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Protein endocytosis by a kidney tubule suspension: metabolic requirements

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Abstract

Endocytosis in the renal tubular cell is a permanent process serving the role of saving nitrogen from plasma peptides that are continuously cleared away by kidney glomerulus. Since small proteins appear in urine after strenuous exercise, it was hypothesized that renal ischemia impairs the tubular endocytic reabsorption of proteins. The aim of this paper is to describe a simple in vitro model of renal endocytosis and to use it in studies of endocytic metabolic requirements. The results show that rabbit renal proximal tubules in suspension are able to take up ^{125}I-lysozyme, as well as RITC-lactalbumin. The fluorescent protein was taken up only by the ends of the everted tubule fragments, and accumulated into intracellular vesicles, demonstrating the luminal pathway of endocytosis. The amount of ^{125}I-lysozyme taken up was equivalent to that taken up by isolated perfused tubules (Nielsen et al. (1986) Am. J. Physiol. 251, F822–F830). Anoxia decreased 12-fold the intracellular accumulation of ^{125}I-lysozyme; however, the time-course of inhibition shows that only the late steps of endocytic accumulation are energy-dependent. Substrate deprivation studies suggest a specific role of glucose to sustain endocytosis. Lastly, renal uptake of ^{125}I-lysozyme was shown to be strongly depressed by chloroquine, an alkalinizing agent of endosomes and lysosomes. We conclude that (1) renal tubules in suspension are a satisfactory model for endocytic studies in kidney; (2) suppressing oxygen and substrate supplies to kidney impairs endocytic tubular reabsorption of proteins.

Key words: Endocytosis; Lysozyme; Lactalbumin; Anoxia; Proteolysis; Confocal microscopy; (Kidney)

1. Introduction

Endocytosis in the renal proximal convoluted tubular cell is a permanent process serving the role of saving nitrogen from active plasma peptides that are continuously cleared away by kidney glomerulus [1]. This process has been shown to be poorly selective with regard to the protein species [2,3], and the affinity of the luminal membrane for proteins, is in the low range of that of an enzyme for its substrate ($K_m = 10^{-6}$ M [2]); however, its great capacity allows the kidney to reabsorb virtually all of the small proteins from the tubular lumen. Small proteins appear in urine only in a few circumstances such as proximal tubule injury [4,5] and after strenuous exercise [6], during which renal plasma flow may be decreased to 20% of normal value [7]; hence, it was hypothesized that renal ischemia impairs the tubular endocytic reabsorption of small proteins [6]. Energy dependence of endocytosis in kidney was also suggested by the fact that the metabolic inhibitors maleate and iodoacetate are able to inhibit or totally suppress the tubular reabsorption of lysozyme, a small plasma protein with bacteriolytic properties which is excreted during tubular proteinuria [1,8,9]. However, though the different steps of endocytosis in kidney have been described in detail [1,8,10–12], no information is available on the substrate or oxygen requirements for endocytosis in this organ or on metabolic factors modulating tubular uptake of proteins.

The problem is that it is not possible to study renal endocytosis in vitro with isolated renal cells in suspension since they loose their polarity [13]; the best model

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for endocytosis in kidney, the isolated perfused tubule, suffers the shortcomings of being time-consuming and of yielding a very small amount of tissue. This paper shows that a suspension of proximal tubules is able to take up ^{125}I-lysozyme, as well as RITC-lactalbumin, a protein with the same low molecular weight (14000) and a close primary structure [14]; the use of fluorescence image processing and of confocal microscopy after RITC-lactalbumin uptake allowed to ascertain and localize the endocytic capacity in tubular fragments; the measurement of tubule-associated radioactivity allowed to obtain quantitative data on ^{125}I-lysozyme uptake and to perform a time-course study.

In addition, our model allowed the investigation of the effects of anoxia and of alanine, glucose, or glutathione depletion on endocytosis and storage of ^{125}I-lysozyme.

2. Materials and methods

2.1. Protein tracers

Human milk lysozyme (Sigma) was labelled with ^{125}I (IMS 30 Amersham, UK) using the iodine monochloride method of Izzo et al. [15]. The iodination mixture contained 4 mol of ICl and 0.008 mol ^{125}I per mol of lysozyme. After 5 min at room temperature, free iodine was removed by passage on a Sephadex G25M column (PD 10 Pharmacia, Uppsala); then, albumin (pure fraction, fatty acid free, Sigma) was added to minimize lysozyme autoirradiation at storage (5 mg/mg of lysozyme). In five iodinations, 90% lysozyme was recovered with a labelling efficiency of 27% and a free iodine content of 0.6%; specific activity was 0.3 mCi/mg lysozyme.

Rhodamine isothiocyanate-α-lactalbumin (RITC-lactalbumin) from bovine milk was obtained from Sigma (St. Louis, MO, USA). Approximate content in RITC was 0.15–0.55 μmol/mg. The labelled protein was dialysed overnight against 0.9% NaCl before use.

Protein analysis was made by the method of Lowry et al. [16].

