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Inhibition of alkaline phosphatase activity and D-glucose uptake in rat renal brush-border membrane vesicles by aminoglycosides

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The binding of aminoglycoside antibiotics to, and their effects on, the plasma membrane were studied using isolated rat renal brush-border membrane vesicles. Dibekacin was noted to bind with brush-border membrane vesicles having a single class of many binding sites. ^{3}H-labeled dibekacin binding was inhibited competitively by unlabeled dibekacin, gentamicin or amikacin. The inhibition constants obtained from the Dixon plots followed the order of gentamicin ≃ dibekacin > amikacin. The alkaline phosphatase activity of brush-border membrane vesicles was inhibited by gentamicin significantly, as was also observed by a histochemical study. Sodium-dependent D-glucose uptake by brush-border membrane vesicles was significantly inhibited by the addition of gentamicin.

Introduction

The clinical use of aminoglycoside antibiotics is limited, due to their nephrotoxic effects [1,2]. Though the detailed mechanisms of nephrotoxicity are still unclear, nephrotoxicity initially starts by accumulation of antibiotics in renal proximal tubular cells [3]. Aminoglycosides have been found to be taken up by tubular cells by pinocytosis through the brush-border membrane [4,5]. In a previous report of ours [6], the binding of dibekacin to the brush-border membrane and basolateral membrane vesicles was demonstrated, and aminoglycosides shown to accumulate to proximal tubular cells after passing through the brush-border membrane. This membrane of renal proximal tubular cells is important for secretion and reabsorption processes. Many kinds of enzymes and carriers are located in the brush-border membrane to maintain various complicated functions.

In the present paper, an examination was made of the affinity of aminoglycosides toward brush-border membrane vesicles, the effects of gentamicin on the membrane-bound enzyme, alkaline phosphatase, and the uptake of D-glucose or inorganic phosphorus (P_i) by brush-border membrane vesicles.

Materials and Methods

Materials. Dibekacin and ^{3}H-labeled dibekacin (66.0 μCi/mg) were kindly provided by Meiji Seika Kaisha Ltd. (Tokyo). Gentamicin and amikacin were obtained from Shionogi Pharmaceutical Co. Ltd. (Osaka) and Banyu Pharmaceutical Co. Ltd. (Tokyo), respectively. ^{14}C-labeled D-glucose (14.4 mCi/nmol) and [^{32}P]orthophosphoric acid (carried free) were purchased from New England Nuclear. All other

Abbreviations: Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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reagents were of the best grade available. Male Wistar rats (200–250 g) were purchased from Shizuoka Agricultural Co. (Shizuoka).

Preparation of membrane vesicles. Brush-border membrane vesicles were prepared from rat kidney cortex by the calcium precipitation method [7]. The activity of the marker enzyme, alkaline phosphatase, was enriched by about 9 times that of the homogenate. The activities of cytochrome *c* oxidase, *N*-acetyl-β-D-glucosaminase and glucose-6-phosphatase activities in the brush-border membrane fraction were lower than those in the homogenate. $(Na^+ + K^+)$-ATPase activity was very low and, consequently, contamination of the basolateral membrane was negligible. The membrane preparation was suspended in 20 mM Tris-Hepes buffer (pH 7.0) containing 250 mM mannitol.

Binding study. The binding affinity of aminoglycosides toward brush-border membrane vesicles was determined by observing the inhibitory effects of cold aminoglycosides on the binding of ^{3}H-labeled dibekacin, using the rapid filtration technique. 30 μl of a brush-border membrane vesicle suspension were incubated at 37°C for 30 min with 30 μl of ^{3}H-labeled dibekacin and an equal volume of unlabeled aminoglycosides (final concn., 10, 20, 50, 100 and 200 μM). 2 ml of ice-cold Tris-Hepes buffer were added followed by rapid filtration (Millipore, HA-type, 0.45 μm). The vesicles trapped on the filter were washed twice with 2.0 ml of the ice-cold buffer. The filter was dried and the radioactivity was counted by a liquid scintillation counter (Aloka 903) using toluene cocktail as the scintillator. Non-specific binding of the radioactivity to the filter was determined separatedly and subtracted from each experimental value.

