Characterization of Protamine Uptake by Opossum Kidney Epithelial Cells

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Protamine, a mixture of polypeptides that is rich in arginine, has been used clinically as an antidote to heparin overdoses and a complexing agent in a long-acting insulin preparation. When protamine is administered intravenously, its abundant accumulation in the kidneys has been reported. However, the renal uptake mechanism for protamine is not clear. In this study, we examined the transport mechanism for protamine in opossum kidney (OK) cells, a suitable *in vitro* model for renal proximal tubular epithelial cells. Flow cytometric analysis revealed that the association of fluorescein isothiocyanate (FITC)-labeled protamine from salmon (FITC-protamine) by OK cells was inhibited by unlabeled protamine in a concentration-dependent manner. The association of FITC-protamine was temperature- and energy-dependent. Confocal microscopy analysis showed that the fluorescence was localized in the cytoplasm and nucleus of OK cells. In addition, FITC-protamine association was inhibited by cationic drugs such as polycationic gentamicin and polymyxin B, but it was increased by a basic amino acid, arginine. Inhibitors for clathrin- and caveolin-dependent endocytosis showed inhibitory effects on FITC-protamine association. Pretreatment with heparinase III partially but significantly decreased the association of FITC-protamine. These results suggest that protamine may be taken up by OK cells *via* receptor-mediated endocytosis, which may result in its localization in the cytoplasm and nucleus of the cells.

Key words protamine; endocytosis; renal proximal tubular cell; opossum kidney cell; polycationic drug

Protamines are small nuclear proteins that are very basic due to their high arginine contents. They are usually isolated from the sperm of salmon and certain other fish species. Protamine binds to heparin, which is strongly acidic, through an electrostatic interaction, and forms a stable salt that abolishes the anticoagulant activity of heparin. Therefore, protamine sulfate is regularly used following a cardiopulmonary bypass and cardiac catheterization to reverse the anticoagulant activity of heparin.¹⁾ In addition, it is prevalently used as a complexing agent in the formulation of long-acting insulin products.²⁾ Furthermore, protamine is known to complex and condense DNA from an extended conformation to highly compact structures, and to possess several amino acid sequences resembling that of a nuclear localization signal. Therefore, protamine has been developed as a carrier for gene delivery.^{3–5)} Thus, protamine has been employed for various purposes in clinical and preclinical stages.

In spite of the clinical application of protamine sulfate for decades, there have only been a few reports on the pharmacokinetics and tissue distribution of injected protamine.^{6–8)} DeLucia *et al.*⁶⁾ showed that the radioactivity per gram tissue was highest in the kidneys at 3 min after an intravenous administration of ¹²⁵I-labeled protamine to rats, the blood half-life being 24 min. However, it is not clear whether the radioactivity in tissues after an injection of radiolabeled protamine results from the distribution of an intact form of protamine or its degraded products in tissues.

We recently reported that protamine, as well as other cationic proteins and peptides, inhibits the uptake of a nephrotoxic drug, gentamicin, which is highly accumulated in the kidneys, by cultured opossum kidney (OK) epithelial cells.⁹⁾ OK is a renal tubular epithelial cell line expressing megalin and

cubilin,¹⁰⁾ multiligand endocytic receptors that play important roles in renal endocytic uptake of low-molecular weight proteins, albumin, and cationic drugs such as gentamicin passing through the glomerulus.^{11,12)} Therefore, we suggested that protamine might decrease the uptake of gentamicin by interacting with the endocytic receptor that is involved in gentamicin binding to the plasma membrane surface.⁹⁾

Protamine and its low-molecular weight fragments are reported to exhibit membrane-translocating activity comparable to those of other cell-penetrating peptides (CPPs) such as the human immunodeficiency virus (HIV) TAT peptide.^{5,13,14)} Reynolds *et al.*⁵⁾ reported strong nuclear localization of fluorescent-labeled protamine in HeLa cells and Caco-2 cells, a human cervical cancer cell line and a human colon carcinoma cell line, respectively. Park *et al.*¹³⁾ showed that low-molecular weight fragments derived from protamine accumulated in the cytoplasm and nucleus of 293T human embryonic kidney transformed cells. However, the molecular mechanisms underlying the cellular uptake of protamine have not been fully elucidated.