2.2. Preparation of purified proximal tubules suspension

In each experiment, one male New Zealand rabbit (Elevage des Dombes, Châtillon sur Chalaronne, France) fed with a standard diet was killed by i.v. infusion of sodium pentobarbital (Sanofi, France). The kidneys were rapidly removed and placed in ice-chilled Krebs-Henseleit saline buffer (KHS) containing (in mM): NaCl 118; KCl 4.7; $MgSO_4$ 1.18; KH_2PO_4 1.18; $CaCl_2$ 2.5; $NaHCO_3$ 24.9; gassed with O_2 95%/CO_2 5%. Unless otherwise specified, all further steps were performed at + 4°C, and all buffers and incubation media were gassed with O_2 95%/CO_2 5%. Proximal tubules suspensions were obtained by the method of Vinay et al. [17] modified by Gesek et al. [18]. Briefly, the kidneys were decapsulated and the cortex was dissected and cut into thin slices with a Stadie-Riggs microtome. After washing in gassed KHS buffer, slices were resuspended in 15 ml KHS buffer containing 30 mg collagenase A from *Clostridium histolyticum* (Boehringer, Germany), and 50 mg bovine serum albumin (fraction V, Sigma), in a 250 ml flask coated with silicon (Sigmacote, Sigma). The stoppered flask was gassed with O_2 95%/CO_2 5%, then incubated 45 min at 37°C in a shaking bath. Digestion was stopped by addition of 100 ml ice-cold KHS buffer deprived of calcium and the rough tubule suspension obtained was filtered on a stainless steel strainer, then washed three times by centrifugation at $60 \times g$ for 2 min and resuspension in KHS buffer without calcium. The tubules were resuspended for 5 min in 50 ml KHS buffer without calcium previously adjusted to 300 mosmol/kg and containing 50 mg/ml of serum albumin. After centrifugation at $60 \times g$, tubules were resuspended in Percoll (Pharmacia, Uppsala) diluted to 45% in KHS buffer without calcium and adjusted to 300 mosmol/kg as advised and discussed by Gesek and colleagues [18]. After centrifugation for 30 min at $12500 \times g$, the suspension was separated into four bands, the lowest of which contained a pure tubular suspension enriched in proximal fragments. This band was pipetted out and washed three to four times in standard KHS buffer by centrifugation and resuspension as described above, and the final tubule concentration was grossly adjusted to 5 mg dry weight/ml by absorbance measurement. An aliquot was saved for further dry weight measurement.

2.3. Unpurified tubule suspensions

In some experiments, unpurified tubules were prepared as previously described [19]: collagenase digestion was stopped by standard KHS buffer, and the rough tubule suspension obtained was filtered on a stainless steel strainer, then washed three times by centrifugation at $60 \times g$ for 2 min and resuspension in KHS buffer. The final concentration was adjusted to 25 mg (dry weight)/ml.

2.4. Determination of lysozyme uptake and degradation by tubule suspensions

Tubules were prepared freshly for each experiment and used as soon as possible. Incubations of lysozyme with tubules were performed in 25 ml Erlenmeyer flasks coated with silicon, then with serum albumin (fraction V, 20 g/l in distilled water during 1 h, then rinsed three times in distilled water and dried overnight). Likewise, glass tubes containing lysozyme

solutions were coated with albumin in order to avoid lysozyme adsorption to glassware. The incubation procedure was as follows.

Standard assay with purified proximal tubules. 0.2 ml tubule suspension and 0.7 ml incubation buffer were introduced into a coated flask at + 4°C. The flask, closed with a rubber stopper, was gassed through the stopper with either O_2 95%/CO_2 5% (standard condition) or N_2 95%/CO_2 5% (anoxia) during 5 min, and then stored in the cold until the time of utilization (up to 1 h). The flask was equilibrated 12 min at 37°C in a shaking bath; then 0.1 ml lysozyme solution was introduced by injection through the stopper and the incubation pursued at 37°C in the shaking bath. The final content of incubation mixture was (in KHS buffer): glucose 5 mM, alanine 5 mM, glutathione (reduced) 2 mM, lysozyme 150 nM, albumin (pure fraction, brought with labelled lysozyme) 12.5 μg/ml, tubules about 1 mg dry weight/ml. Gassed incubation buffer was adjusted to pH 7.42 before addition to the flask. In some experiments, glucose and alanine or glutathione were omitted. After 5 to 120 min, incubation was stopped by injection of 4 ml ice-cold stop-solution containing choline chloride (150 mM), Hepes (10 mM, pH 7.4) and bovine serum albumin (20 g/l).

Scaled up assay. The sensitivity of the assay was sometimes improved by scaling the total volume of incubation up to 2.5 ml and using unpurified tubules with a tissue concentration of 5 mg/ml. At the end of the incubation time, 1 ml incubation mixture was introduced into a tube containing 4 ml ice-cold stop-solution; then a 10000-fold excess of unlabelled lysozyme was added to the remaining medium in the flask, the incubation pursued during 5 min, and 1 ml incubation mixture was transferred into a tube containing 4 ml ice-cold stop-solution for determination of irreversible uptake.

Uptake was determined by immediate filtration of 2 ml diluted incubation mixture on a cellulose acetate filter of porosity 1.2 μm, previously soaked in stop-solution; the filter was rinsed by 2 ml stop-solution then counted in a gamma scintillation Packard spectrometer. Preliminary experiments showed that uptake was proportional to tubule content in the range of concentrations we used; reducing porosity did not increase the amount of radioactivity retained by the filter but lowered the filtration rate. In standard assays filter radioactivity in blank assays without tubules averaged 9% of total uptake value at 90 min.

Degradation was sometimes determined in the same assay by adding 1 ml TCA 20% (w/v) to 1 ml diluted incubation mixture; after centrifugation, the radioactivity of 1 ml supernatant was assessed and further referred to as the radioactivity of degradation products. Control experiments with freezing-thawing or Potter-Elvejhem grinding of precipitated incubation mixture showed that TCA extracted the total amount of non-TCA-precipitable activity present inside or outside the tubules.

Results were corrected for total volume in the flask and expressed in percent of total radioactivity in the flask per mg of tubular dry weight. Total radioactivity was assessed by counting 1 ml of diluted incubation mixture and correcting for total volume. Statistical analysis was made with the Student's *t*-test or by the non parametric *U* test of Mann and Whitney for small series.

2.5. Uptake of RITC-lactalbumin

Unpurified tubules (5 mg/ml) were incubated with 150 nM RITC-lactalbumin and 12.5 μg/ml bovine serum albumin with the same protocol as that used for the measurement of ^{125}I-lyzozyme uptake; at the end of the incubation time, 1 ml incubation mixture was transferred into 4 ml ice-cold stop solution (operation in duplicate) and immediately centrifuged at $60 \times g$ during 30 s. The pellet was suspended in 0.3 ml phosphate-buffered saline, (PBS), then fixed by the addition of 4.7 ml paraformaldehyde, 4.4% in PBS. After 1 h, the fixed tubule suspension was washed three times with PBS by centrifugation at $60 \times g$ during 30 s and stored at + 4°C.