Histochemical staining of alkaline phosphatase. The rats were injected with gentamicin (120 mg/kg per day) of saline (control) intraperitoneally on each of 7 days, and then killed. The kidney was removed and fixed with 10% formaline/1% calcium chloride solution at 4°C for 24 h, and then washed several times with 30% sucrose containing arabic gum (1%) at 4°C. The cortex sections, 4 μm thickness, were incubated for 10 min at room temperature in a reaction mixture containing 0.05 M 2-amino-2-methyl-1,3-propandiol (pH 9.75), 0.05% (w/v) 3-hydroxy-2-anthranoic acid 2-methyl anilide phosphate, 0.25% (v/v) dimethyl formamide and 0.1% (w/v) Fast blue RR salt. The sections were washed thoroughly with distilled water, post-stained with 1% methyl green in 0.1 M veronal acetate buffer (pH 4.0), dehydrated by ethanol and embedded in gelatin.

Transport of glucose and phosphorus. The effects of gentamicin on the uptake of D-glucose or P_i by brush-border membrane vesicles in the presence and absence of a sodium gradient were studied by rapid filtration at 25°C. In the sodium gradient ($[Na^+]_{in} < [Na^+]_{out}$)-dependent uptake studies, 30 μl of the pre-incubated brush-border membrane vesicle suspension was added to 170 μl of the incubation medium consisting of 20 mM Tris-Hepes (pH 7.0), 50 mM mannitol and 100 mM NaCl containing ^{14}C-labeled D-glucose (final concn., 50 μM) or ^{32}P (final concn., 100 μM). In the equilibrated-sodium concentration ($[Na^+]_{in} = [Na^+]_{out}$) uptake studies, the vesicles were suspended in the incubation medium and pre-incubated for 30 min so as to equilibrate the sodium concentration across the membrane. The pre-incubated suspension (30 μl) was added to 170 μl of the incubation medium containing ^{14}C-labeled glucose (final concn., 50 μM) or ^{32}P (final concn., 100 μM). At the specified time, incubation was terminated by the addition of 2.0 ml of ice-cold

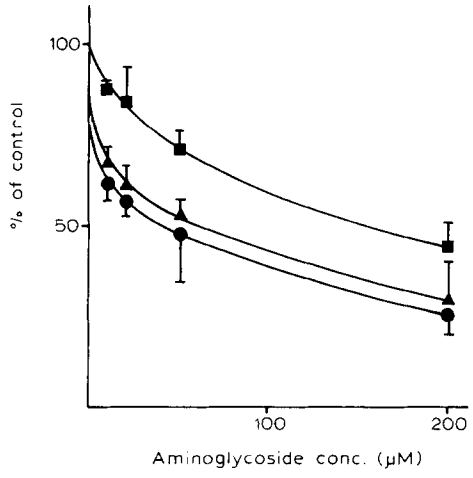

Fig. 1. Effect of unlabeled aminoglycosides on ^{3}H-labeled dibekacin binding to brush-border membrane vesicles. Values shown represent the mean ± S.E. of three experiments. ●, gentamicin; ▲, dibekacin; ■, amikacin.

