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Expression of Chloride Channel, ClC-5, and Its Role in Receptor-Mediated Endocytosis of Albumin in OK Cells

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By using Western blot and RT-PCR analyses, the expression of ClC-5, a member of the ClC family of voltage-gated chloride channels, and its mRNA was detected in OK cells. The effect of chloride channel inhibitors on receptor-mediated endocytosis of albumin was examined in OK cells and compared to that of vacuolar H^+-ATPase inhibitors. Accumulation of fluorescein-isothiocyanate (FITC)–albumin, a receptor-mediated endocytosis marker, was inhibited by 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), a chloride channel inhibitor, in a concentration-dependent fashion. In contrast, uptake of FITC–inulin, a fluid-phase endocytosis marker, was not affected by NPPB. Other chloride channel inhibitors, 4,4′-diisothiocyanatostilbene-2-2′-disulfonic acid and diphenylamine-2-carboxylic acid, also inhibited FITC–albumin uptake. NPPB, as well as a vacuolar H^+-ATPase inhibitor bafilomycin A_1, caused a decrease in the affinity and in the maximal velocity of FITC–albumin uptake. These results suggest that chloride channel, most likely ClC-5, plays an important role in the receptor-mediated endocytosis of albumin in OK cells. © 2001 Academic Press

Key Words: **receptor-mediated endocytosis; albumin; chloride channel; culture cell; vacuolar H^+-ATPase; Dent's disease.**

Low-molecular-weight proteins filtered by the glomerulus are reabsorbed in the proximal tubules, and it is generally agreed that most proteins are taken up by endocytosis at the luminal membrane surface (1). Some proteins, including β_2-microglobulin, insulin, lysozyme, immunoglobulin light chain, insulin-like growth factor II (IGF-II), and native and modified serum albumin, are reabsorbed by receptor-mediated endocytosis in the renal proximal tubule. Recently, it has been reported that megalin/LRP-2 (low-density lipoprotein receptor-related protein-2), an endocytic receptor abundantly expressed in the renal proximal tubules, plays an important role in the tubular uptake of filtered plasma proteins (2).

The endosomal low pH plays an important role in the dissociation of receptor–ligand complexes and the recycling of unoccupied receptors to cell surface sequential to the internalization of receptor-bound ligands by receptor-mediated endocytosis (3–6). The proton gradient across the endosomal membrane is established by a vacuolar type proton-translocating adenosine triphosphatase (V-H^+-ATPase) (5, 6). V-H^+-ATPase translocates the protons from the cytoplasm into the endosomal lumen without direct molecular coupling to other cations or anions and generates a positive membrane potential within endosomal vesicles. The interior positive membrane potential prevents the continued translocation of protons into the endosomal vesicles. Therefore, the membrane potential must be dissipated by the influx of external anions or the efflux of internal cations in order to maintain electroneutrality and to generate the proton gradient. It has been suggested that the pH gradient formation in endosome may be regulated by the cooperative function of chloride channels coexisting with V-H^+-ATPase in endosomal membrane (7).

Dent's disease is an X-linked renal tubular disorder, which is characterized by low-molecular-weight proteinuria, hypercalciuria, nephrolithiasis, and renal failure (8). The renal tubular disorder is due to mutations of the *CLCN5* gene, which is located on chromosome Xp11.22 and encodes an outwardly rectifying chloride channel ClC-5 (8–12). In the renal proximal tubular cells, ClC-5 expression is highest below the brush border in a region densely packed with endocytic vesicles, where ClC-5 colocalizes with V-H^+-ATPase and Rab4 (a marker of recycling early endosome) (13). In addition, the localization of ClC-5 overlaps the distribution of endocytosed proteins such as albumin and

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β_2-microglobulin (13, 14). Therefore, ClC-5 is suggested to be a potent candidate for the chloride channel of early endosomes in proximal tubular cells and ClC-5 dysfunction in Dent's disease leads to an impairment in the reabsorption of low-molecular-weight proteins.

So far, it has been reported that bafilomycin A_1, a specific inhibitor of vacuolar H^+-ATPase, reduces the receptor-mediated endocytosis of albumin by endosomal alkalinization (15). However, evidences showing that ClC-5 regulates the activity of receptor-mediated endocytosis in the renal proximal tubules are lacking. Therefore, in the present study, the involvement of chloride channel in receptor-mediated endocytosis was examined by investigating the effect of chloride channel inhibitors on the receptor-mediated endocytosis in OK cells. OK cell is an established cell line derived from the American opossum kidney (16), and is used as a suitable model with which to study endocytosis in the renal proximal tubules (17).