Fluorescence microscopy. The same day, after a few seconds of decantation on bottom of a Petri dish, the fixed tubular suspension was observed on an Axiovert 35 M inverted microscope from Zeiss, with an objective ×32. Fluorescence images were taken with a low light camera (LHESA); epifluorescence analyses were performed using the following filter combinations from Zeiss: BP512–560/LP590. Images were numerized with the Sapphire image processor from Quantel (Paris, France), stored on a Bernouilli box, and photomicrographs were made using the UP5000P videoprinter from Sony.

Confocal microscopy. The duplicate fixed tubular suspension, decanted on bottom of a Petri dish, was observed the next day on the LSM Zeiss confocal straight microscope, with a water immersion objective ×40 (NA: 0.75), in the Centre Commun de Quantimétrie (Dr. Catherine SOUCHIER, Lyon). The excitation was made with a He/Ne laser at 543 nm, the detection with a long pass filter at 590 nm; images were taken with a zoom 80.

3. Results

3.1. RITC-lactalbumin uptake

Morphological examination of tubules after 90 min of incubation either with ^{125}I-lysozyme or with RITC-

lactalbumin showed tubular fragments with everted ends, the lumen seeming to be collapsed (Fig. 1a). When tubules were incubated with RITC-lactalbumin during 90 min under standard conditions (a representative experiment is shown in Fig. 1a), bright spots of fluorescence, suggesting intracellular accumulation of the label inside endocytic vesicles, were found associated with the everted ends of the tubular fragments, where the luminal membrane is exposed to the medium; pale fluorescence, uniformly distributed along the tubule was probably the result of extracellular binding of the protein to the basolateral side of the tubule. In order to assess the intracellular localization of the fluorescent spots, the same material was examined with a confocal microscope; Fig. 2a and b shows a series of views focused on two or three cells of the end of a tubular fragment, with steps of 2 μm on the z-axis between two consecutive views; spots of fluorescence are localized inside the cytoplasm, since some of them are well resoluted on the x or y-axis (image plane), and separated from the cell surface, at each level on the z-axis through the cell (Fig. 2a and b). Incubation during the same time at 0°C totally abolished any trace of fluorescence associated with tubules (Fig. 1b).

3.2. ^{125}I-lysozyme uptake

Normal plasma content of lysozyme is 350 nM [4], 75% of which enter the tubular fluid [9]. When purified proximal tubules were incubated for 90 min with 150 nM ^{125}I-lysozyme under standard conditions, i.e., KHS buffer containing 5 mM Glc, 5 mM Ala, 2 mM glutathione, gassed with 95% O_2, 5% CO_2, 9.5 $\pm$3.9% of radioactivity was associated with tubules (1 mg dry weight in 1 ml) (data drawn from 17 experiments shown in Figs. 3, 5 and 6). The time-course study (Figs. 3, 4 and 6) shows a progressive accumulation of the label by a seemingly saturable process; however, the steady-state was not reached even after 2 h. Degradation products appeared in the flask only after at least 10 min of incubation and the degradation time-course became linear only after a delay of 30 min (Fig. 6). Part of the radioactivity associated with tubules could be rapidly displaced by a 10000-fold excess of unlabelled

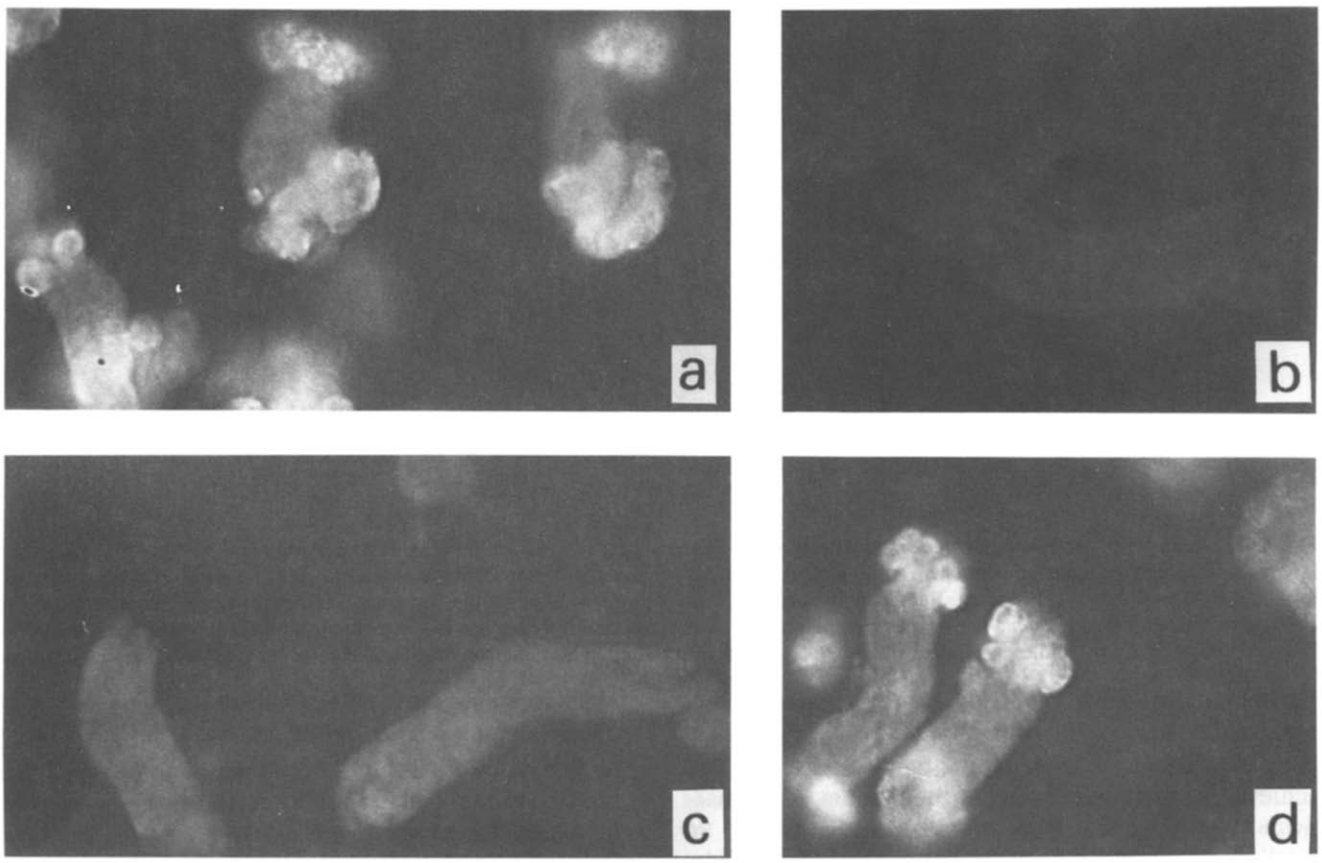
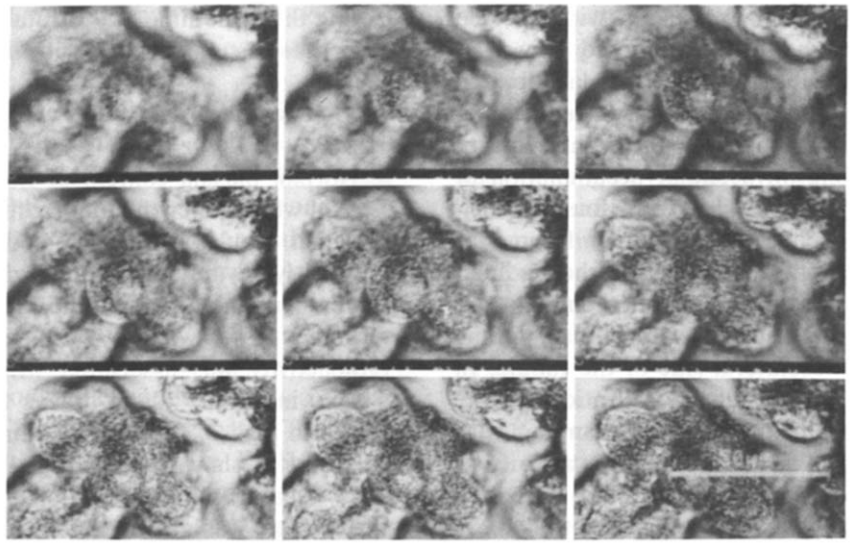


