive control. B: histogram showing calculated mean fluorescence for 5 random fields. Values are means ± SE (n = 5).
In Vitro Cell Line Experiments

We (6) have shown previously that WKPT cells endocytose insulin and β2gpI, that uptake is temperature dependent, and that these two proteins do not colocalize. To confirm that insulin and β2gpI do not compete for uptake, we incubated Cy2-β2gpI in the presence of excess unlabeled insulin or β2gpI (Fig. 5A, a–c). A 10-fold molar excess of β2gpI blocked Cy2-β2gpI endocytosis (Fig. 5Ab), whereas the same concentration of insulin did not affect Cy2-β2gpI uptake (Fig. 5Ac). In the reciprocal experiment, a 10-fold molar excess of β2gpI did not block Cy2-insulin uptake (Fig. 5B, d vs. e). A 10-fold molar excess of insulin did not block uptake of Cy2-insulin, but appeared to enhance it (Fig. 5B, d vs. f). These results indicate that insulin and β2gpI are endocytosed independently and bind to different sites on the surface of WKPT cells. In separate experiments, we had examined whether their endocytic compartments could be differentiated based on staining with EEA1, a marker for early endosomes. We found that EEA1 partially colocalizes with Cy2-β2gpI but not with Cy2-insulin (6).

Analysis by flow cytometry was used to quantify the uptake of 5 μM Cy2-insulin in the presence of increasing amounts of unlabeled insulin (Fig. 6). The mean fluorescence of the sorted cells increased in a linear manner and nearly doubled from 23.8 ± 1.19 arbitrary units (AU) when no unlabeled insulin was added, to 51.0 ± 2.44 AU with 100 μM unlabeled insulin, before reaching a plateau with 200 μM unlabeled insulin (50.5 ± 0.93 AU). The opposite trend was observed with β2gpI (Fig. 7): in cells exposed to 1 μM Cy2-β2gpI for 2 h, the mean fluorescence decreased from 13.3 ± 0.03 AU to the control value (4.6 ± 0.47 AU) in the presence of 10 μM β2gpI.

L-Lysine did not seem to affect insulin uptake in vivo (although it did prevent the priming effect), so we tested its effect in vitro (Fig. 8). Again, addition of 40 μM unlabeled insulin significantly enhanced Cy2-insulin uptake compared with the positive control; however, neither 40 μM β2gpI (as expected) nor 40 μM l-lysine affected insulin uptake (although we were unable to test the priming effect in the same way we did in vivo).

In another set of experiments, WKPT cells were primed with increasing amounts of insulin for 1 h, washed, and then incubated with 5 μM Cy2-insulin. After 2 h of incubation, insulin uptake was measured in the insulin-primed cells: Cy2-insulin uptake was enhanced (Fig. 9). Together, these results suggest that endocytosis of insulin is regulated by a positive feedback mechanism, possibly involving the upregulation of its endocytic receptor. This is in contrast to β2gpI uptake, where excess β2gpI competes effectively with labeled β2gpI, indicating that the uptake system is saturable.

In L2 cells, which express high levels of megalin, β2gpI binds to megalin and RAP is known to inhibit its uptake (16). Insulin internalization is also mediated by megalin, but RAP has no effect on insulin uptake in L2 cells (13). To study the effect of RAP on insulin and β2gpI endocytosis in WKPT...
cells, we monitored the uptake of Cy2-insulin or Cy2-β2gpI in the presence of various concentrations of RAP (Fig. 10). RAP integrity was tested by SDS-PAGE (Fig. 10A), and because BSA was present in the RAP buffer, a BSA control was used in the uptake assay (Fig. 10, B and C. +BSA). RAP did not inhibit insulin uptake (Fig. 10B) or β2gpI internalization, which is at odds with findings in L2 cells and suggests that in WKPT cells, β2gpI internalization is not mediated by megalin.

