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Gentamicin inhibits Na^+-dependent D-glucose transport in rabbit kidney brush-border membrane vesicles

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We studied the effect of gentamicin on Na^+-dependent D-glucose transport into brush-border membrane vesicles isolated from rabbit kidney outer cortex (early proximal tubule) and outer medulla (late proximal tubule) in vitro. We found the same osmotically active space and nonspecific binding between control and gentamicin-treated brush-border membrane vesicles. There was no difference in the passive permeability properties between control and gentamicin-treated brush-border membrane vesicles. Kinetic analyses of D-glucose transport into 1 mM gentamicin-treated brush-border membrane vesicles demonstrated that gentamicin decreased V_{max} in the outer cortical preparation, while it did not affect V_{max} in the outer medullary preparation. With regard to K_m, there was no effect of gentamicin in any vesicle preparation. When brush-border membrane vesicles were incubated with higher concentrations of gentamicin, Na^+-dependent D-glucose transport was inhibited dose-dependently in both outer cortical and outer medullary preparations. Dixon plots yield inhibition constant $K_i = 4$ mM in the outer cortical preparation and $K_i = 7$ mM in the outer medullary preparation. These results indicate that the Na^+-dependent D-glucose transport system in early proximal tubule is more vulnerable to gentamicin toxicity than that in late proximal tubule.

Introduction

Gentamicin has been widely used in the clinical management of gram-negative infection, but it has been known to be potentially nephrotoxic [1–3]. Functional correlates of gentamicin nephrotoxicity are a decrease in urine-concentrating capacity, tubular proteinuria, enzymuria and alterations of epithelial transport processes of proximal tubule. Gentamicin also induces tubular cell necrosis, which is confined exclusively to proximal convoluted and straight tubules [4–6]. Electron microscopy reveals a decrease in the incidence and height of brush-border, myeloid bodies, swelling of mitochondria and so on. Recent studies have clearly shown that luminal uptake of gentamicin occurs along both proximal convoluted and straight tubule [7–10]. The majority of the evidence, therefore, suggests that gentamicin affects some transport events across the brush-border membrane of proximal tubule.

In this paper, we used brush-border membrane vesicles isolated from rabbit kidney and investigated the effects of gentamicin on Na^+-dependent D-glucose transport into brush-border membrane vesicles in vitro. The initial event in the interaction of gentamicin with the renal proximal tubular

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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cell is its binding to the brush-border membrane [11,12]. The kinetic approach to the effect of gentamicin on D-glucose transport is of considerable interest from the pathophysiological point of view, since this provides a basis for understanding how gentamicin affects such transport.

It has been known that there is nephron heterogeneity of glucose transport sites [13]. Segment 1 and segment 2 in proximal tubule are more susceptible than segment 3 to gentamicin nephrotoxicity [4,14]. Therefore we prepared brush-border membrane vesicles from outer cortex (segment 1 and segment 2), and outer medulla (segment 3) to compare the degree of gentamicin toxicity between both vesicle preparations.

Materials and Methods

Vesicle preparation

Male albino rabbits, weighing about 2 kg, were killed by decapitation. The kidneys were perfused with Dulbecco's phosphate-buffered saline until blood-free. They were then removed, placed in ice-cold Dulbecco's phosphate-buffered saline. We took slices of up to 0.5 mm thick from the surface of the kidney using a tissue slicer, and obtained outer cortical tissue. Then the residual kidney was sectioned transversely into up to 2 mm thick slices. Strips of outer stripe tissue in the outer medulla were trimmed by microdissecting scissors under a microscope. Thus we always took both outer cortical and outer medullary tissue from the same animal and two preparations were done in parallel. Brush-border membrane vesicles were prepared from each tissue by a Ca^{2+} precipitation method [13]. The final vesicle preparation was resuspended in 10 mM Tris-Hepes (pH 7.4)/100 mM mannitol/100 mM KSCN. Each vesicle preparation was divided into two fractions. Gentamicin was added to the one fraction and the vehicle was added to the other. Brush-border membrane vesicles were then incubated at 37°C for 60 min before use.