Fig. 1. Uptake of RITC-lactalbumin; observation with an inverted fluorescence microscope. Unpurified tubules (12.5 mg/2.5 ml) were incubated during 90 min with 150 nM RITC-lactalbumin as indicated in Materials and methods, then fixed by 4% paraformaldehyde. (a) Standard conditions of incubation (37°C, KHS buffer, Glc 5 mM, Ala 5 mM, GSH 2 mM, gassed with O_2 95%/CO_2 5%). (b) Incubation performed at 0°C. (c) Incubation with N_2 instead of O_2. (d) Incubation medium without glucose. The gain of the microscope and the image numerization were the same for each condition.



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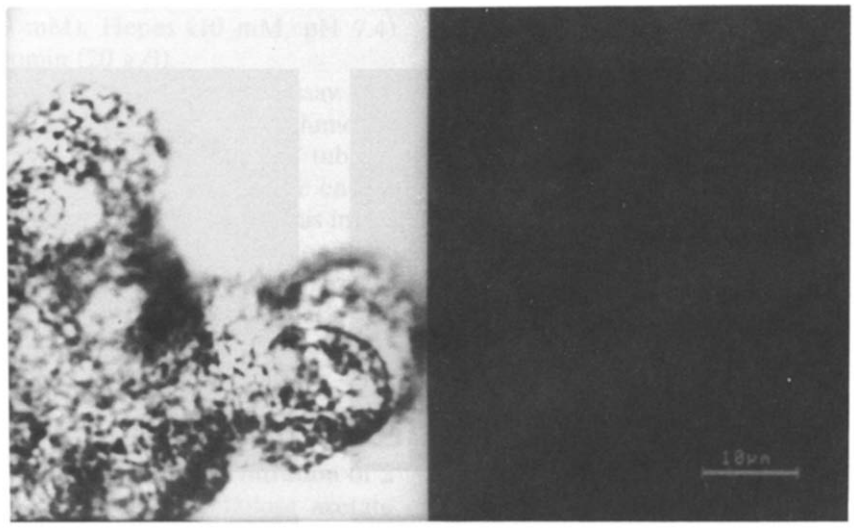
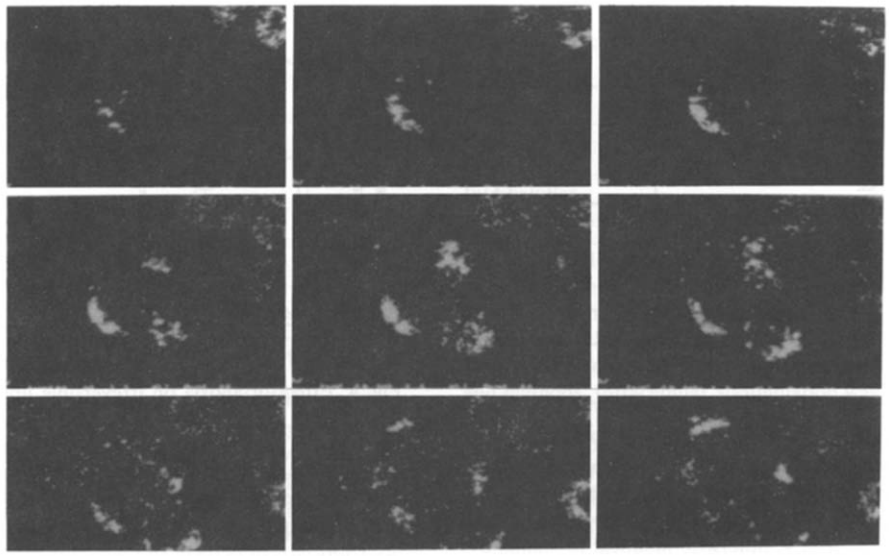


c

Fig. 2. Uptake of RITC-lactalbumin; confocal microscopy views of the experiment shown in Fig. 1. (a and b) Standard conditions of incubation; (a) transmitted light microscopy views across the end of a tubular fragment; the step between two consecutive views is 2 μm on the z-axis (orthogonal to the plane); (b) confocal fluorescence microscopy views corresponding to Fig. 2a; each view is an average of eight frames. (c) Tubules incubated with N_2 instead of O_2; left, transmitted light; right, confocal fluorescence in the same position on the z-axis; one frame with excitation during 8 s. (d) Tubules incubated without glucose; left, transmitted light; right, confocal fluorescence. Bars: (a) and (b): 50 μm; (c) and (d): 10 μm.