DISCUSSION

In Vivo Experiments

An important aim of our study was to devise a method for quantifying labeled insulin uptake in renal tubules in vivo by using conventional single-laser confocal microscopy and to see whether we could inhibit it and/or detect any change in uptake. Video-rate, long-wavelength confocal microscopy is a powerful technique for this purpose because of its optical sectioning property at increased depth within the intact functioning kidney in both reflection and fluorescence modes. Renal tubule morphology is readily discriminated in reflection mode, and insulin labeled with a fluorescent dye is observable in fluorescence mode. When capturing a set of images and analyzing them by computer, we can get a spatiotemporal distribution of insulin molecules in renal tubular cells.

In previous work exploiting the advantages of real-time video-rate confocal scanning light microscopy, we used a Noran Odyssey scanner coupled to an upright Nikon microscope to study subcapsular layers in the rat kidney (3, 26). The preparation of the animal for kidney imaging is as for renal micropuncture experiments. In the original configuration (3) using an argon ion laser and the dominant 488-nm laser line, we identified proximal and distal tubule segments by their morphology, and we imaged capillary flow in reflection mode and mitochondrial autofluorescence in the yellow-orange spectral range. A number of fluorescent substances were used to visualize capillary plasma, highlighting red cells in negative contrast, prelabeled white cells, and passage into and transit through the tubular lumen (3). The strong mitochondrial autofluorescence in the yellow-orange spectral range limited the utility of the fluorescence approach with dyes excited by the 488-nm laser line (3). Therefore we modified the instrument by fitting a 633-nm HeNe laser with the expectation that the reduction in light scattering at the longer wavelength would give a useful improvement in reflection mode imaging, which we used to study diuretic-induced changes in renal tubular diameter (26). A similar in vivo approach also has been described, though using dual-photon microscopy (8), which has some advantages (particularly the ability to use more than one fluorophor), although it is more costly and less widely available. This technique has also been widely described (25), and usually without parallel or complementary in vitro studies, although attempts have been made to make more quantitative analyses of glomerular filtration and permeability (12, 36).

In the present study, we have shown that we can visualize native Cy5 and Cy5-labeled substances introduced into the circulation and monitor their immediate binding, rapid uptake, and passage through the renal tubule in real time, in vivo. Indeed, this pattern in the PT was confirmed on immuno-EM of kidney sections taken at early and late time points after insulin injection. However, we were faced with the challenge of quantifying the fluorescence signal to measure uptake of fluorescent label. Difficulties arose because of 1) the nonunifor-

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**Fig. 10.** Receptor-associated protein (RAP) does not inhibit uptake of either insulin or β2gpI. A: analysis of recombinant RAP (0.5, 1.0, and 2.0 μg) by SDS-PAGE. BSA samples (0.5, 1.0, 2.0, and 3.0 μg) were loaded as a control, since BSA was present in the RAP. RAP (40 kDa) is indicated on the gel. B and C: confluent WKPT cells were preincubated with 0.2, 0.5, or 1.0 μM RAP for 1 h at 37°C, and 5 μM Cy2-β2gpI (B) or Cy2-insulin (C) was subsequently added to the cells and incubated for 2 h at 37°C. The fluorescence of the cells was measured by confocal microscopy. For each condition, the mean fluorescence for 10 random fields was quantified. Values are means ± SE (n = 10).
nity of fluorescence excitation within the field of view, which we solved by calibrating every pixel address separately; and 2) a longer-term bleaching effect for bound Cy5 label, solved by knowing the irradiation time for each field and an empirically determined bleaching rate, and by applying an appropriate correction. On the basis of our solutions, we have been able to show that the method can be used for relative quantification of physiological measurement of fluorescent label uptake in vivo.

Previous studies have shown that L-lysine can inhibit tubular reabsorption of filtered peptides (14), including albumin (33); lysine was also recently shown to alter endocytic receptor trafficking (30). We tested lysine’s effect on insulin uptake by infusing L-lysine intravenously for 90 min, followed immediately by a first injection of labeled insulin; the second injection followed after 140 min. In control experiments, we found that the first injection of insulin stimulated its uptake following a second injection, a priming effect, and that this effect was blunted after lysine pretreatment.