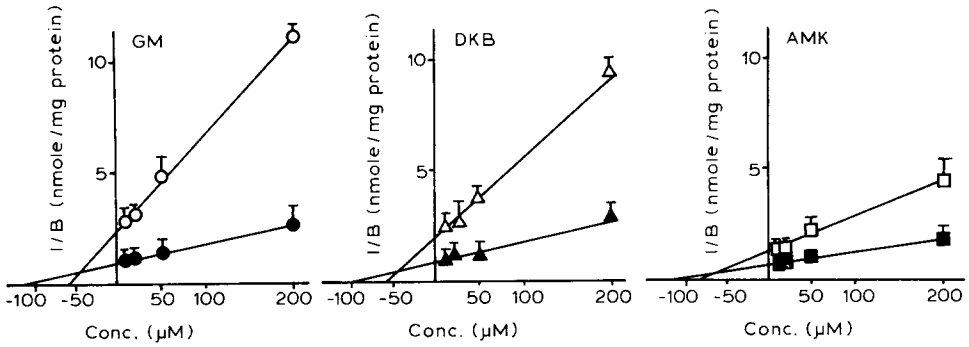

Fig. 2. Dixon plots of the effect of unlabeled gentamicin (GM), dibekacin (DKB) and amikacin (AMK) on 10 (○, △, □) and 20 μM (●, ▲, ■) ^{3}H-labeled dibekacin binding to brush-border membrane vesicles. Values shown represent the mean ± S.E. of three experiments. The inhibition constants (K_i) were 41.82 ± 6.16 for gentamicin, 42.66 ± 11.32 for dibekacin and 59.37 ± 3.03 μM for amikacin. Statistical difference of K_i was calculated by Student's t-test, and K_i values between gentamicin and amikacin were significantly different ($P < 0.05$).

incubation medium and was filtered off rapidly.

Analytical methods. Protein content was determined by the method of Lowry et al. [8], using bovine serum albumin as the standard. Alkaline phosphatase activity was assayed with *p*-nitrophenyl phosphate as the substrate [9]. ($Na^+ + K^+$)-ATPase [10], *N*-acetyl-β-D-glucosaminidase [11], cytochrome *c* oxidase [12] and glucose-6-phosphatase [13] activities were measured by methods previously reported.

Results

Effects of cold aminoglycosides on the binding of ^{3}H-labeled dibekacin

The binding of ^{3}H-labeled dibekacin to brush-border membrane vesicles decreased with increasing concentration of unlabeled aminoglycosides, gentamicin, dibekacin and amikacin (Fig. 1). Dixon plots indicated these aminoglycosides to inhibit this binding competitively (Fig. 2).

Inhibition of alkaline phosphatase activity in brush-border membrane vesicles

The effects of gentamicin on the membrane-bound enzyme, alkaline phosphatase, was investigated. The activity of this enzyme was significantly inhibited by 10^{-3} or 10^{-4} M gentamicin (Table I).

Histochemical observation of alkaline phosphatase

The effects of gentamicin administration on the alkaline phosphatase activity of the kidney cortex were studied histochemically. As shown in Fig. 3,

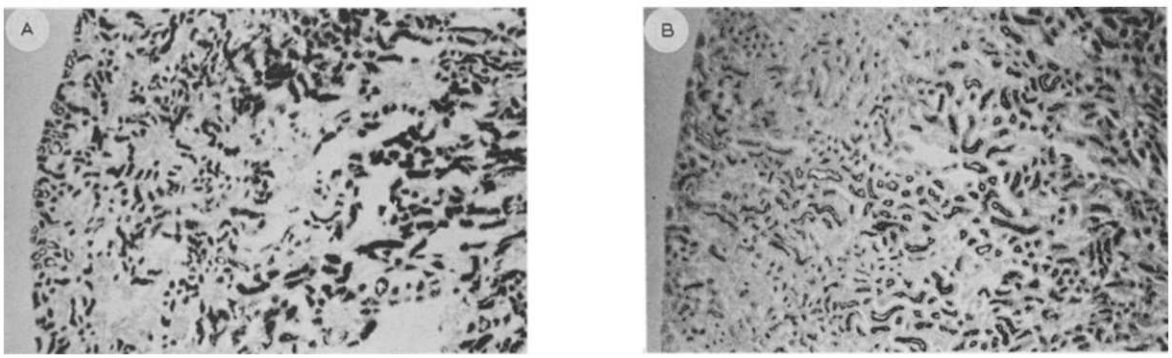

Fig. 3. Light micrographs of alkaline phosphatase activity of kidney cortex (×8). (A) Saline administered, (B) gentamicin (120 mg/kg per day, 7 days) administered.

TABLE I

EFFECT OF GENTAMICIN ON ALKALINE PHOSPHATASE OF BRUSH-BORDER MEMBRANE VESICLES

Values shown represent the mean ± S.E. of three experiments. * $P < 0.05$; ** $P < 0.01$.