lysozyme; Table 1 shows that a new steady-state was achieved within 5 min and that the amount of radioactivity reversibly bound to tubules averaged 1.8% of total content in the flask. Tubule-associated radioactivity which was not rapidly displaced was supposed to represent internalized label. In order to improve accuracy of the results, purified tubules were replaced in some experiments by unpurified tubules, the very much higher yield of which allowed to scale up the assay to 5 mg dry weight/ml; in addition, the total volume was increased to 2.5 ml in order to determine total and irreversible uptake with the same flask. These conditions allowed to lower standard deviation of short times measurements (Fig. 4); irreversible uptake of lysozyme per mg after 90 min was similar: $8.3 \pm 3.5\%$ ($n = 4$, see also Fig. 4), whereas binding was higher:



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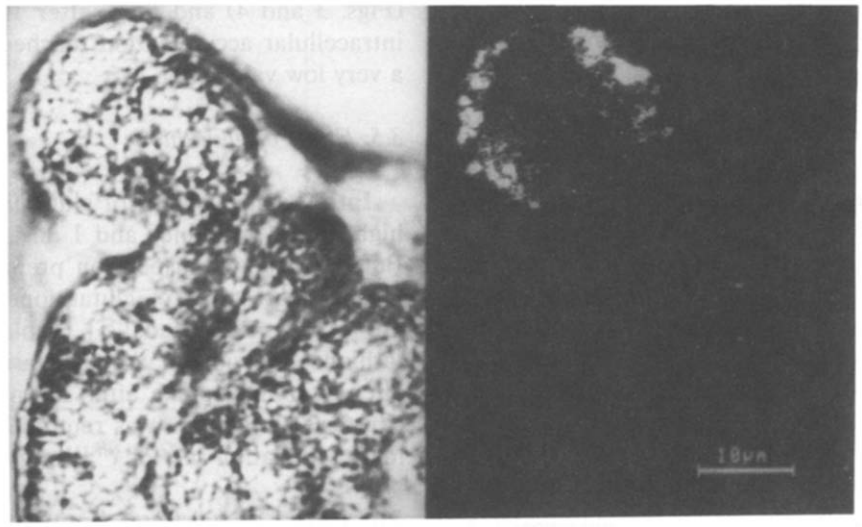

d

Fig. 2 (continued).

3.5 ± 0.6 ($n = 4$); the time-course study of irreversible uptake (Fig. 4) showed a progressive accumulation of the label, seeming to evolve toward a steady state.

3.3. Effect of chloroquine

In order to know if the observed uptake follows the endocytic-lysosomal route, lysozyme degradation and uptake were measured in the presence of chloroquine; this compound has been shown to inhibit lysosomal proteolysis in different cell types and organs [20,21] including kidney [22], by means of its accumulation into the acidic organelles, inducing a local alkalinisation [23]. Fig. 6 shows that lysozyme degradation by a tubular suspension is strongly, although not completely, inhibited by chloroquine. This experiment shows in addition that the uptake of lysozyme was strongly depressed by chloroquine as soon as the first 5 min; chloroquine decreased by 75% the amount of lysozyme accumulated after 90 min of incubation (Fig. 6).

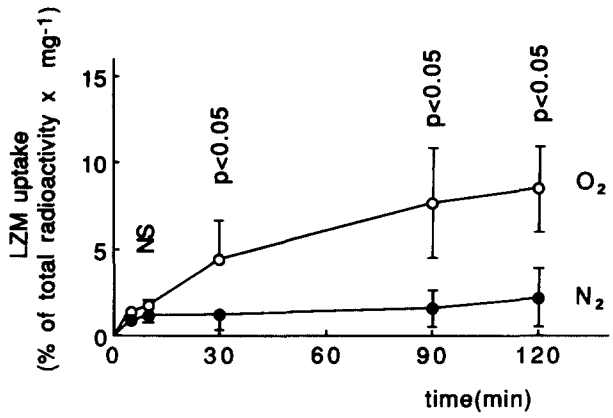

Fig. 3. Effect of anoxia on total uptake of lysozyme. Purified proximal tubules (1 mg/ml) were incubated with 150 nM labelled LZM (i) under standard conditions (37°C, KHS buffer, Glc 5 mM, Ala 5 mM, GSH 2 mM, gassed with O_2 95%/CO_2 5%, ○) or (ii) in anoxia (gassed with N_2 95%/CO_2 5%, ●, otherwise identical to standard). Uptake was measured at different times as indicated in Materials and methods. Results are expressed in percent of total radioactivity in the incubation flask per mg of tubular dry weight, mean ± S.D. (four experiments, each point in triplicate). Statistical analysis was made with the *U* test of Mann and Whitney, * $P < 0.05$.