The detailed compositions of the various media used in experiments are given in the figure legends. In general 10 mM Tris-Hepes (pH 7.4)/100 mM mannitol/100 mM KSCN was used as the basis for all media. In this way 100 mM KSCN was present in equilibrium across the vesicle membrane at all times. As proved by Turner and Moran [13], 100 mM KSCN in equilibrium with 12.5 μg valinomycin per mg vesicle protein is sufficient to short-circuit transmembrane electrical potential difference.

Measurements of D-glucose uptake

Unless otherwise noted, the procedure for uptake measurements was as follows. A 50 μl aliquot of vesicles (3–4 mg protein per ml) was placed in a glass test tube and at time zero a 100 μl aliquot of incubation medium containing D-[^{3}H]glucose and other constituents as required was added. After one second the reaction was terminated by the addition of a 10-fold dilution of ice-cold stop solution using a fast sampling apparatus. The apparatus consists of an electric timer, a solenoid, a syringe which contains the stop solution and an Eppendorf pipette with a microswitch. After addition of the stop solution the vesicles were applied to a Millipore filter (HAWP 0.45 μm) under light suction. The filter was then washed with a further 4.5 ml of the stop solution. The filter which retained brush-border membrane vesicles was dissolved in scintillation fluid and counted along with samples of the incubation medium and appropriate standards. Thus we measured an initial flux of D-glucose transport. The Na^+-dependent component of D-glucose flux was calculated from the total flux by subtracting the Na^+-independent flux measured with choline replacing sodium. The stop solution contained 150 mM NaCl, 1 mM phlorizin and sufficient mannitol to compensate for intravesicular osmolarity, in 10 mM Tris-Hepes (pH 7.4).

All experimental points were carried out, at least in triplicate, at 25°C. The errors indicated in the tables and figures (providing they are large enough to illustrate) are standard deviations. Results of representative experiments are shown.

Chemicals. D-[^{3}H]Glucose and L-[^{3}H]glucose were from New England Nuclear Corp. (Boston, MA). Choline chloride (3 × crystallized) was from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of the highest purity available from commercial sources.

Student's independent *t*-test was used in the statistical evaluation of data and *p* values of less than 0.05 were taken to indicate statistically significant differences.

Results

Enzymatic characterization of vesicle preparations

The average enzymatic activities of outer cortical and outer medullary homogenates and the relative enrichments in the final vesicle preparations are given in Table I. It is obvious that both vesicle preparations were enriched more than 8.5-times in brush-border membrane enzyme markers and were only slightly contaminated by enzymes characteristic of antiluminal membrane and intracellular organelles. Comparing our data to those of Turner and Moran [13] who have prepared brush-border membrane vesicles from both outer cortical and outer medullary tissue by the same method, we found essentially identical enrichments for brush-border membrane, antiluminal membrane, mitochondria and endoplasmic reticulum markers.

Distribution spaces of D-glucose

Fig. 1 shows the results of an experiment in which the distribution spaces of D-glucose in control and 1 mM gentamicin-treated brush-border membrane vesicles were measured as a function of extravesicular osmolarity. The osmolarity of the extravesicular medium was varied by changing its mannitol concentration. We measured uptakes for 5 min at 37°C in the presence of sodium, by which time D-glucose uptake is close to its equilibrium value [15]. The plots of uptake vs. inverse osmolarity for both control and gentamicin-treated brush-border membrane vesicles are linear and superimposable in either outer cortical or outer medullary vesicle preparations. This indicates that D-glucose is equilibrating with the same osmolarity active space. The non-zero intrcept on the vertical axis represents 'uptake' when the extravesicular osmolarity is extrapolated to infinity. This component of 'uptake' is probably due to nonspecific binding and trapping by the membranes and filters.