Concn. (M)	% of control
Control	100.0 ± 5.3
10^{-6}	96.5 ± 3.4
10^{-5}	95.4 ± 2.9
10^{-4}	84.0 ± 7.5 *
10^{-3}	71.2 ± 6.9 **

there was activity in the brush-border membrane of the proximal tubule (Fig. 3 (A)). It decreased with the administration of gentamicin to the rat (Fig. 3 (B)). This activity was clearly inhibited, particularly near the renal capsule.

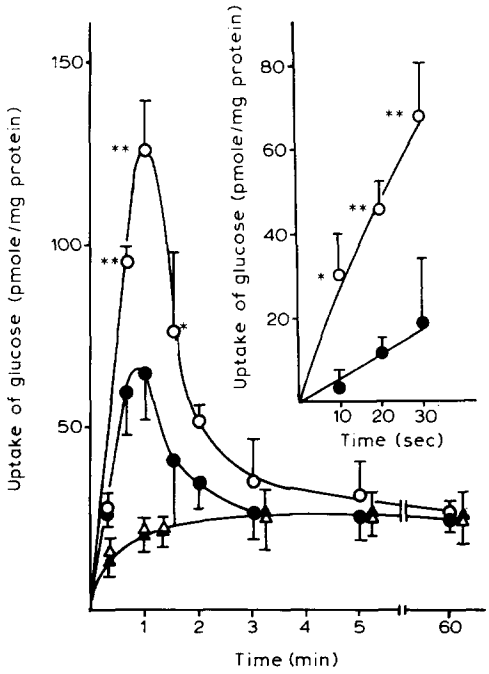

Fig. 4. D-Glucose uptake by brush-border membrane vesicles in the presence (●, ▲) and absence (○, △) of 1 mM gentamicin. Values shown represent the mean ± S.E. of three experiments. ●, ○, presence of sodium gradient ($[Na^+]_{in} < [Na^+]_{out}$); ▲, △, equilibrated sodium ($[Na^+]_{in} = [Na^+]_{out}$) * $P < 0.05$; ** $P < 0.01$.

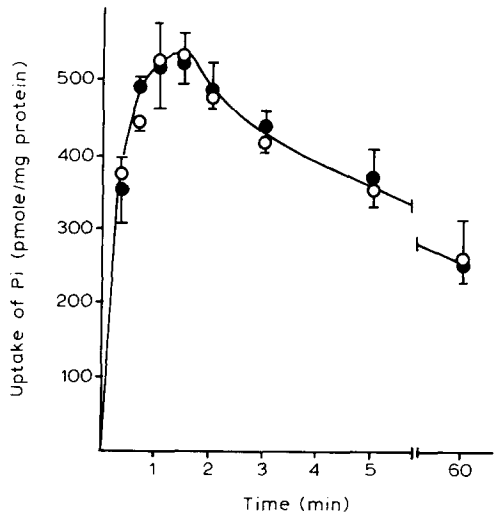

Fig. 5. P_i uptake by brush-border membrane vesicles in the presence (●) and absence (○) of 1 mM gentamicin under the sodium gradient ($[Na^+]_{in} < [Na^+]_{out}$).

Effects of gentamicin on D-glucose and P_i transport

The effects of gentamicin on the transport of D-glucose and P_i across brush-border membrane vesicles were studied. The overshoot phenomenon of D-glucose uptake was detected in the presence of a sodium gradient ($[Na^+]_{in} < [Na^+]_{out}$), but the extent of which decreased by about 50% in the presence of gentamicin (Fig. 4 (A)). Further, the initial uptake of D-glucose in the presence of the sodium gradient decreased significantly with the addition of gentamicin (Fig. 4 (B)). However, the D-glucose uptake by the vesicles at the equilibrated sodium concentration ($[Na^+]_{in} = [Na^+]_{out}$) was not influenced by gentamicin. On the other hand, with or without gentamicin, no significant difference could be observed in P_i uptake in the presence of the sodium gradient, $[Na^+]_{in} < [Na^+]_{out}$ (Fig. 5).