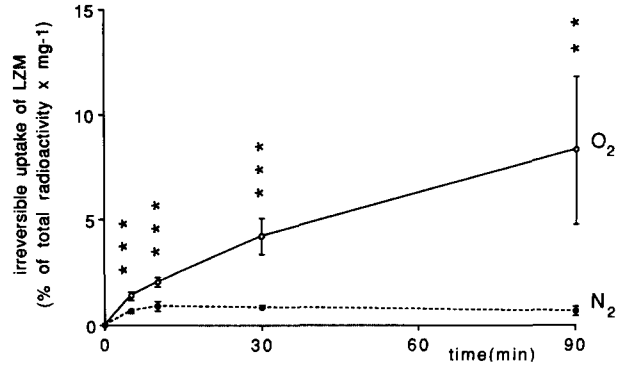

Fig. 4. Effect of anoxia on irreversible uptake of lysozyme. Unpurified tubules (12.5 mg/2.5 ml) were incubated with 150 nM labelled lysozyme in oxygenated buffer (○) or in anoxia (△) similarly to experiments shown in Fig. 3, except that, at the end of the incubation time, extracellular labelling was chased during 5 min by the addition of a 10000-fold excess of unlabelled lysozyme into the flask. Results are expressed in percent of total radioactivity in the flask per mg of tubular dry weight, mean ± S.D., $n = 4$; ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test for paired data).

3.4. Effect of anoxia on the uptake of RITC-lactalbumin and ^{125}I-lysozyme

Under anoxic conditions, transmitted light or fluorescence microscopy showed that tubular fragments were less everted than in the presence of oxygen (Fig. 1c); Fig. 2c shows that the access to the luminal membrane was still possible. After 90 min of incubation with RITC-lactalbumin, no spot of fluorescence was detectable within the tubules, neither by confocal nor by simple fluorescence microscopy (Figs. 1c and 2c). When tubules were incubated with ^{125}I-lysozyme under anoxic conditions, intracellular accumulation after 90 min of incubation was decreased 12-fold (Fig. 4); however, lysozyme uptake was not totally suppressed but the total and irreversible tubule-associated radioactivity were only decreased during short incubation times (Figs. 3 and 4) and then, after 10 min of incubation, intracellular accumulation reached a steady-state with a very low value (Fig. 4).

Table 1
Chase of taken up radioactivity by unlabelled lysozyme

Time of chase (min)	Radioactivity taken up by tubules	Radioactivity displaced by unlabelled lysozyme
0	8.2 ± 2.7	–
0.5	7.7 ± 2.6 (NS)	−0.5 ± 0.8
1	6.5 ± 2.3 *	−1.7 ± 0.9
5	6.4 ± 2.1 *	−1.8 ± 0.8

Purified tubules were incubated with labelled lysozyme during 90 min under standard conditions (see Fig. 3 for details), then unlabelled lysozyme in 10000-fold excess was added into the flask and incubation pursued during the indicated time. Results are expressed in percent of total radioactivity in the flask per mg dry weight, mean ± S.D. ($n = 4$ tubule preparations, each point in triplicate) *$P < 0.05$ (Student's *t*-test for paired data, comparison with time 0). Radioactivity displaced by unlabelled lysozyme is the mean of differences between labelled lysozyme uptake before and after chase.

3.5. Effect of glutathione

Intratissular content of glutathione is normally very high, 3 mM in kidney and 1 mM in whole blood [24]. But, in a tubule suspension prepared in KHS buffer, intracellular stores of glutathione or free aminoacids are readily exhausted [25]. Table 2 shows that glutathione omission in the incubation medium did not alter lysozyme uptake after 120 min of incubation; by contrast, glutathione was required to obtain the maximal degradation rate of ^{125}I-lysozyme.

3.6. Effect of substrate deprivation on the uptake of RITC-lactalbumin and ^{125}I-lysozyme

Energy substrate requirement for endocytosis was investigated by omitting glucose and alanine, the two

Table 2
Effect of glutathione depletion on lysozyme uptake and degradation

	GSH 2 mM	GSH 0 mM	
Uptake	8.2 ± 3.6 ($n = 5$)	8.6 ± 4.5 ($n = 5$)	NS
Degradation	4.0 ± 1.3 ($n = 5$)	2.0 ± 1.1 ($n = 5$)	***

Procedure of incubation is described in Materials and methods: tubules and lysozyme were incubated during 120 min in the presence of 95% O_2, 5% CO_2, 5 mM glucose, 5 mM alanine, in KHS buffer, with (GSH 2 mM) or without (GSH 0 mM) glutathione 2 mM. Results are given in percent of total radioactivity per mg of tubule dry weight. *** $P < 0.001$ (*t*-test for paired data).

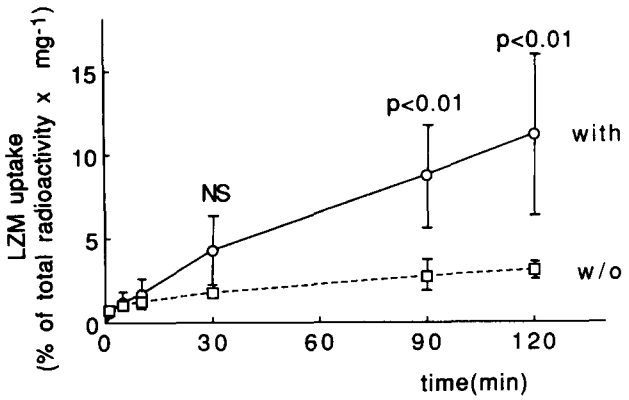

Fig. 5. Effect of glucose and alanine deprivation on lysozyme uptake. Purified proximal tubules (1 mg/ml) were incubated with 150 nM labelled lysozyme as described in Materials and methods (37°C, O_2 95%, CO_2 5%). The incubation medium contained either KHS buffer, Ala 5 mM, Glc 5 mM, GSH 2 mM (with), or KHS buffer, GSH 2 mM (w/o). Uptake is expressed in percent of total radioactivity in the flask per mg of tubular dry weight, mean ± S.D. (five experiments, each point in triplicate). Statistical analysis was made with the *U* test of Mann and Whitney, ** $P < 0.01$.