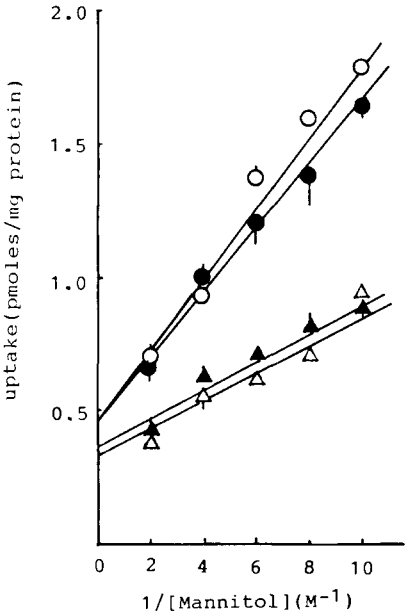

Fig. 1. Effect of extravesicular osmolarity on the equilibrium uptake of D-glucose. Vesicles (○, outer cortical control; ●, outer cortical gentamicin-treated, △, outer medulalry control; ▲, outer medullary gentamicin-treated brush-border membrane vesicles) were prepared in 10 mM Tris-Hepes containing 100 mM NaCl, 100 mM mannitol and 100 mM KSCN plus valinomycin. The incubation medium was the same buffer containing 0.1 mM D-[^{3}H]glucose and 100–500 mM mannitol (final concentration). Uptake was measured after 5 min of incubation.

TABLE I

ENZYME ACTIVITIES OF HOMOGENATE AND BRUSH-BORDER MEMBRANE VESICLES

Values are means ± S.D. for seven or eight independent determinations. Activites are given as micromoles substrate consumed per milligram protein per hour. Hom, homogenate; BBMV, brush-border membrane vesicles.

Enzyme	Outer cortex			Outer medulla		
	Hom	BBMV	BBMV/Hom	Hom	BBMV	BBMV/Hom
Alkaline phosphatase	11.0 ±2.6	106.0 ±25.4	9.64	11.0 ±2.7	98.2 ±27.4	8.93
Maltase	2.1 ±0.6	19.2 ± 8.1	9.14	1.9 ±0.5	16.2 ± 3.6	8.53
(Na^+-K^+)-ATPase	3.1 ±1.2	0.9 ± 0.6	0.29	4.2 ±1.3	1.8 ± 1.2	0.43
Succinic dehydrogenase	1.19±0.12	0.08± 0.06	0.07	1.37±0.10	0.18± 0.10	0.13
Glucose-6-phosphatase	7.7 ±1.0	4.3 ± 1.0	0.56	5.2 ±1.0	4.1 ± 0.4	0.79

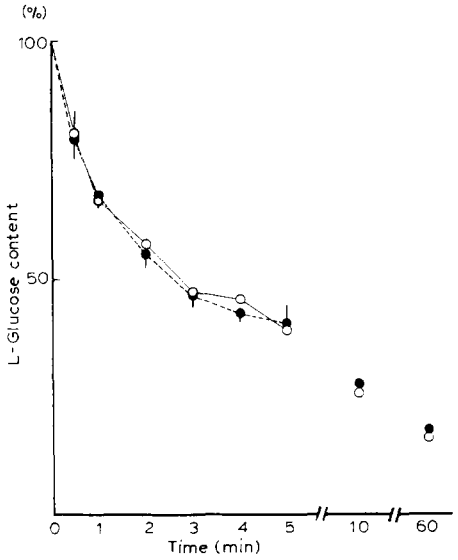

Fig. 2. The time dependence of L-glucose efflux from brush-border membrane vesicles. Vesicles (○, control; ●, gentamicin-treated brush-border membrane vesicles) were preloaded with 2.4 μM L-[^{3}H]glucose in 10 mM Tris-Hepes containing 100 mM mannitol, and then diluted (1 : 6) into the same medium without L-glucose. L-Glucose retained in these vesicles was measured as a function of time.