The aim of this study was to characterize the uptake of protamine by OK cells, which are widely employed as an *in vitro* model of renal proximal tubular epithelial cells.^{9,10,15–17)} The present findings indicated that protamine is taken up in energy- and temperature-dependent manners, and that its internalization is mediated *via* receptor-mediated, and partially *via* clathrin- and caveolin-dependent, endocytosis.

MATERIALS AND METHODS

Materials Protamine sulfate from salmon, 2,4-dinitrophenol, gentamicin sulfate, polymyxin B sulfate, and chlorpromazine hydrochloride were obtained from Nacalai Tesque (Kyoto, Japan). Fluorescein isothiocyanate, fluorescein isothiocya-

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nate (FITC)-labeled bovine serum albumin (FITC-albumin), phenylarsine oxide, nystatin, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA), cytochalasin D and heparinase III from *Flavobacterium heparinum* were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). L-Arginine monohydrochloride, γ -globulin from human serum, methyl- β -cyclodextrin and Hoechst 33342 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D-Mannitol was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All other chemicals used in the experiments were commercial products of the highest purity available.

Preparation of FITC-Protamine Labeling of protamine with FITC was performed as follows. Briefly, 37 mg FITC and 400 mg protamine sulfate were dissolved in 0.1 M borate buffer (pH 9.0). After incubation for 4 h at room temperature, the pH of the mixture was adjusted to 7.5 with 0.8 M boric acid. The solution was then dialyzed with a cellulose membrane for 90 h at 4°C and then concentrated by freeze-drying. To remove unbound FITC completely, the lyophilized products were subjected to gel filtration on a Sephadex G-15 column (2.1 by 40 cm), using 0.1% trifluoroacetic acid (TFA) in distilled water as the mobile phase. The fractions that were collected by measuring fluorescence intensity (Ex. 500 nm, Em. 520 nm) were dialyzed to remove TFA with a cellulose membrane for 24 h at 4°C, and then concentrated by freeze-drying. The lyophilized proteins were used as FITC-protamine and were stored in a -30°C freezer until use. The mass spectra of unlabeled protamine and FITC-protamine are shown in Supplemental Fig. 1.

Cell Culture OK cells were cultured in medium 199 (Gibco BRL, Life Technologies, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum without antibiotics, under an atmosphere of 5% CO₂-95% air at 37°C, and subcultured every 7 d using 0.05% trypsin and 0.02% ethylenediamine-tetraacetic acid (EDTA). The medium was replaced with fresh medium every 2 d, and the cells were used for the experiments at 5–7 d after seeding.

Fig. 1. Effect of Unlabeled Protamine on the Association of Fluorescein Isothiocyanate-Labeled Protamine (FITC-Protamine) by OK Cells

OK cells were incubated with FITC-protamine (30 μ g/mL) for 60 min at 37°C in the absence (control, open circle) or presence (closed circles) of unlabeled protamine at various concentrations (0.1, 0.3, 1 and 3 mg/mL). Then, the cells were washed, harvested with trypsin/EDTA and analyzed by flow cytometry. The effect of unlabeled protamine on FITC-protamine association is shown relative to the fluorescence in the absence of unlabeled protamine (control). Each symbol represents the mean $\pm$ S.E. of three determinations. * p < 0.05, significantly different from the value in the absence of unlabeled protamine (control).