MATERIALS AND METHODS

Materials. Fluorescein-isothiocyanate (FITC)-labeled bovine serum albumin (FITC–albumin), FITC-labeled inulin (FITC–inulin), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4,4′-diisothiocyanatostilbene-2-2′-disulfonic acid (DIDS) and bafilomycin A_1 were purchased from Sigma Chemical Co. (St. Louis, MO). Diphenylamine-2-carboxylic acid (DPC) was obtained from Across Organics (NJ). *N*-Ethylmaleimide was obtained from Nacalai Tesque (Kyoto, Japan). Phenylmethylsulfonyl fluoride (PMSF) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals used were of the highest purity available.

Cell culture. OK cells were cultured in medium 199 (Gibco BRL, Life Technologies, NY) containing 10% fetal bovine serum (Biological Industries, Israel) without antibiotics, in an atmosphere of 5% CO_2–95% air at 37°C, and subcultured every 7 days using 0.02% EDTA and 0.05% trypsin (18). OK cells were used between passages 84 and 93.

Uptake studies. Uptake of FITC–albumin and FITC–inulin was measured in OK cells attached to the culture dishes (35-mm) as described previously (19). Briefly, fresh medium was replaced every 2 or 3 days, and the cells were used on the 5th or 6th days after seeding. Experiments were performed in Dulbecco's phosphate-buffered saline (PBS buffer containing in mM, 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1.5 KH_2PO_4, 1 $CaCl_2$, and 0.5 $MgCl_2$) supplemented with 5 mM D-glucose. After removal of the culture medium, each dish was washed and preincubated with PBS buffer. Then, PBS buffer containing FITC–albumin or FITC–inulin was added to each dish and the cells were incubated at 37°C (uptake) or 4°C (binding) for a specified period. At the end of the incubation, the uptake buffer was aspirated and the dishes were rinsed rapidly 3 times with 1 mL of ice-cold PBS buffer. The cells were scraped with rubber policeman into 1 mL of ice-cold PBS buffer and the dishes were rinsed again with 1 mL of ice-cold PBS buffer to improve the recovery of the cells. The cells were centrifuged 4°C for 5 min at 1000 rpm and the supernatant was aspirated. The cell pellet was resuspended gently in 1 mL of ice-cold PBS buffer and centrifuged again. The pellet was homogenized in PBS buffer containing Triton X-100 (0.1% v/v), and the homogenate was used for fluorescence and protein assays.

Cell treatment. NPPB, DIDS, DPC, and bafilomycin A_1 were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO during exposure was 0.25–0.5%. OK cells were pretreated with each compound for 30 min at 37°C in PBS buffer containing 5 mM D-glucose. The control cells were incubated with the same concentration of DMSO in each experiment. Finally, the cell monolayers were washed three times with PBS buffer before measuring uptake of FITC–albumin and FITC–inulin.

Preparation of antiserum against ClC-5. Rabbit antiserum against human ClC-5 was raised against the synthetic 18-amino-acid peptide, SELISELFNDCGLLDSSK (corresponding to residues 382–399) (13). The synthetic peptide was produced with the peptide synthesizer (PSSM-8, Shimadzu, Japan). The peptide was linked to a carrier protein, keyhole limpet hemocyanin (Sigma Chemical Co.), with a coupling agent, *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Sigma Chemical Co.). The cross-linked peptide was mixed with an equal volume of Freund's complete adjuvant (Wako Pure Chemicals, Osaka, Japan) and the emulsion was injected intradermally into New Zealand White female rabbits (2.0–2.5 kg) on day 0. Subsequent immunization was performed with incomplete Freund's adjuvant (Wako Pure Chemicals) at four intervals between days 21 and 79. Serum samples were tested for reactivity with the antigen peptide by dot-blot analysis, using a horseradish peroxidase-conjugated goat antibody to rabbit IgG (ICN Pharmaceuticals Inc., OH) as a secondary antibody. At day 21 after the start of immunization, antibodies reactive with synthetic peptide were detected by dot-blot analysis. All animal experiments were performed in accordance with the Guide for Animal Experimentation, Hiroshima University and the Committee of Research Facilities for Laboratory Animal Sciences, School of Medicine, Hiroshima University.