In one experiment, we did try to combine PT micropuncture with confocal microscopy to visualize the uptake of insulin injected directly into the tubule lumen. Although this approach allowed more accurate dosage, as well as localized delivery of insulin (or any other fluorescently labeled substance), it proved technically demanding because of the short working distance of the high-aperture objective lenses needed for confocal microscopy, which made insertion of the micropipette under the objective difficult (see Supplemental Video).

**In Vitro Experiments**

We have also confirmed in vitro that insulin and β2gpI (which was not tested in vivo) are endocytosed by two pathways in WKPT cells: insulin and β2gpI do not compete for binding and do not colocalize in the same intracellular compartment after uptake; β2gpI endocytosis is constitutive and can be blocked with excess β2gpI, whereas insulin uptake is again upregulated (primed) in the presence of increasing amounts of insulin.

We have shown recently that the uptake of both insulin and β2gpI is mediated by clathrin and that insulin internalization occurs via the adaptor complex AP-2, although AP-2 expression is not required for β2gpI uptake; moreover, uptake of β2gpI is widespread and insulin uptake is predominantly apical (6). The lack of interference of insulin endocytosis by insulin was previously noted in 1980 when 125I-labeled insulin was injected in the whole animal: quantitative autoradiography showed extensive labeling over proximal convoluted tubules, which was not depressed by coinjection of unlabeled insulin (1). Our finding of the effect of insulin appears to relate to the increase in its binding sites: once cells have been sensitized to insulin, insulin can be removed, and the cells still show enhanced uptake. This suggests that insulin can stimulate signal transducing pathways that can mobilize sequestered proteins from the interior of the cell to the plasma membrane for the enhanced uptake of insulin.

Two previous studies in yolk sac L2 cells (which express high levels of the endocytic receptor megalin) reported that β2gpI and insulin were endocytosed by binding to megalin. However, whereas β2gpI uptake was Ca2+ dependent and completely blocked by RAP (16), insulin binding to megalin did not require Ca2+, and insulin uptake was not inhibited by the megalin binding protein RAP (23). In the WKPT cell line, the expression level of megalin is low and similar to that in the LLCPK cell line. RAP did not inhibit endocytosis of insulin or β2gpI, which suggests that uptake of β2gpI and insulin does not depend on megalin. The observation that megalin-dependent uptake is mainly apical, whereas we observed that β2gpI uptake is more widespread, suggests an alternative receptor. Although β2gpI uptake is clathrin mediated, the adaptor is unlikely to be AP-2, since depletion of the μ2-subunit of AP2, AP-50, had no effect on endocytosis; in contrast, insulin uptake is apical and is inhibited by depletion of AP-50 protein (6).

Retinol binding protein (RBP) uptake in vivo is also mediated almost entirely by megalin, as suggested by the absence of RBP-containing granules in the PT of megalin knockout mice (7). In immortalized rat renal proximal tubule (IRPT) cells, RBP transcytosis is also mediated by megalin, but RBP transport to the lysosome and subsequent degradation (55% of internalized RBP) are independent of megalin (13). Therefore, in the IRPT model, other megalin-independent mechanisms of endocytosis must account for RBP degradation: other non-megalin receptors, or fluid-phase pinocytosis, has been suggested.

Finally, we have shown that it is not only possible to image the distribution of filtered fluorescently labeled substances in real time but also that it is feasible to develop methods for relative quantification of the fluorescence signal in vivo; moreover, in the case of insulin, there is a novel priming effect on its uptake in the early PT following initial exposure, although the mechanism of this phenomenon is not apparent from our experiments.

Several questions need to be addressed in future studies. What is the mechanism underlying the priming effect observed with insulin in vivo and in vitro, and does it have any functional significance for the effects of insulin? Does it extend to other filtered proteins or peptides? Does it have any implication for protein handling by the proximal tubule?

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