Flow Cytometry Uptake studies were performed on the confluent cells attached to 12-well plates. Experiments were performed in Dulbecco's phosphate-buffered saline (PBS buffer containing, in mM, 137 NaCl, 3 KCl, 8 Na₂HPO₄, 1.5 KH₂PO₄, 1 CaCl₂ and 0.5 MgCl₂). PBS buffer containing 5 mM D-glucose [PBS(G) buffer] was used as an incubation buffer. After removal of the culture medium, each well was washed and preincubated with PBS(G) buffer. Then, PBS(G) buffer containing FITC-protamine or FITC-albumin was added to each well, and the cells were incubated at 37°C or 4°C for a specified period. At the end of the incubation, the cells were rinsed rapidly three times with 1 mL of ice-cold PBS buffer. Then, the cells were incubated with 500 μ L of phosphate buffer containing 0.05% trypsin and 0.02% EDTA for 20 min at 37°C to detach the cells and to remove surface-bound compounds. The cells were centrifuged at 4°C for 5 min at 10000 rpm and the supernatant was discarded. The cells were suspended in 500 μ L of a buffer [10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.5), 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂], and analyzed immediately with a flow cytometer (EPICS XL, Beckman Coulter, Tokyo, Japan) using EXPO™32 MultiCOMP software (Beckman Coulter). In each assay, the fluorescence of 10000 cells was analyzed.

Confocal Laser Scanning Microscopy OK cells were cultured in 35-mm glass-bottom dishes for 5 d. The cells were incubated with FITC-protamine (500 μ g/mL) for 60 min at 37°C or 4°C. At 30 min before the end of the incubation, Hoechst 33342 (10 μ M) was added to the incubation buffer containing FITC-protamine. After being washed with ice-cold PBS buffer three times, the live cells were viewed using a confocal laser scanning fluorescence microscope (LSM510 invert., Carl Zeiss, Göttingen, Germany).

Cell Treatment To examine the effects of various compounds on the uptake of FITC-protamine, OK cells were preincubated with PBS(G) buffer in the absence or presence of each compound at 37°C. Pretreatment with 2,4-dinitrophenol (1 mM) was performed for 15 min, followed by coincubation with FITC-protamine for 60 min. Pretreatment with phenylarsine oxide (10 μ M) was performed for 15 min, but it was not present during the incubation with FITC-protamine. Pretreatment with chlorpromazine (50 or 100 μ M), nystatin (10, 25 or 50 μ M), methyl- β -cyclodextrin (5 mM), and EIPA (50 or 100 μ M) was performed for 30 min, followed by coincubation with FITC-protamine for 60 min. Pretreatment with cytochalasins B and D (20 μ M) was performed for 120 min, followed by coincubation with FITC-protamine for 60 min. Pretreatment with heparinase III (10, 30 or 100 mU) was performed for 60 min, but it was not coincubated with FITC-protamine. After the preincubation, the cells were washed three times, and the uptake assay was performed as described above. For hypertonic conditions, 400 mM mannitol was added to the incubation buffer containing FITC-protamine. The control cells were treated with the same concentration of dimethyl sulfoxide (DMSO) (0.5%) in each experiment.

Preparation of Brush-Border Membranes from Rat Kidney Experiments involving animals were performed in accordance with the Guide for Animal Experimentation, Hiroshima University, and the Committee of Research Facilities for Laboratory Animal Sciences, Graduate School of Biomedical & Health Sciences, Hiroshima University. Renal

brush-border membranes were isolated from the renal cortices of male Wistar albino rats (190–220 g). The isolation procedure for renal brush-border membranes was based on the Mg/ethylene glycol bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) precipitation method described previously.¹⁸ The isolated membranes were suspended in a buffer comprising 100 mM mannitol and 10 mM HEPES/Tris (pH 7.5), and stored in liquid nitrogen until use.