Western blot analysis. OK cells and renal cortex of male Wistar rat (220–240 g) were homogenized for 2 min with an IKA T25 Basic dispenser (IKA Labortechnik, Germany) in an ice-cold buffer (150 mM NaCl, 1 mM, EDTA, 1 mM PMSF with 20 mM Tris, pH 7.4), and were subsequently homogenized with a glass/Teflon Potter homogenizer with 10 strokes at 1000 rpm. The homogenate was centrifuged at 3000*g* for 10 min at 4°C in an Avanti 30 Compact Centrifuge with rotor F0630 (Beckman Instruments, Inc., CA). The supernatant was centrifuged at 40,000*g* for 30 min at 4°C. The pellet (crude membrane fraction) was resuspended in the ice-cold buffer containing 1% Triton X-100, and centrifuged at 14,000*g* for 15 min at 4°C. The supernatant was heated for 5 min at 95°C in a loading buffer; 2% SDS, 62.5 mM Tris–HCl, 7% glycerol with 5% 2-mercaptoethanol. These samples were subjected to SDS–polyacrylamide gel electrophoresis with 10% polyacrylamide gels, and the proteins were transferred for 60 min to polyvinylidene difluoride membrane (Immun-Blot, Bio-Rad) at 4°C. The membrane was blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris–HCl, pH 7.5) with 0.05% Tween 20 (TBS-T) overnight at 4°C. The membranes were washed three times for 10 min in TBS-T and were incubated with the anti-rat ClC-5 rabbit antiserum (1:5000 dilution). The membranes were washed three times in TBS-T, and were incubated with the horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:2000 dilution), washed three times in TBS-T, and visualized with enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK).

RT-PCR analysis. Total RNA was extracted from OK cells using MagExtractor RNA kit (TOYOBO, Osaka, Japan). Primers were synthesized chemically on the basis of the sequence of human ClC-5: sense strand, 5′-ACCATGAACATTGTTGCTGGAAC-3′; antisense strand, 5′-CTGCCAGCACCAAGGTGATGG-3′ (13). RT-PCR was performed using a ReverTra Dash RT-PCR kit (TOYOBO, Osaka, Japan) according to the manufacturer's instruction. Amplification was performed according to the following profile: denaturation at 95°C for 1 min, annealing at 56°C for 30 s, extension at 60°C for 30 s, 40 cycles. The PCR products were separated by electrophoresis in 1.5% agarose gels, and amplified bands were detected by ethidium bromide staining.

Analytical methods. The intracellular fluorescence was measured by using a Hitachi fluorescence spectrophotometer F-3000 (Tokyo, Japan) at an excitation wavelength of 500 nm and an emission wavelength of 520 nm. Protein was determined by the method of Lowry *et al.* (20) with bovine serum albumin as the standard.