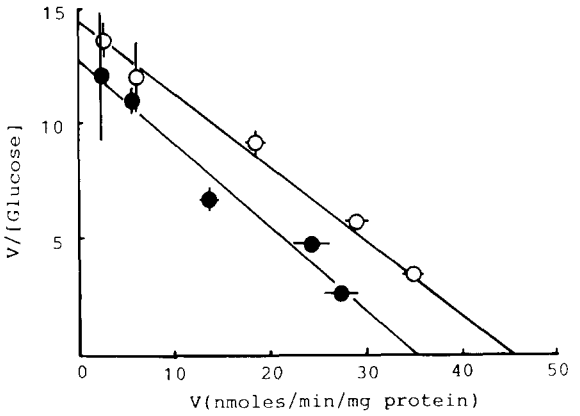

Fig. 3. Eadie-Hofstee plots of Na^+-dependent components of the D-glucose flux into outer cortical brush-border membrane vesicles. The sodium-dependent component of D-glucose flux was measured as a function of D-glucose concentration. Data were from a single experiment. ○, control; ●, gentamicin-treated outer cortical brush-border membrane vesicles. Least-squares fits to these plots yield K_m = 5.45 and 5.58 mM, and V_{max} = 201.0 and 167.4 nmol/min per mg protein, respectively.

Permeability of brush-border membrane vesicles

Fig. 2 shows the passive permeability properties of control and 1 mM gentamicin-treated whole cortical brush-border membrane vesicles as evi-

TABLE II

KINETIC PARAMETERS OF OUTER CORTICAL (A) AND OUTER MEDULLARY (B) BRUSH-BORDER MEMBRANE VESICLES

Expt. no.	Control		1 mM Gentamicin	
	K_m (mM)	V_{max} (nmol/min per mg protein)	K_m (mM)	V_{max} (nmol/min per mg protein)
A 1	4.60	84.0	4.78	73.8
2	3.52	115.2	3.36	90.0
3	3.12	45.6	2.72	35.4
4	3.82	69.0	3.46	51.6
5	3.76	94.2	2.95	62.4
Mean ± S.D.	3.73 ± 0.54	81.6 ± 26.2	3.45 ± 0.80	62.6 ± 20.8 *
B 1	0.81	37.2	0.77	39.0
2	1.10	55.82	1.09	46.8
3	0.71	26.4	0.71	22.2
4	0.91	28.8	1.11	33.6
5	0.63	40.8	0.97	52.8
Mean ± S.D.	0.83 ± 0.18	37.8 ± 11.6	0.93 ± 0.18	38.9 ± 11.9 *

* $P < 0.05$.

denced by the time dependence of L-glucose efflux from the brush-border membrane vesicles in the absence of sodium. The permeability of gentamicin-treated brush-border membrane vesicles was the same as that of control brush-border membrane vesicles as reflected in the rate of L-glucose efflux. These data indicate that there is no significant change in vesicle membrane permeability properties between control and gentamicin-treated brush-border membrane vesicles.

Kinetics of Na^+-dependent D-glucose transport

The kinetics of Na^+-dependent D-glucose uptake into control and 1 mM gentamicin-treated outer cortical brush-border membrane vesicles under zero trans sodium and glucose conditions at 25°C is illustrated in Fig. 3. The data are shown as modified Eadie-Hofstee plots. It is obvious from Fig. 3 that the kinetics of D-glucose transport are different in the two vesicle fractions. The least squares fit to the data from 1 mM gentamicin-treated brush-border membrane vesicles yields much the same apparent K_m as that from the control, while V_{max} in the former is less than that in the latter. Table IIA and B shows the summary of each of the five independent experiments in outer cortical and outer medullary preparations. In the control sets we demonstrate two distinct D-glucose transport sites. Outer cortical data indicate a low-affinity site with $K_m = 3.73 \pm 0.54$ mM and $V_{max} = 81.6 \pm 26.2$ nmol/min per mg protein, whereas outer medullary data indicate a high-affinity site with $K_m = 0.83 \pm 0.18$ and $V_{max} = 37.8 \pm 11.6$ nmol/min per mg protein. Gentamicin only affects the outer cortical D-glucose transport site, decreasing V_{max} from 81.6 ± 26.2 to 62.6 ± 20.8 nmol/min per mg protein ($P < 0.05$).