Western Blot Analysis and Ligand Blotting The crude membrane fraction derived from OK cells was prepared on the sixth day after seeding as described below. Briefly, after removal of the culture medium, each dish was washed with ice-cold PBS buffer and the cells were collected with a rubber policeman. The cell suspension was homogenized for 2 min with an IKA T25 Basic disperser (IKA LABORTECHNIK, Germany) in an ice-cold preparation buffer (150 mM NaCl, 5 mM, EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris, pH 7.4), and was subsequently homogenized with a glass/Teflon Potter homogenizer, 10 strokes, at 1000 rpm. The homogenate was centrifuged at 3000×*g* for 15 min at 4°C. The supernatant was centrifuged at 15000×*g* for 15 min at 4°C, and the resulting supernatant was centrifuged at 100000×*g* for 1 h at 4°C. The pellet was resuspended in an ice-cold preparation buffer comprising 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 1% sodium deoxycholate, and then left for 30 min on ice with vortex mixing about every 10 min. Then, the lysate was centrifuged at 8000×*g* for 3 min at 4°C. The supernatant, which contained crude membrane fractions of OK cells, was mixed with a loading buffer. The brush-border membranes of rat renal cortices were used for comparison. The samples (100 µg/lane for OK cells, 22.5 µg/lane for rat renal brush-border membrane) were subjected to SDS-polyacrylamide gel electrophoresis on 4–15% precast linear gradient polyacrylamide gradient gels (Bio-Rad Laboratories, Hercules, CA, U.S.A.), and the proteins were transferred for 75 min to polyvinylidene difluoride (PVDF) membranes at 4°C. For Western blotting, each membrane was blocked in 5% non-fat dry milk in Tris-buffered saline (TBS, 150 mM NaCl, 20.5 mM Tris, pH 7.4) containing 0.05% Tween 20 (TBS-T) overnight at 4°C. The membrane was washed twice for 5 min in TBS-T and once for 5 min in TBS. Then, the membrane was incubated with anti-cubilin goat antibodies (T-16, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) (1:100 dilution). The membrane was washed three times in TBS-T, and then incubated with horseradish peroxidase-conjugated anti-goat donkey immunoglobulin G (IgG) antibodies (Santa Cruz Biotechnology, Inc.) (1:500 dilution), washed three times in TBS-T, and visualized with enhanced chemiluminescence (Pierce Western Blotting Substrate Plus, Thermo Scientific, Rockford, IL, U.S.A.). The blot was visualized with a luminescent image analyzer (LAS 4000plus, GE Healthcare Japan Corporation, Tokyo, Japan). For ligand blotting with FITC-protamine, the PVDF membrane was blocked in 40 mg/mL bovine serum γ-globulin in TBS-T overnight at 4°C. The membrane was then washed twice for 5 min in TBS-T and once for 5 min in TBS. The membrane was incubated with PBS buffer containing 10 µg/mL FITC-protamine at 37°C for 1 h. The membrane was then washed twice for 5 min in TBS-T and once for 5 min in TBS. The blot was visualized using a Typhoon FLA 7000 imaging system (GE Healthcare Japan Corporation). Protein was determined by the method of

Bradford¹⁹ with bovine serum albumin as the standard.

Data Analysis The half-maximal inhibitory concentration (IC_{50}) value was determined by means of the Hill equation using the KaleidaGraph™ program (Version 3.08, Synergy Software, PA, U.S.A.) for curve-fitting as described previously.²⁰ Statistically significant differences were evaluated by one-way analysis of variance with Tukey–Kramer's test for post hoc analysis. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

Effect of Unlabeled Protamine on FITC-Protamine Association by OK Cells First, we examined whether or not FITC-protamine is taken up by OK cells *via* a specific uptake system. Unlabeled protamine inhibited the association of FITC-protamine (30 µg/mL) at 37°C for 60 min in a concentration-dependent manner, the IC_{50} value being 16.4 µg/mL (Fig. 1). This observation indicates that FITC-protamine was internalized into the cells *via* a specific transport pathway. Furthermore, by employing the data shown in Fig. 1, the Michaelis constant K_m value of the association of protamine was estimated to be 202 µg/mL (see Supplemental Fig. 2).