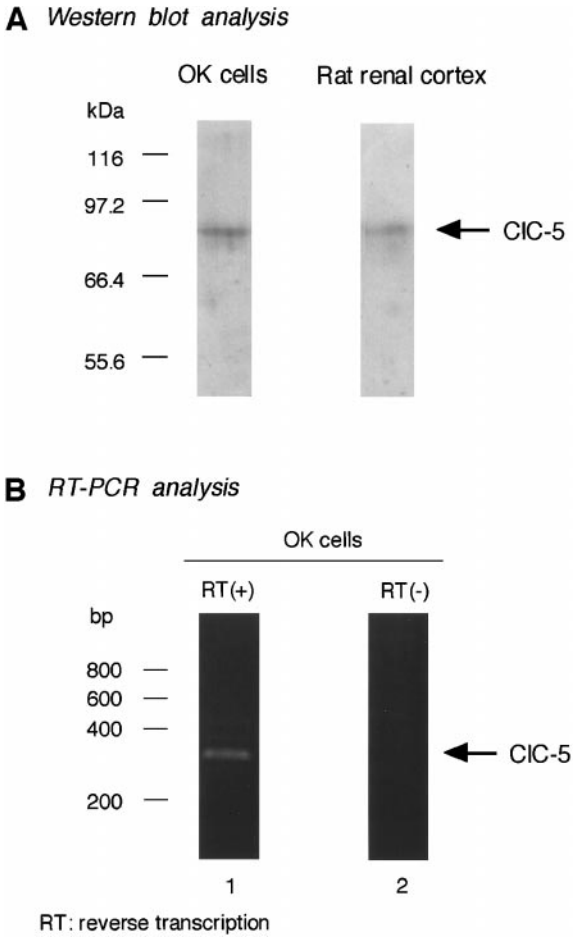

FIG. 1. Western blot analysis of ClC-5 in crude membranes from OK cells and rat renal cortex (A) and detection by PCR amplification of ClC-5 mRNA in OK cells (B). (A) Ten (OK cells) or two (rat renal cortex) micrograms of crude membranes were separated in 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane. ClC-5 was detected by Western blotting with anti-ClC-5 antiserum (1:5000 dilution). (B) Total RNA from OK cells was reverse-transcribed and first-strand cDNA synthesized was amplified with a set of specific primers described in the text. The PCR products with (lane 1) or without (lane 2) reverse transcription were separated by electrophoresis through a 1.5% agarose gels and stained with ethidium bromide.

RESULTS

Expression of ClC-5 in OK Kidney Epithelial Cells

Western blot analysis was performed to detect the ClC-5 protein in OK cells used in the present study. With the rabbit antiserum raised against the synthetic peptide for human ClC-5, the protein with the apparent molecular mass of 78 kDa was detected on the crude membrane from OK cells as well as from rat renal cortex (Fig. 1A). The band was not detected when the pre-immune serum was used, and when the antiserum was preadsorbed with the synthetic antigen peptide, indicating that the antiserum would specifically recognize the ClC-5 protein (data not shown). The expression of ClC-5 mRNA in OK cells was also investigated by RT-PCR with ClC-5-specific primers. PCR amplification with reverse transcription of total RNA from OK cells gave a product of the expected size (315 bp), whereas no band was detected when the total RNA from OK cells was subjected to PCR without reverse transcription (Fig. 1B).

Effect of Chloride Channel Inhibitors on Endocytic Uptake of FITC–Albumin and FITC–Inulin

FITC-labeled albumin and inulin were used as a marker for receptor-mediated endocytosis and for fluid-phase endocytosis, respectively. Figure 2 shows the effect of chloride channel inhibitors (NPPB, DIDS, DPC) and vacuolar H^+-ATPase inhibitors (bafilomycin A_1 and NEM) on FITC–albumin uptake in OK cells. Chloride channel inhibitors as well as vacuolar H^+-ATPase inhibitors inhibited FITC–albumin uptake in OK cells. Effect of various concentrations of NPPB on the receptor-mediated endocytosis and the fluid-phase endocytosis in OK cells were examined. The OK cells were treated with various concentrations of NPPB (10^{-6} to 10^{-3} M) or bafilomycin A_1 (10^{-9} to 10^{-7} M) for 30 min, then the endocytic uptake of FITC–albumin and FITC–inulin were measured. As shown in Fig. 3, both NPPB and bafilomycin A_1 inhibited FITC–albumin uptake in concentration dependent manner with an apparent half-maximal inhibitory concentration (IC_{50}) of 324 μM and 71.2 nM, respectively. DIDS also

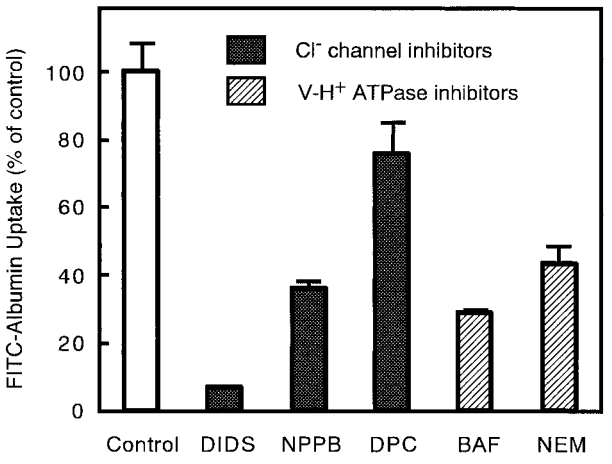

FIG. 2. Effect of chloride channel inhibitors and vacuolar H^+-ATPase inhibitors on fluorescein-isothiocyanate (FITC)–albumin uptake in OK cells. Confluent monolayers were incubated for 30 min with 4,4′-diisothiocyanatostilbene-2-2′-disulfonic acid (DIDS, 10 μM), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB, 1 mM), diphenylamine-2-carboxylic acid (DPC, 2 mM) or bafilomycin A_1 (BAF, 100 nM), except for 1 min with *N*-ethylmaleimide (NEM, 1 mM). After washing the cells, uptake of FITC–albumin (30 μg/mL) for 30 min was measured at 37°C. FITC–albumin uptake in the untreated cells (control) was 3.77 ± 0.27 μg/mg protein/30 min. Each column is the mean ± SE of three to five monolayers. V-H^+-ATPase, vacuolar type proton-translocating adenosine triphosphatase.