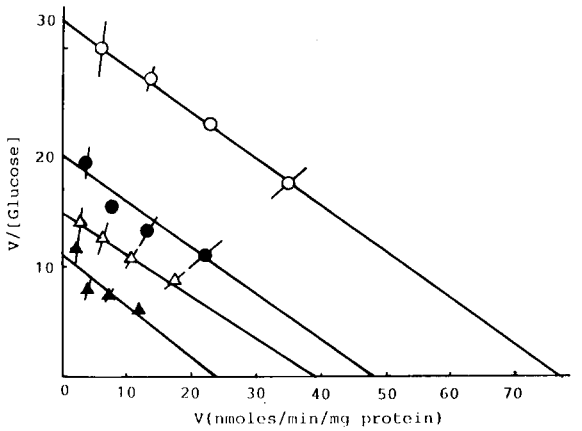

Fig. 4. Eadie-Hofstee plots of Na^+-dependent components of the D-glucose flux into outer cortical brush-border membrane vesicles. The sodium-dependent components of the D-glucose flux into outer cortical brush-border membrane vesicles. The sodium-dependent component of D-glucose flux was measured as a function of D-glucose concentration. Gentamicin was added to the vesicles to the appropriate concentration (○, control; ●, 3.3 mM gentamicin; △, 6.6 mM gentamicin; ▲, 9.9 mM gentamicin). Least-squares fits to these plots yield parallel regression lines, indicating that gentamicin decreases V_{max} in a dose-dependent manner, while it does not change K_m values.

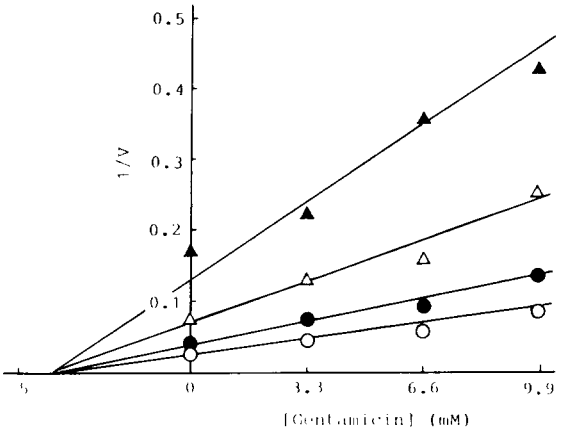

Fig. 5. Dixon plots of Na^+-dependent components of the D-glucose flux into outer cortical brush-border membrane vesicles. Gentamicin was added to the vesicles to appropriate concentration. Incubation medium contained different concentrations of (D-glucose (○, 2.0 mM; ●, 1.0 mM; △, 0.5 mM; ▲, 0.2 mM). The sodium-dependent component of D-glucose flux was measured as a function of gentamicin concentration. Least-squares fits to these plots yield regression lines which cross to a point, and the inhibition constant (K_i) is about 4 mM.