Time-Course of FITC-Protamine Association by OK Cells The time-course of FITC-protamine association was examined, as shown in Fig. 2A. The association of FITC-protamine increased with time until 30 min, and thereafter it lev-

Fig. 2. Time Courses of the Association of FITC-Protamine and FITC-Albumin by OK Cells

OK cells were incubated with FITC-protamine (30 µg/mL) at 37°C for 15, 30, 45 and 60 min (A), or FITC-albumin (30 µg/mL) at 37°C for 30, 60, 120 and 180 min (B). Then, the cells were washed, harvested with trypsin/EDTA and analyzed by flow cytometry. Each symbol represents the mean±S.E. of three determinations.

eled off. On the other hand, the association of FITC-albumin, a ligand that is taken up *via* receptor-mediated endocytosis by renal proximal tubular epithelial cells, reached the maximum level at around 180 min (Fig. 2B). Thus, the maximal association of FITC-protamine was observed within a shorter incubation period as compared to that of FITC-albumin.

Temperature- and Metabolic Energy-Dependent Association of FITC-Protamine by OK Cells In order to examine the effect of incubation temperature on FITC-protamine uptake, cells were incubated with buffer including 30 μ g/mL FITC-protamine at 37°C or 4°C. As shown in Fig. 3, the association of FITC-protamine at 37°C was significantly greater than that at 4°C. Furthermore, the effect of DNP, an uncoupler

Fig. 3. Effects of 2,4-Dinitrophenol (DNP) and Temperature on the Association of FITC-Protamine by OK Cells

OK cells were incubated with FITC-protamine (30 μ g/mL) at 37°C or 4°C for 60 min in the absence (open columns) or presence (gray columns) of 1 mM DNP. Then, the cells were washed, harvested with trypsin/EDTA and analyzed by flow cytometry. Each column represents the mean $\pm$ S.E. of three determinations. * p < 0.05, significantly different from the value in the absence of DNP at each incubation temperature. † p < 0.05, significantly different from the value under each conditions at 37°C.

of oxidative phosphorylation, on FITC-protamine uptake was examined. Treatment with 1 mM 2,4-dinitrophenol (DNP) significantly inhibited the association of FITC-protamine at 37°C, but not at 4°C (Fig. 3). These results indicate that the uptake of FITC-protamine is dependent on temperature and metabolic energy.

Intracellular Localization of FITC-Protamine Taken Up by OK Cells Intracellular localization of FITC-protamine was examined by confocal laser scanning microscopy (Fig. 4). After OK cells had been incubated with FITC-protamine at 37°C or 4°C for 30 min, confocal microscopy analysis was performed on live OK cells in order to avoid fixation artifacts that were suggested in uptake studies on CPPs.^{21,22} When FITC-protamine was incubated with OK cells at 37°C, punctate and diffuse fluorescence was observed in the cytoplasm of the cells. In addition, staining of the nuclear membrane and nuclei was clearly observed. The fluorescence in the nuclei was not spread evenly but concentrated in certain spots on them. In contrast, little intracellular accumulation of fluorescence was observed when the OK cells were incubated at 4°C for 30 min. However, interestingly, clear fluorescence was observed on the plasma membranes of the cells that had been incubated at 4°C, but not at 37°C.

Effects of Endocytosis Inhibitors on FITC-Protamine Association by OK Cells We characterized the molecular mechanisms underlying the internalization of FITC-protamine by OK cells. First, the effects of inhibitors of clathrin-dependent endocytosis on FITC-protamine were examined (Table 1). The inhibitors for clathrin-dependent endocytosis employed in this study were phenylarsine oxide, an inhibitor of clathrin-coat formation, and chlorpromazine, a cationic amphiphilic drug that induces the redistribution of a clathrin-coated pit component AP-2 to endosomes. The two clathrin-dependent endocytosis inhibitors significantly decreased the association of FITC-protamine. Furthermore, we examined the effect of hypertonicity, which inhibits clathrin-coated pit

Fig. 4. Confocal Laser Scanning Micrographs of OK Cells Incubated with FITC-Protamine