Discussion

The kidney is one of the major sites for the toxicity of aminoglycosides, and nephrotoxicity appears to start through interactions between aminoglycosides and the plasma membrane of renal proximal tubular cells [1,2]. Membrane vesicles have been widely used as a model system for studying renal functions and properties [14–16]. This approach helps to obviate complications en-

countered in studying nephrotoxicity in vivo or in whole cell systems.

^{3}H-labeled dibekacin binding to brush-border membrane vesicles has already been examined as a function of concentration, and saturation in regions of high dibekacin concentrations was observed [6]. Discrimination between ^{3}H-labeled dibekacin binding and uptake into brush-border membrane vesicles has been attempted by changing the medium osmolarity. About 50% of ^{3}H-labeled dibekacin was found to bind to the surface of the brush-border membrane and about 50% to be incorporated into the vesicles [6].

In this study, we examined the affinity of aminoglycosides to the brush-border membrane and changes brought about in the functions of this membrane by aminoglycosides. Dixon plots of ^{3}H-labeled dibekacin binding to brush-border membrane vesicles in the presence of unlabeled gentamicin, dibekacin or amikacin indicated that these compounds inhibited the binding competitively (Fig. 2). It thus appears that they must have the same binding site(s) on brush-border membrane vesicles. Affinity toward the binding site of the brush-border membrane was in the order of gentamicin $\simeq$ dibekacin > amikacin (Fig. 2). This order is consistent with that of aminoglycoside nephrotoxicity as reported on the basis of in vivo experimental results [17,18]. Differences in affinity toward the brush-border membrane may reflect the intensity of aminoglycoside nephrotoxicity.

Alkaline phosphatase has been reported to be localized in the brush-border membrane, tightly interacting with phosphatidylinositol, and to be liberated from brush-border membrane by phospholipase C [19,2]. Sastrasinh et al. [21] have reported that aminoglycosides have high affinity to phosphatidylinositol. Thus, gentamicin probably binds to the phosphatidylinositol of the brush-border membrane and inhibits the neighbouring alkaline phosphatase activity (Table I). In consideration of this, inhibition of alkaline phosphatase in Table I may require a gentamicin concentration higher than that expected from the affinity of gentamicin toward the binding site (Fig. 2). This inhibition was also observed by a histochemical study (Fig. 3). The physiological role of alkaline phosphatase in the brush-border membrane still remains unclear. Previously, Peticlere and Plante [22] proposed the hypothesis that alkaline phosphatase performs some roles in the renal transport of P_i. However, some investigators report alkaline phosphatase to be incapable of this, since its enzymatic removal from the brush-border membrane by phosphatidylinositol-specific phospholipase C has no effect on P_i uptake [19]. Recently, Beliveau and Brunette [23] presented evidence that alkaline phosphatase may be a phosphate-binding protein. P_i is reabsorbed from the renal proximal tubule by a sodium-dependent, carrier-mediated transport system [24], and its transport by isolated renal brush-border membrane vesicles has been studied by Hoffmann et al. [25]. Thus, we investigated the effects of gentamicin on P_i uptake in brush-border membrane vesicles. On treating the vesicles with gentamicin, no change could be noted in the sodium-dependent P_i uptake (Fig. 5).

The initial uptake of sodium-dependent D-glucose decreased significantly by gentamicin, but at 60 min it was essentially the same irrespective of the sodium gradient ($[Na^+]_{in} < [Na^+]_{out}$ and $[Na^+]_{in} = [Na^+]_{out}$ (Fig. 4). There is thus the possibility that gentamicin does not alter the intravesicular volume of brush-border membrane vesicles. Two explanations are possible: (1) gentamicin directly interacts with the glucose carrier in the brush-border membrane to inhibit the glucose uptake, or (2) gentamicin affects D-glucose uptake indirectly through stimulation of sodium uptake. However, since sodium-dependent P_i uptake was not changed by gentamicin treatment, the latter possibility appears unlikely. That D-glucose uptake into brush-border membrane vesicles decreased by gentamicin is consistent with the pathogenesis that glycosuria developed following gentamicin administration to dogs [26]. The uptake of D-glucose decreased by gentamicin may possibly be due to failure of the D-glucose carrier to function in brush-border membrane or some alteration in the membrane structure that would affect the carrier function.