We further investigated a relationship between the concentration of gentamicin and the inhibition of Na^+-dependent D-glucose transport. Fig. 4 shows the Eadie-Hofstee plots of the data from outer cortical preparations incubated with different concentrations of gentamicin (0, 3.3, 6.6, 9.9 mM). Least squares fits to these data yield parallel regression lines, indicating that gentamicin decreases V_{max} in a dose-dependent manner, while it does not change K_m values. Fig. 5 shows the Dixon plots of the data from outer cortical preparations. The regression lines crossed one another on the abscissa, providing an inhibition constant

(K_i) of about 4 mM. Moreover the Dixon plots indicated that gentamicin behaves as a noncompetitive inhibitor of outer cortical D-glucose transport. In the same manner we carried out the experiment in outer medullary brush-border membrane vesicles, and Fig. 6 shows the Eadie-Hofstee plots of the data from outer medullary preparations. Least squares fits to these data yield parallel regression lines as observed in the outer cortical preparation, indicating that gentamicin decreases V_{max} in a dose-dependent manner not only in outer cortical but also in outer medullary brush-border membrane vesicles. We obtained a K_i value of about 7 mM for outer medullary preparation from Dixon plots of these data.

We employed the equilibrium exchange method that we reported previously [17] to assess the effect of gentamicin on glucose transport without a sodium gradient. Vesicles were preloaded in 10 mM Tris-Hepes (pH 7.4)/100 mM mannitol/60 mM NaCl/2 mM D-glucose. Incubation medium contained the same constituents and trace amounts of D-[^{3}H]glucose. There is no net flux of D-glucose but the flux of D-[^{3}H]glucose into the vesicles can be measured. We measured the uptakes of D-[^{3}H]glucose for 10, 20 and 30 s in control and 3.3 mM gentamicin-treated vesicles. There was no significant change in the uptake between control and gentamicin-treated vesicles prepared from outer cortex and outer medulla (data not shown).

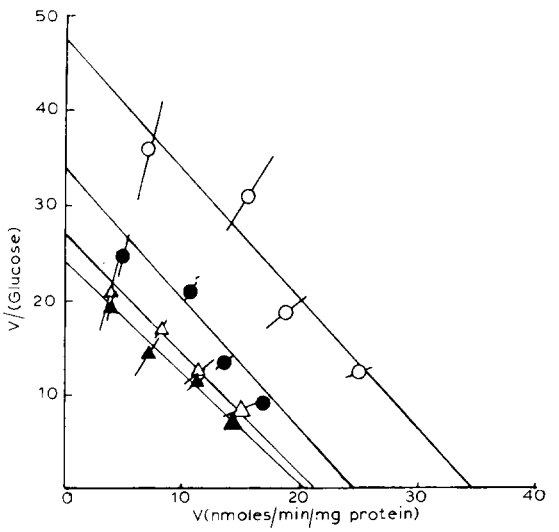

Fig. 6. Eadie-Hofstee plots of Na^+-dependent components of the D-glucose flux into outer medullary brush-border membrane vesicles. Gentamicin was added to the vesicles to the appropriate concentration (○, control; ●, 3.3 mM gentamicin; △, 6.6 mM gentamicin; ▲, 9.9 mM gentamicin). Least-squares fits to these plots yield parallel regression lines, indicating that gentamicin decreases V_{max} in a dose-dependent manner, while it does not change K_m values.

Discussion

This paper presents the results of a series of experiments which investigate gentamicin toxicity on Na^+-dependent D-glucose transport system in vitro.

We found the same osmotically active space and nonspecific binding for control and gentamicin-treated brush-border membrane vesicles (Fig. 1). There was no difference in the passive permeability properties between control and gentamicin-treated brush-border membrane vesicles, measured by the time dependence of the L-glucose efflux from brush-border membrane vesicles (Fig. 2). Thus we cannot find any alteration in the integrity or the membrane properties of gentamicin-treated brush-border membrane vesicles.

Knauss et al. [16] reported that the activity of alkaline phosphatase in brush-border membrane vesicles isolated from gentamicin-administered rat kidneys was less than that from normal rat kidneys. However we could not find any significant difference in the activities of this enzyme between control and 1 mM gentamicin-treated brush-border membrane vesicles (data not shown). Thus gentamicin does not affect alkaline phosphatase in either outer cortical or outer medullary brush-border membrane vesicles during incubation for 60 min at 37°C.