OK cells were incubated with FITC-protamine (500 μ g/mL) at 37°C (A, B and C) or 4°C (D, E and F) for 60 min, including coincubation with 10 μ M Hoechst33342 for 30 min. After being washed, the live cells were observed by confocal laser scanning microscopy. (A, D) FITC-protamine (green), (B, E) Hoechst33342 (blue), (C, F) merge. (Color images were converted into gray scale.)

formation, on FITC-protamine association (Table 1). A high concentration of mannitol (400mM) significantly decreased the association of FITC-protamine. Thus, these observations indicate that FITC-protamine association by OK cells is, at least in part, mediated by clathrin-dependent endocytosis. Next, we examined the effects of inhibitors of caveolin-de-

Table 1. Effects of Hypertonic Conditions, Various Endocytosis Inhibitors and Heparinase III on FITC-Protamine Association by OK Cells

	FITC-protamine association (% of control)
10 μ M Phenylarsine oxide	54.5 $\pm$ 2.6*
50 μ M Chlorpromazine	63.8 $\pm$ 2.7*
100 μ M Chlorpromazine	14.9 $\pm$ 0.5*
400mM Mannitol	57.1 $\pm$ 4.6*
10 μ M Nystatin	121.6 $\pm$ 7.0
25 μ M Nystatin	130.8 $\pm$ 5.1
50 μ M Nystatin	18.1 $\pm$ 0.2*
5mM Methyl- β -cyclodextrin	42.4 $\pm$ 2.6*
50 μ M EIPA	73.2 $\pm$ 2.1*
100 μ M EIPA	53.6 $\pm$ 4.3*
20 μ M Cytochalasin B	111.1 $\pm$ 3.6
20 μ M Cytochalasin D	113.8 $\pm$ 4.5
10mU/mL Heparinase III	105.5 $\pm$ 2.7
30mU/mL Heparinase III	86.5 $\pm$ 2.7*
100mU/mL Heparinase III	71.8 $\pm$ 4.5*

OK cells were treated with various compounds as described under Materials and Methods. The association of FITC-protamine (30 μ g/mL) after 60 min at 37°C was analyzed by flow cytometry. Values represent the means $\pm$ S.E. of three determinations. * p < 0.05, significantly different from the value without compound tested (control). EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride.

pendent endocytosis on FITC-protamine association (Table 1). Nystatin is a cholesterol-binding drug that inhibits caveolin-mediated endocytosis. Methyl- β -cyclodextrin is a cholesterol-sequestering drug that disrupts the caveolae integrity. The association of FITC-protamine was significantly inhibited by these caveolin-dependent endocytosis inhibitors. Thus, it is likely that caveolin-dependent endocytosis is also involved in FITC-protamine association by OK cells. In addition, the effects of macropinocytosis inhibitors on FITC-protamine association were examined (Table 1). EIPA, an analogue of an ion-exchange inhibitor, amiloride, decreased the association of FITC-albumin in a concentration-dependent manner, but other macropinocytosis inhibitors, cytochalasins B and D, did not inhibit FITC-protamine association. Thus, EIPA might decrease FITC-protamine association by indirectly or directly affecting a multitude of endocytic processes.²³⁾

Effects of Cationic Compounds and Serum γ -Globulin on FITC-Protamine Association by OK Cells Since we reported that protamine inhibited gentamicin uptake by OK cells,⁹⁾ the effect of gentamicin on FITC-protamine association was examined. As shown in Fig. 5A, gentamicin inhibited the association of FITC-protamine in a concentration-dependent manner. In addition, polymyxin B, a cationic peptide antibiotic that binds to megalin,^{24,25)} greatly decreased the association of FITC-protamine (Fig. 5B). Furthermore, human serum γ -globulin, which decreases FITC-IgG uptake by OK cells,²⁰⁾ also inhibited the association of FITC-protamine in a concentration-dependent manner (Fig. 5C). In contrast, arginine, a basic amino acid, did not inhibit, but rather enhanced the association of FITC-protamine (Fig. 5D).