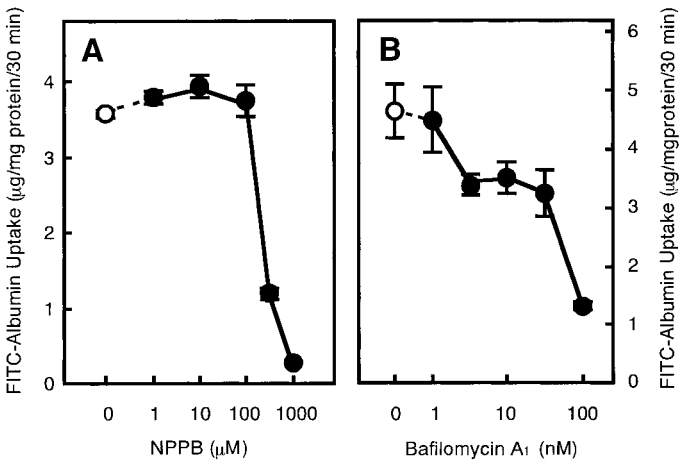

FIG. 3. Dose-dependent effect of NPPB (A) and bafilomycin A_1 (B) on FITC–albumin uptake in OK cells. Confluent monolayers were incubated for 30 min with various concentrations of NPPB (10^{-6} to 10^{-3} M) or bafilomycin A_1 (10^{-9} to 10^{-7} M), and uptake of FITC–albumin (30 μg/mL) for 30 min was measured at 37°C. Each point is the mean ± SE of three to five monolayers.

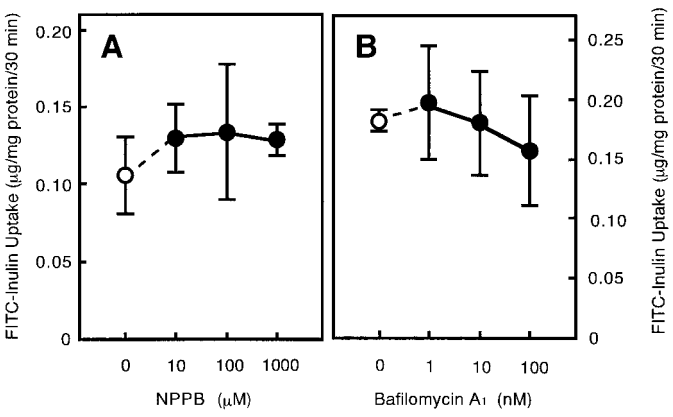

FIG. 5. Effect of NPPB (A) and bafilomycin A_1 (B) on FITC–inulin uptake in OK cells. Confluent monolayers were incubated for 30 min with various concentrations of NPPB (10^{-6} to 10^{-3} M) or bafilomycin A_1 (10^{-9} to 10^{-7} M), and uptake of FITC–inulin (500 μg/mL) for 30 min was measured at 37°C. Each point is the mean ± SE of three to five monolayers.

inhibited the FITC–albumin uptake in a concentration-dependent manner, and the IC_{50} was 2.9 μM (Fig. 4). The IC_{50} value of DPC on FITC–albumin uptake was estimated to be approximately 3 mM (data not shown). In contrast, FITC–inulin uptake was not inhibited by the pretreatment with NPPB and bafilomycin A_1 (Fig. 5).

The effect of NPPB on the binding of FITC–albumin to the apical membrane surface of OK cells was examined. NPPB (300 μM) did not affect the binding of FITC–albumin in OK cells (Fig. 6). Similarly, bafilomycin A_1 (100 nM) did not affect the binding of FITC–albumin to the apical membrane of OK cells (Fig. 6).