During the preparation of this manuscript, Horio et al. [27] reported gentamicin to inhibit sodium-dependent D-glucose transport in rabbit kidney brush-border membrane vesicles. This finding may possibly help elucidate the mechanism(s) of nephrotoxicity induced by aminoglycosides.

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References

1 Kaloyanides, G.J. and Pastoriza-Munoz, E. (1980) Kidney Int. 18, 571–582
2 Morin, T.P., Viotte, J., Vandewalle, A., Hoof, F.V., Tulkens, P. and Fillastre, J.P. (1980) Kidney Int. 18, 583–590
3 Pastoriza-Munoz, E., Bowman, R.L. and Kaloyanides, G.J. (1979) Kidney Int. 16, 440–450
4 Silverblatt, F.J. and Kuehn, C. (1979) Kidney Int. 15, 335–345
5 Wedeen, R.P., Batuman, V., Cheeks, C., Marquet, E. and Sobel, H. (1983) Lab. Invest. 48, 212–223
6 Aramaki, Y., Takahashi, M., Inaba, A., Ishii, Y. and Tsuchiya, S. (1986) Biochim. Biophys. Acta 862, 111–118
7 Malathi, P., Preiser, H., Fairclough, P., Mallett, P. and Crane, R.K. (1979) Biochim. Biophys. Acta 554, 259–263
8 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
9 Bessey, O.A., Lowry, O.H. and Brock, M.J. (1946) J. Biol. Chem. 164, 321–326
10 Jorgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
11 Niebes, P. and Ponard, G. (1975) Biochem. Pharmacol. 24, 905–909
12 Wharton, D.C. and Tzagoloff, A. (1967) Methods Enzymol. 10, 245–250
13 Aronson, N.N. and Touster, O. (1974) Methods Enzymol. 31, 90–102
14 Hesney, R.W., Sacktor, B. and Rowen, R. (1973) J. Biol. Chem. 248, 2182–2191
15 Lipsky, J.J., Cheng, L., Sacktor, B. and Lietman, P.S. (1980) J. Pharmacol. Exp. Ther. 215, 390–393
16 Hori, R., Takano, M., Okano, T., Kitazawa, S. and Inui, K. (1982) Biochim. Biophys. Acta 692, 97–100
17 Viotte, G., Olier, B., Morin, J.P., Hemet, J. and Fillastre, J.P. (1983) Drugs Exptl. Clin. Res. 9, 735–747
18 Brion, N., Barge, J., Godefroy, I., Dromer, F., Dubois, C., Contrepois, A. and Carbon, C. (1984) Antimicrob. Agents Chemother. 25, 168–172
19 Schali, C., Vaughn, D.A. and Fanestill, D.D. (1984) Biochim. Biophys. Acta 769, 277–283
20 Yusufi, A.N.K., Low, M.G., Turner, S.T. and Dousa, T.P. (1983) J. Biol. Chem. 258, 5695–5701
21 Sastrasinh, M., Knauss, T.C., Weinberg, J.M. and Humes, H.D. (1982) J. Pharmacol. Exp. Ther. 222, 350–358
22 Peticlere, C. and Plante, G.E. (1981) J. Physiol. Pharmacol. 59, 311–319
23 Beliveau, R. and Brunette, G. (1983) Kidney Int. 23, 95
24 Cheng, L. and Sacktor, B. (1981) J. Biol. Chem. 256, 1556–1564
25 Hoffmann, N., Thees, M. and Kinne, R. (1976) Pflugers Arch. 362, 147–156
26 Cronin, R.E. (1979) Clin. Nephrol. 11, 251–256
27 Horio, M., Fukuhara, Y., Orita, Y., Akanishi, T., Nakahara, H., Moriyama, T. and Kumada, T. (1986) Biochim. Biophys. Acta 858, 153–160