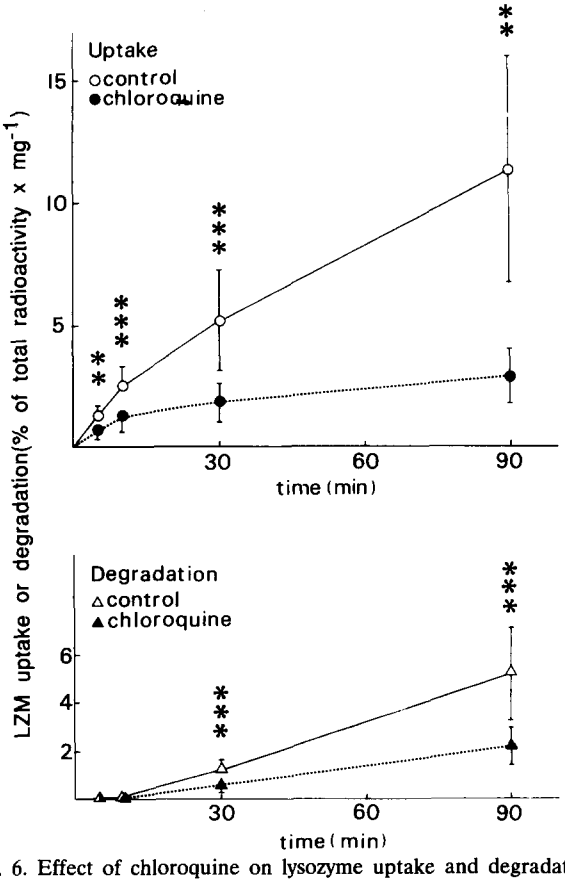

Fig. 6. Effect of chloroquine on lysozyme uptake and degradation. Purified proximal tubules (1 mg/ml) were incubated with 150 nM labelled LZM in standard conditions as described in Materials and methods, in the absence (control, ○, △) or in presence of 0.5 mM chloroquine (●, ▲). Uptake and degradation are expressed in percent of total radioactivity in the flask per mg tubular dry weight, mean ± S.D. (7 to 8 experiments, each point in triplicate). Statistical analysis was made with the *t*-test of Student for paired data; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 3
Effect of glucose deprivation on lysozyme accumulation by renal tubules

	Complete medium	Glucose omitted	
Total uptake	8.2 ± 2.0	3.7 ± 1.1	*
Binding	1.4 ± 1.0	1.5 ± 0.6	NS
Intracellular accumulation	6.3 ± 1.8	2.1 ± 1.1	*

Purified tubules were incubated with labelled lysozyme during 90 min as described in Materials and methods. The incubation medium contained either KHS buffer, Ala 5 mM, Glc 5 mM, GSH 2 mM (complete) or KHS buffer, Ala 5 mM, GSH 2 mM (glucose omitted). Uptake of labelled lysozyme was measured as indicated in Materials and methods. Intracellular accumulation was determined after 5 min chase by a 10000-fold excess of unlabelled lysozyme. Lysozyme binding to tubules was determined by the difference between total uptake and intracellular accumulation. Results are percent of total radioactivity in the flask (mean ± S.D., five experiments, each point in triplicate); * $P < 0.05$ (Student's *t*-test for paired data).

main substrates, in the incubation medium; however glutathione (2 mM) was maintained in order to preserve the capacity of ^{125}I-lysozyme degradation. In spite of the presence of this amino acid producer [25], omission of alanine and glucose divided uptake of ^{125}I-lysozyme by three after 90 min (Fig. 5), and the tubule-associated radioactivity plateaued after 5 min. Glucose was in great part responsible for this substrate requirement since, when glucose alone was omitted, lysozyme uptake was still divided by two (Table 3). Table 3 shows in addition that only intracellular storage of the label required the presence of glucose, whereas reversible binding was not modified by glucose depletion. However, confocal microscopy analysis of fluorescence after RITC-lactalbumin uptake showed that, when glucose was omitted in the incubation medium, there is no qualitatively detectable difference in the uptake process (Fig. 2d).

4. Discussion

The present study shows that isolated renal tubules in suspension are able to accumulate lysozyme as does the kidney in vivo [1,26]. The endocytic capacity of tubules in suspension was assessed by the use of a commercially available fluorescent protein, RITC-lactalbumin. The two proteins most probably share the same endocytic pathway since protein reabsorption is not a selective process [1–3,27], and lysozyme and lactalbumin have close structures [14]; however, it cannot be excluded that some differences exist concerning their tubular handling; indeed, the basic protein lysozyme was shown to be particularly resistant to lysosomal proteolysis with a renal half-life of 100 min [1,9], whereas other proteins have a half-life of 10 to 30 min [1]; moreover, slight discrepancies were found in

the time-course of competition between proteins for tubular reabsorption [27].

In this paper, confocal microscopy study after RITC-lactalbumin uptake yields a definite evidence for the capacity of our model to internalize a protein into endocytic vacuoles. Fluorescence microscopy showed that renal tubules in suspension take up the label by the very end of the everted fragment, where the luminal membrane is exposed to the incubation medium; this spatial limitation of uptake shows that the endocytic uptake of RITC-lactalbumin is performed by a luminal route and thus confirms the data available until now on endocytosis in kidney [1]. It must be pointed out that, in this work, the use of recent techniques confocal microscopy and fluorescence image processing allowed to obtain rapidly informations which were untill now drawn only from electron microscopy.

The amount of lysozyme taken up by our model is in the same range as that obtained with perfused tubules by Nielsen et al. [11]; thus, luminal perfusion pressure is not an absolute requisite for tubular endocytosis. However, in our experiments, uptake of lysozyme was only 10% of total radioactivity after 90 min, with 1 ml handled volume and 1 mg tissue dry weight; in vivo 100% of filtered lysozyme is taken up; assuming that the glomerular filtration rate of 1 kg body weight is 2.5 ml/min, and since the average dry weight of renal tubules per kg body weight in rabbit is 250 mg (measurements from our laboratory), the in vivo uptake capacity would be at least 10-times greater than the uptake rate measured in the present experiments. This discrepancy can be explained by the fact that in our tubule suspensions only the ends of tubular fragments were able to internalize a protein. The endocytic nature of lysozyme uptake in the present study is demonstrated by three observations: (i) the main part of taken up lysozyme is not rapidly reversible, suggesting an intracellular localization of the label; (ii) there is a lag-time of about 10 min before the appearance of degradation products, which is the same as that observed in renal slices during endocytic uptake of lysozyme [10]; this strongly suggests that, in the present experiments, lysozyme had to be internalized before being degraded; (iii) degradation and uptake were sensitive to chloroquine, an inhibitor of the endosomal-lysosomal pathway; however, it cannot be excluded that, due to cathepsins released by disrupted tubules, some additional extracellular degradation occurred.