Influence of NPPB on Kinetic Parameters of FITC–Albumin Uptake

The effect of NPPB on the kinetic parameters of FITC–albumin uptake was examined (Fig. 7). The kinetic constants of FITC–albumin uptake in OK cells were analyzed by measuring the uptake of FITC–albumin over a concentration range of 30–500 μg/mL. FITC–albumin uptake was saturable and inhibited by NPPB at all concentrations of FITC–albumin exam-

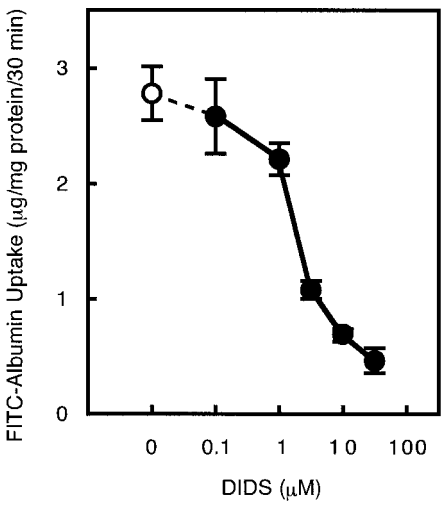

FIG. 4. Dose-dependent effect of DIDS on FITC–albumin uptake in OK cells. Confluent monolayers were incubated for 30 min with various concentrations of DIDS (10^{-7} to 3×10^{-5} M), and uptake of FITC–albumin (30 μg/mL) for 30 min was measured at 37°C. Each point is the mean ± SE of five to nine monolayers.

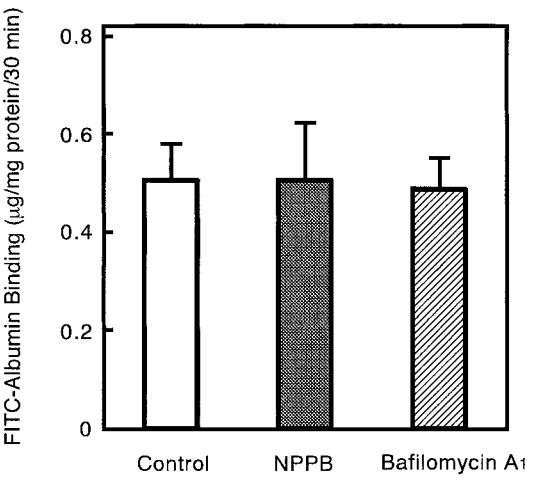

FIG. 6. Effect of NPPB and bafilomycin A_1 on FITC–albumin binding to the apical membrane surface of OK cells. Confluent monolayers were incubated for 30 min at 37°C with NPPB (300 μM) or bafilomycin A_1 (100 nM), and binding of FITC–albumin (100 μg/mL) for 30 min was measured at 4°C. Each column is the mean ± SE of three to five monolayers.

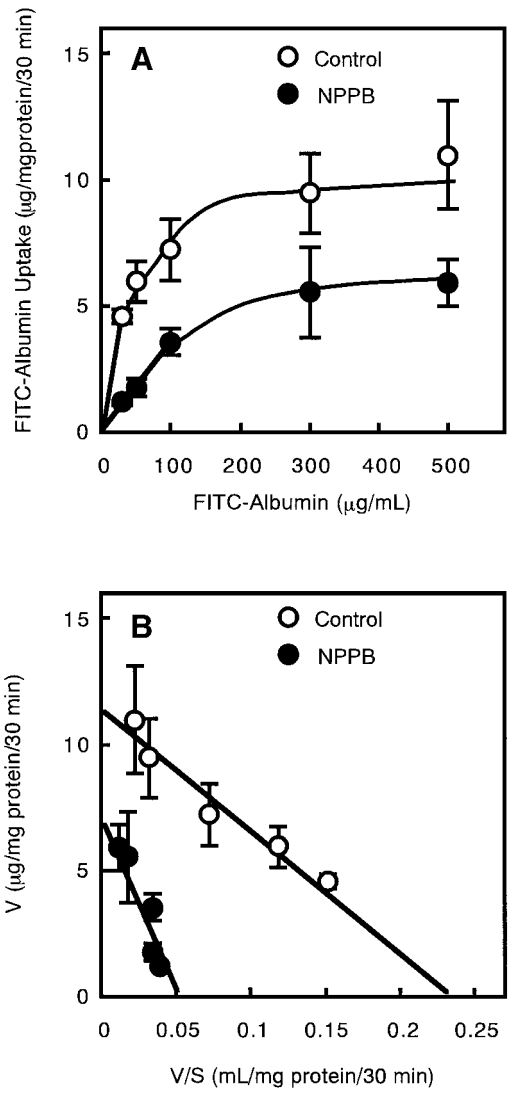

FIG. 7. Influence of NPPB on kinetic parameters of FITC–albumin uptake in OK cells. Confluent monolayers were incubated for 30 min without (open circles) or with (closed circles) NPPB (300 μM), and uptake of FITC–albumin (30 μg/mL) for 30 min was measured at 37°C (A). Eadie–Hofstee plots of the data (B). Each point is the mean ± SE of three to five monolayers.