In control preparations the kinetic parameters of D-glucose transport are different in outer cortical and outer medullary brush-border membrane vesicles and there are two distinct D-glucose transport sites along the proximal tubule. Kinetic analyses of D-glucose transport into 1 mM gentamicin-treated brush-border membrane vesicles demonstrated that gentamicin decreased V_{max} in outer cortical preparations, while it did not affect it in outer medullary preparations. With regard to K_m, there was no effect of gentamicin in both vesicle preparations (Table I). Thus 1 mM

gentamicin only affects the outer cortical D-glucose transport site. However, the experiments using higher concentrations of gentamicin (3.3, 6.6, 9.9 mM) showed a dose-dependent decrease of V_{max} in both outer cortical and outer medullary brush-border membrane vesicles. Figs. 4 and 6 clearly demonstrated that gentamicin inhibited Na^+-dependent D-glucose transport in a noncompetitive manner. Dixon plots (Fig. 5) yield an inhibition constant (K_i) of about 4 mM in outer cortical and about 7 mM in outer medullary brush-border membrane vesicles.

In our previous reports, we characterized the effect of anhydro-4-epitetracycline, which is the most toxic substance formed upon tetracycline degradation, on Na^+-dependent D-glucose transport [15,17]. The same inhibitory effct as mentioned above was observed in anhydro-4-epitetracycline-administered rabbits. It is of interest that there was no alteration of the transport system when brush-border membrane vesicles were incubated with anhydro-4-epitetracycline in vitro.

In equilibrium exchange conditions, there was no significant change in D-glucose uptake between control and gentamicin-treated vesicles, while gentamicin inhibited the Na^+-dependent D-glucose transport in zero trans conditions. These results suggest the hypothesis that gentamicin may stimulate the sodium permeability across membranes and rapidly dissipate the Na^+ gradient, the driving force of D-glucose transport. We are, however, reluctant to accept this hypothesis, because gentamicin has low solubility in lipids [18] and there has been no report of an ionophore-like effect of gentamicin. It is known that kinetic parameters of Na^+-dependnet D-glucose transport obtained in zero trans conditions are different from those obtained in equilibrium exchange conditions, possibly because of the difference in the manner of carrier movements [19]. We cannot exclude the possibility that gentamicin inhibits Na^+ or glucose binding to the carrier in zero trans conditions. In equilibrium exchange conditions, the carrier is loaded with glucose and Na^+ and this carrier-substrate complex may behave in a quite different manner. It is well established that gentamicin is absorbed by pinocytosis from the proximal tubular lumen and that pinocytotic vesicles then fuse with lysosomes [9]. The first step leading to cellular uptake of cationic gentamicin is binding to anionic sites on brush-border membranes. The anionic binding sites appear to be made up of one or more phospholipids (predominantly phosphatidylinositol) [16]. Thus, gentamicin may alter the fluidity of the lipid bilayer leading to the inhibition of carrier-mediated transport only in zero trans conditions.

The characteristic binding of gentamicin to membrane phospholipids may be a factor leading to development of its toxicity. It has been known that segment 1 and segment 2 are more vulnerable to gentamicin nephrotoxicity than segment 3 [4,14]. This is partly due to a much higher gentamicin load in segments 1 and 2 than in segment 3. We, however, demonstrate in vitro that 1 mM gentamicin alters the D-glucose transport system only in outer cortical brush-border membrane vesicles (segment 1 and segment 2) and that the K_i value in the outer cortical preparation is smaller than that in the outer medullary one (segment 3). These results indicate that two distinct D-glucose transport sites may have different susceptibilities to gentamicin toxicity or that the phosphatidylinositol contents in the outer cortical and outer medullary brush-border membranes may differ. The latter suggestion is supported by the evidence that diabetic rats with lower renal cortical phosphatidylinositol content are more resistant to gentamicin nephrotoxicity than normal rats [20].

Acknowledgements

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