Fig. 5. Effects of Cationic Compounds and Serum γ -Globulin on the Association of FITC-Protamine by OK Cells

OK cells were incubated with FITC-protamine (30 μ g/mL) for 60 min at 37°C in the absence (control, open circle) or presence (closed circles) of various concentrations of gentamicin (A), polymyxin B (B), human serum γ -globulin (C), or L-arginine (D). Then, the cells were washed, harvested with trypsin/EDTA and analyzed by flow cytometry. The effect of each compound on FITC-protamine association is shown relative to the fluorescence in the absence of its compound (control). Each symbol represents the mean $\pm$ S.E. of three determinations. * p < 0.05, significantly different from the value in the absence of compound tested (control).

Fig. 6. Ligand Blotting with FITC-Protamine and Western Blotting with Anti-cubilin Antibodies for Crude Membranes from OK Cells and Brush-Border Membranes from Rat Renal Cortices

The crude membranes from OK cells and brush-border membranes from rat renal cortices were prepared as described under Materials and Methods. The membrane samples were separated by 4–15% gradient SDS-PAGE, and then transferred polyvinylidene difluoride (PVDF) membranes. Each PVDF membrane was incubated with FITC-protamine (10 μ g/mL) (A) or anti-cubilin antibodies (1 : 100 dilution) (B). The bands on ligand blotting (A) and Western blotting (B) were visualized with a fluorescence imaging system and enhanced chemiluminescence, respectively.

Effect of Heparinase Treatment on FITC-Protamine Association by OK Cells Heparan sulfate proteoglycans (HSPGs) are suggested to be involved in the internalization of CPPs.^{26,27} Therefore, we investigated the effect of heparinase III, a heparin-degrading lyase that recognizes the ubiquitous cell-surface HSPGs, on FITC-protamine association. Pretreatment with heparinase III for 60 min decreased the association of FITC-protamine in a concentration-dependent manner (Table 1).

Ligand Blotting for Crude Membrane Fraction from OK Cells with FITC-Protamine To investigate the presence of membrane proteins that exhibit binding affinity for FITC-protamine, ligand blot analysis was performed using crude membrane fractions derived from OK cells and brush-border membranes from rat renal cortices. As shown in Fig. 6A, there were two major bands corresponding to apparent molecular sizes of 410 and 100 kDa, which were detected for membrane fractions from both OK cells and rat renal cortices. Figure 6B shows Western blot analysis of the two membrane fractions with anti-cubilin antibodies. Cubilin was detected in both membrane fractions, the bands corresponding to almost the same size as that of the higher molecular size observed on ligand blotting (Fig. 6B).

DISCUSSION

Here, a quantitative cellular uptake study was performed by flow cytometry. For the flow cytometric analysis, OK cells were detached from culture plates by trypsinization after

incubation with FITC-protamine. Therefore, the fluorescence intensity that was detected with the flow cytometer is considered to represent the amount of FITC-protamine that was internalized into the cells since the FITC-protamine adsorbed to the plasma membrane would be removed during trypsinization, as suggested for the protocol of flow cytometry analysis of translocation mechanisms for CPPs.^{21,22,27} Furthermore, live cells without fixation were employed for the confocal microscopic analysis, which was performed to investigate the intracellular localization of FITC-protamine, because the cell fixation procedure is reported to lead to artificial redistribution of CPPs into the nucleus.^{21,22,27}

In this study, the concentration-dependent inhibition of FITC-protamine association by unlabeled protamine indicated that FITC-protamine is taken up *via* a specific pathway. In addition, when the time-course of FITC-protamine association was examined, the amount of FITC-protamine became maximum at up to 30 min after the start of incubation. The period necessary for the uptake amount to reach the maximum was shorter than that in the case of FITC-albumin (about 180 min), a ligand which is taken up *via* receptor-mediated endocytosis. In the case of gentamicin uptake, which is inhibited by protamine, the uptake of [³H]gentamicin by OK cells increased with time up to 80 min.⁹ In addition, Jones *et al.*²⁸ reported that the maximal uptake of some CPPs such as antennapedia, TAT and polyarginine occurred between 1 and 3 h. Thus, it is likely that the uptake of protamine is relatively more rapidly saturated than those of other ligands that are reported to be internalized *via* endocytosis.