Taken as a whole, our results show that renal tubules in suspension provide a satisfactory model for studying endocytosis; due to the high amount of tissue available, it is well-suited for studies with a number of different metabolic conditions. The use of unpurified tubules allows the upscaling of the assay and the improvement of the sensitivity of the lysozyme uptake measurement, especially in the case of short incubation times, without changing the absolute value of irreversible uptake; this is due to the fact that proximal tubules are the main endocytic site in kidney [1]. However, reversible binding to plasma membranes was increased with unpurified tubules and therefore unpurified tubules are not a valuable model for proximal tubule binding measurements. We used unpurified tubules in fluorescence microscopy experiments since proximal tubules are easy to identify morphologically.

4.1. Metabolic requirements

There is a general agreement that endocytosis requires energy [28], however, energy supply for endocytosis has been reported to differ according to specific metabolic features of the cell considered; leucocyte phagocytosis is not inhibited by anoxia and requires glucose as attested by the effect of glucose deprivation, iodoacetate, NaF and 2-deoxyglucose [29]. By contrast, a combination of glycolysis inhibitors and of respiration inhibitors is required to achieve a maximal decrease in endocytosis in fibroblasts and hepatocytes [28].

The present work shows that, in renal tubules, anoxia causes a dramatic reduction of lactalbumin uptake and of lysozyme irreversible uptake. This is consistent with the fact that, in kidney proximal cell, the energy metabolism as well as cytoskeleton stability is strongly dependent on oxygen supplies [30–32]. However, the use of ^{125}I-lysozyme allowed us to demonstrate that storage after long periods of incubation is much more inhibited than uptake after short times of incubation. This suggests that energy is not required for the first step of internalization but for a further step and corroborates previous studies on hepatic uptake of asialoproteins [33]; as a matter of fact, hepatocytes have been shown to be able to internalize asialoglycoprotein receptors during almost total ATP depletion; under this condition, however, receptor recycling, which is required for continuous endocytosis, was inhibited [33]. The first step of internalization of insulin receptors was also shown to be insensitive to metabolic poisons [21], whereas ATP was shown to activate endosomal fusion in a cell-free fusion assay [34] and to be implicated in uncoating of coated pitts [35].

The main energy substrates of the proximal tubule in vivo are lactate, citrate, fatty acids and amino acids [31]. Glucose may be metabolized either to lactate or to CO_2, in spite of a relatively low level of hexokinase in this segment [31,36]; it contributes to 10–25% of the total oxygen consumption in rabbit renal slices [31]. This work shows that, in isolated proximal tubules, when glucose alone was omitted, irreversible uptake of ^{125}I-lysozyme was divided by three; during these experiments, energy could be provided by oxidative pathways from alanine [19], glutathione [25] and triacylglycerol [31] metabolism; intracellular stores of glycogen might

also in part compensate for glucose deprivation [31]; in this case, the real inhibitory effect of glucose depletion would be even greater. These data clearly show a specific requirement of kidney tubular endocytosis of lysozyme for glucose, either by means of a localized cytoplasmic supply of energy, or by activating some metabolic pathway. A specific role for glucose in proximal tubule has been noticed for many years since, in spite of its rather modest contribution to cellular energy metabolism, glucose was shown to improve cell function [31], especially Na-K-ATPase activity, independently of overall ATP production, and possibly by means of the specific compartmentation of glycolysis in the cytoplasm [37]. A role for glucose in proximal tubule endocytosis was also for long suggested by the surprising inhibition of lysozyme uptake in perfused rat kidney by iodoacetate (an inhibitor of thiol groups that blocks the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase), in spite of the presence of non-glycolytic energy sources in the perfusate [9]; iodoacetate was shown to be a strong depressor of endocytosis in numerous cell types [1,9,28,29]. RITC-lactalbumin uptake was less sensitive to glucose depletion; this is probably due in great part to the poor capacity of quantitative discrimination of fluorescence microscopy when compared to radioactivity measurements on tissue extracts; however, it is also possible that differences between lactalbumin and lysozyme exist, either in their sensitivity to glucose or in the rate of some step of their renal handling, since no intracellular fluorescence was detectable before 90 min of incubation.

A mechanistic study of the activating role of glucose on endocytosis is beyond the scope of this study, but these results sustain the hypothesis that exercise-induced proteinurias are due to renal ischemia, as a result of either substrate deprivation or local anoxia. However, due to the difficulty of local P_{O_2} measurements near proximal tubules during strenuous exercise, it is not known whether the combination of reduced renal blood flow and the high oxygen avidity of proximal tubule leads to total anoxia in this site.

The activating effect of glutathione on proteolysis of an exogenous protein has already been observed in kidney isolated lysosomes [38,39]. In addition, this tripeptide was shown to protect the tubule against endogenous protein loss due to anoxia by means of glycine it contains, possibly by a regulatory mechanism [25,40]. Glutathione, thus, seems to exert a key role in tubular cell metabolism.

To our knowledge, this work is the first report of chloroquine inhibition of lysozyme uptake by a renal model. This inhibition cannot be a feed-back regulatory response linked to the output of degradation products since the inhibitory effect was detectable within the first 5 min. Moreover, it was possible to reduce proteolysis to the same extent without altering uptake rate, by omitting glutathione in the medium. The mechanism could rather be related to lysosomal or endosomal proton gradient since cellular acidification or nigericin pH clamping were shown to inhibit endocytosis [41,42], possibly by means of clathrin modification [42]; by contrast, in adipocytes, chloroquine increases insulin-receptors accumulation by means of a reduced lysosomal proteolysis [21].

In conclusion, this study has shown that renal tubules in suspension provide a convenient model for studying renal endocytosis in vitro; it allowed to investigate some of the metabolic features of endocytosis, to demonstrate that oxygen and energy substrates are required, and to suggest a specific role for glucose in renal protein handling.

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