ined (Fig. 7A). Eadie–Hofstee plots were linear in control and NPPB-treated cells (Fig. 7B). The Michaelis constants (K_m) in the cells incubated without and with NPPB were 35.4 ± 13.7 and 147.1 ± 15.2 μg/mL, and maximum uptake rates (V_{max}) in the cells incubated without and with NPPB were 10.1 ± 3.1 and 4.7 ± 2.0 μg/mg protein/30 min, respectively (means ± SE of three experiments). Thus, NPPB caused a significant decrease in the affinity as well as in the maximal velocity of FITC–albumin uptake in OK cells, indicating that the inhibition is apparently a mixed-type. The apparent mixed-type inhibition of FITC–albumin uptake was also observed in the OK cells pretreated with bafilomycin A_1 (data not shown).

DISCUSSION

Acidification of the early endosome plays an important role in receptor-mediated endocytosis process. The endosomal low pH, which is established by the components of the acidification machinery (vacuolar-H^+-ATPase and chloride channel), leads to the dissociation of receptor-ligand complexes. Therefore, change in the activities of vacuolar H^+-ATPase and chloride channel could affect the overall process of receptor-mediated endocytosis. In fact, Gekle *et al.* (15) have shown that endosomal alkalinization by a specific vacuolar H^+-ATPase inhibitor bafilomycin A_1 reduces the uptake of albumin by receptor-mediated endocytosis in OK cells. On the other hand, little information concerning the effect of chloride channel inhibitor on the endocytosis is available to our knowledge. The purpose of this study was to investigate the role of chloride channel in the receptor-mediated endocytosis of albumin in OK cells.

OK cells, which were established from the American opossum kidney (16), are a useful *in vitro* model system with which to study various transport systems for amino acids, hexoses, proton, inorganic phosphate, and organic anions in renal proximal tubules (21, 22). In addition, OK cells are often used in investigating a receptor-mediated endocytosis of albumin (17, 23, 24). In the preliminary experiments, we also observed that FITC–albumin uptake in OK cells was a temperature- and concentration-dependent. Furthermore, FITC–albumin uptake in OK cells was inhibited by a metabolic inhibitor 2,4-dinitrophenol, and colchicine, an inhibitor of the function of microtubules (data not shown); all of which are characteristics of receptor-mediated endocytosis. In addition, the expression of ClC-5 protein and mRNA was observed in OK cells in the present study. Thus, OK cells used in the present study would be suitable to examine a role of ClC-5 in receptor-mediated endocytosis.

Chloride channel inhibitors such as NPPB, DIDS and DPC may be lacking for the selectivity to a specific type of chloride channels, in contrast to the action of amiloride on Na^+ channels or Ba^{2+} on K^+ channels (25). However, the rank order of the inhibitory effects on the albumin uptake in OK cells by chloride channel inhibitors used in the present study (DIDS > NPPB > DPC) was well correlated with that on the chloride conductance in renal brush-border membrane vesicles (26), in which the expression of ClC-5 as well as V-H^+-ATPase was observed (13, 27). Schmid *et al.* (28) reported that NPPB and DIDS completely inhibited single chloride channels in endosomal vesicles from renal cortex. Furthermore, DIDS and DPC inhibited the chloride current in the ClC-5 transfected CHO-K1 cells (12). Based on these findings, it seems likely that the inhibition of the receptor-mediated endocytosis of albumin in OK cells is due to the decrease in ClC-5 activity by chloride channel inhibitors.

On one hand, the inhibitory potency of each chloride channel inhibitor on the chloride current and receptor-mediated endocytosis needs to be considered further. Steinmeyer *et al.* (11) reported that DIDS (1 mM) did not have significant effect on an outwardly rectifying chloride current by ClC-5 expressed in Xenopus oocytes. In contrast, Sakamoto *et al.* (12) showed that DIDS (1 mM) inhibited the chloride current in ClC-5 transfected CHO-K1 cells by 65%. In addition, the conductive chloride ion flux in renal brush-border membrane, in which ClC-5 would be expressing, was inhibited by DIDS with IC_{50} value of 70 μM (26). Thus, the sensitivities of ClC-5 to DIDS are different among studies. Nevertheless, when compared with these results, the IC_{50} value (2.9 μM) of DIDS on FITC–albumin uptake in the present study seems to be too low. Though the reason is not clear at this moment, it may be due to the effect of DIDS on other functional proteins, in addition to the effect on ClC-5, involved in the receptor-mediated endocytosis. On the other hand, IC_{50} value (324 μM) of NPPB on FITC–albumin uptake in OK cells seems to be somewhat higher compared with those on chloride channels in some experiments; which are between 10 and 100 μM in endosomal membrane from rat kidney cortex (28) and between 100 and 200 μM in porcine renal brush-border membrane (26). In the case of experiments with culture cells, however, intracellular free concentration of NPPB would be important, which may be different from that in the extracellular medium. Taken together, the apparent IC_{50} values of these inhibitors would vary with functions examined and experimental conditions employed.