We observed that FITC-protamine association is energy- and temperature-dependent on flow cytometry and confocal microscopy. In addition, the vesicular distribution of FITC-protamine was observed in the cytoplasm of the OK cells that had been incubated with FITC-protamine for 30 min at 37°C, but not at 4°C. These findings indicated that FITC-protamine is internalized through endocytic pathway(s). Furthermore, FITC-protamine was detected in the nuclei in addition to its intracellular diffuse localization and its binding to the nuclear membranes of OK cells. Therefore, the distribution of FITC-protamine in the nucleus might occur following the diffusion of FITC-protamine from endocytic vesicles such as endosomes and lysosomes into the cytoplasm, though further studies are needed to clarify the precise pathway.

Interestingly, confocal microscopic analysis showed clear fluorescence in the cell membranes of the cells that had been incubated with FITC-protamine at 4°C, whereas there was little fluorescence in the cell membranes in the case of incubation at 37°C. In addition, as described above, the associated amount of FITC-protamine in OK cells was saturated at 30 min at 37°C. Therefore, these observations might indicate down-regulation of the membrane surface receptor that is responsible for the internalization of FITC-protamine. The cell-surface receptors for epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are reported to be down-regulated by the administration of excess ligand,^{29,30} which might support the speculation about the down-regulation in this study.

Endocytic pathways can be mainly divided into four types: macropinocytosis, clathrin-dependent endocytosis, caveolin-dependent endocytosis and clathrin, caveolin-independent endocytosis.³¹ Based on the effects of pharmacological inhibi-

tors of these endocytic pathways, both clathrin-dependent and caveolin-dependent pathways were suggested to be involved in the internalization of FITC-protamine in OK cells. On the other hand, a known macropinocytosis inhibitor, EIPA, significantly inhibited the association of FITC-protamine by OK cells, whereas cytochalasins B and D, other known macropinocytosis inhibitors, did not decrease FITC-protamine association. Since EIPA has been reported to block clathrin-mediated endocytosis,^{32,33)} the inhibitory effect of EIPA on FITC-protamine association might be independent of macropinocytosis.

HSPGs are suggested to function as membrane surface receptors for CPPs such as the TAT peptide.^{26,27,34)} Therefore, we examined the effect of heparinase III, a heparin-degrading lyase, on the association of FITC-protamine by OK cells. Pretreatment with heparinase III significantly inhibited the association of FITC-protamine, but it was not completely abolished even with 100 mU heparinase III (71.8% of control). To obtain more information on the membrane surface binding receptor responsible for protamine uptake, ligand blotting with FITC-protamine was performed using crude membranes from OK cells and brush-border membranes from rat renal cortices. The ligand blot analysis revealed the presence of two major binding proteins with apparent molecular sizes of 410 and 100 kDa in the membrane fractions from both OK cells and rat renal cortices. Gentamicin, which inhibits FITC-protamine uptake, is reported to bind to not only megalin but also cubilin.³⁵⁾ The molecular sizes of megalin and cubilin are approximately 600 and 460 kDa, respectively.^{11,12)} Western blotting of the two membrane samples with cubilin antibodies gave corresponding to almost the same size as that of the larger molecular size detected on ligand blotting. However, as far as we know, whether or not protamine binds to cubilin has not been reported. Additional experiments are needed to identify the membrane surface receptor that plays an important role in protamine uptake in the kidneys.

In conclusion, we found that FITC-protamine is taken up *via* receptor-mediated endocytic pathways in OK cells. The membrane surface receptor that is involved in FITC-protamine uptake might partially overlap that for gentamicin uptake in OK cells. These findings might provide useful information for understanding the renal distribution of protamine exogenously injected.

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