To further examine the mechanisms underlying the inhibitory effect of chloride channel inhibitors on the albumin uptake, effects of NPPB and bafilomycin A_1 on the uptake and binding of albumin in OK cells were examined and compared. Both NPPB and bafilomycin A_1 reduced the uptake of albumin, but these inhibitors did not affect the binding of albumin to the apical surface of OK cells. In contrast, the uptake of inulin by fluid-phase endocytosis was not inhibited by NPPB and bafilomycin A_1. Furthermore, both inhibitors caused decreases in the affinity and in the maximal velocity of the albumin uptake in OK cells. These similarities between the effects of NPPB and bafilomycin A_1 may suggest that the endosomal alkalinization following decrease in ClC-5 activity is involved in the inhibition of the albumin uptake by NPPB, as in the case of bafilomycin A_1.

The effects of bafilomycin A_1 on the kinetic parameters of FITC–albumin uptake and the fluid-phase endocytosis observed in the present study are well correlated with those reported by Gekle *et al.* (15). However, the effect of bafilomycin A_1 on FITC–albumin binding was different. We observed no effect (Fig. 6) but Gekle *et al.* (15) observed the inhibitory effect. The discrepancy between those studies may be due to a difference in the experimental condition. During preincubation with bafilomycin A_1, unlabeled albumin was included in their study, but not in the present study. In the presence of albumin, bafilomycin A_1 would inhibit dissociation of albumin-receptor complex, which is prerequisite for recycling of the receptor to the cell surface, resulting in the decrease in the binding of albumin in their study.

Cui *et al.* (29) reported that a multiligand endocytosis receptor megalin is able to bind and mediate the endocytosis of albumin in renal proximal tubular cells. However, no significant binding of albumin to megalin was detected by surface plasmon resonance (SPR) analysis (2). Recent report by Birn (30) showed that cubilin, identical to intrinsic factor receptor, constitutes an albumin binding protein important for reabsorption of albumin, and that megalin is involved in the reabsorption of the cubilin–albumin complex in renal proximal tubules. In OK cells, the uptake of albumin was reportedly inhibited by both receptor-associated protein (a substrate for megalin) and intrinsic factor–vitamin B_{12} complex (a substrate for cubilin) (31). Interestingly, the K_d of albumin binding to cubilin by SPR analysis (0.63 μM) is in good agreement with the affinity (approximately 0.55 μM) for albumin uptake observed in the present study. Further studies are needed to clarify the expression and function of megalin and cubilin in OK cells.

Dent's disease and the related syndromes (X-linked recessive nephrolithiasis, X-linked recessive hypophosphatemic rickets, the idiopathic low-molecular-weight proteinuria of Japanese children), which are characterized by low-molecular-weight proteinuria, hypercalciuria, nephrolithiasis and renal failure, are an X-chromosome-linked disease (8–10). Recently, the mutation in the ClC-5 gene (chromosomal location Xp11.22) is found to be responsible for all of these diseases (8, 10). Though low-molecular-weight proteinuria in these syndromes has been suggested to be due to the decrease in receptor-mediated endocytosis in the renal proximal tubules, little evidence for involvement of ClC-5 in the receptor-mediated endocytosis process has been shown. The present observations would provide experimental evidences showing that the low-molecular-weight proteinuria in Dent's disease and the related syndromes is due to a decrease in the activity of chloride channel, ClC-5, followed by an inhibition of the receptor-mediated endocytosis activity of filtered proteins.

In conclusion, the inhibitors of chloride channel reduced the receptor-mediated endocytosis of albumin in OK cells, as bafilomycin A_1, a vacuolar H^+-ATPase inhibitor, did. These results would indicate the important role of chloride channel, most likely ClC-5, in the endosomal acidification and the receptor-mediated endocytosis in the renal proximal tubules.

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