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Calcitonin receptor-stimulating peptide-1 regulates ion transport and growth of renal epithelial cell line LLC-PK$_1$

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Abstract

Calcitonin receptor-stimulating peptide-1 (CRSP-1) is a peptide recently identified from porcine brain by monitoring the cAMP production through an endogenous calcitonin (CT) receptor in the renal epithelial cell line LLC-PK$_1$. Here we investigated the effects of CRSP-1 on the ion transport and growth of LLC-PK$_1$ cells. CRSP-1 inhibited the growth of LLC-PK$_1$ cells with a higher potency than porcine CT. CRSP-1 enhanced the uptake of $^{22}Na^+$ into LLC-PK$_1$ cells more strongly than did CT and slightly reduced the $^{45}Ca^{2+}$ uptake. The enhancement of the $^{22}Na^+$ uptake was abolished by 5-(*N*-ethyl-*N*-isopropyl) amiloride, a strong Na^+/H^+ exchanger (NHE) inhibitor for NHE1, even at a concentration of 1×10^{-8} M, although other ion transporter inhibitors did not affect the $^{22}Na^+$ uptake. These results indicate that CRSP-1 enhances the $^{22}Na^+$ uptake by the specific activation of NHE1. Taken together, CRSP-1 is considered to be a new regulator for the urinary ion excretion and renal epithelial cell growth.

Keywords: Calcitonin receptor-stimulating peptide; Calcitonin; Calcitonin receptor; cAMP; LLC-PK$_1$ cell; Na^+/H^+ exchanger; 5-(*N*-Ethyl-*N*-isopropyl) amiloride; cAMP-dependent protein kinase

Calcitonin receptor-stimulating peptide-1 (CRSP-1) is a strong and specific agonist for the calcitonin (CT) receptor, its stimulatory activity for the cAMP production is 10-fold and more than 100-fold stronger than porcine CT in LLC-PK$_1$ cells and COS-7 cells expressing the CT receptor, respectively [1].

Measurement of CRSP-1 concentration in various porcine tissues by radioimmunoassay showed that the pituitary gland and thyroid gland contain the highest levels of CRSP-1 in the pig, although this peptide is widely distributed throughout the central nervous system. In the in vivo experiment, the bolus administration of CRSP-1 into rats significantly reduced the plasma Ca^{2+} level. We assumed that the CRSP-1 secreted from the pituitary gland and thyroid gland into the systemic circulation stimulated the CT receptor and regulated the physiological events in the kidney and the bone. Thus, we focused on the effect of CRSP-1 on the renal function in this study. LLC-PK$_1$ is one of the most characterized renal tubular epithelial cell lines [2–4]. This cell line abundantly expresses the CT receptor [5] and is often used for the analysis of the cell physiological function of CT in the renal epithelial cells. As CRSP-1 stimulates the cAMP production in LLC-PK$_1$ cells more potently than does CT, we examined the effect of CRSP-1 on LLC-PK$_1$ cells to elucidate the cell physiological function of CRSP-1 in the renal epithelial. In this study, therefore, we investigated the effects of CRSP-1 on ion uptake into LLC-PK$_1$ cells and their growth.

Materials and methods

Materials. Synthetic CRSP-1 and salmon CT were prepared and purchased as described previously [1]. ^{125}I-labeled deoxybromouridine (^{125}I-DU), ^{22}NaCl, and $^{45}CaCl_2$ were purchased from Amersham

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Biosciences (Buckingham, UK). Benzthiazide, furosemide, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS), bumetanide, and 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA) were purchased from Sigma (St. Louis, MO, USA).

Cell culture. LLC-PK_1 cells and opossum kidney (OK) cells were cultured with Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium, respectively, supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 U/ml streptomycin in a humidified atmosphere of 95% air–5% CO_2 at 37 °C.

Measurement of cAMP production in LLC-PK_1 cells. LLC-PK_1 cells were harvested, seeded at a density of 1×10^5 cell/well on 48-well plates, and cultured for 24 h. The cells were washed twice with DMEM/Hepes (20 mM, pH 7.4) containing 0.5 mM of 3-isobutyl-1-methyl xanthine (IBMX, Sigma) and 0.05% bovine serum albumin (DMEM/Hepes/IBMX solution), and were incubated in the same medium for 30 min at 37 °C. The incubation medium was then replaced with 150 μl medium, in which the sample of interest was dissolved, and further incubated at 37 °C for another 30 min. Aliquots (100 μl) of the incubation media were succinylated, evaporated, and then submitted to radioimmunoassay for cAMP, as reported previously [1].

Measurement of ^{125}I-DU uptake into LLC-PK_1 cells. The cells were harvested, seeded at a density of 2×10^4 cell/well on 24-well plates, and cultured for 48 h. The cells at 70% confluence were washed with 0.5 ml serum-free DMEM, replaced with DMEM containing 10% FBS and the peptide of interest, and incubated for 2 h at 37 °C. Then, ^{125}I-DU (4×10^5 cpm/50 μl in the DMEM) was added and further incubated for 5 h at 37 °C. Following the incubation, the cells were washed twice with ice-cold phosphate-buffered saline, incubated on ice for 30 min with 5% trichloroacetic acid, washed twice with 99.5% ethanol, and then solubilized in a buffer containing 0.1 M NaOH, 2% Na_2CO_3, and 1% SDS (500 μl/well). The radioactivity in each well was counted using a γ counter (ARC-1000, Aloka, Tokyo, Japan).

Measurement of intracellular cAMP accumulation in OK cells. OK cells were harvested, seeded at a density of 2×10^5 cell/well on 24-well plates, and cultured for 24 h. Porcine CT receptor cDNA ligated into pcDNA 3.1 expression vector (Promega, Madison, WI, USA) was transfected into the OK cells using Lipofectamine Plus (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol, and further incubated for 24 h. The cells were washed twice with DMEM/Hepes/IBMX solution and incubated in the same medium for 30 min at 37 °C. The incubation medium was then replaced with 250 μl medium, in which the sample of interest was dissolved, and further incubated at 37 °C for another 10 min. Following the incubation, the medium was replaced with 99.5% ethanol, and the cells were frozen at −80 °C for 24 h. The cells were lysed by repeated pipetting, and the debris of the lysate was removed by centrifuging at 12,000*g* for 5 min. The supernatant was evaporated, and the resulting pellet was dissolved in DMEM/Hepes/IBMX solution. Aliquots (100 μl) of the incubation media were succinylated, evaporated, and then submitted to radioimmunoassay for cAMP as reported previously [1].

Measurement of $^{45}Ca^{2+}$ uptake into LLC-PK_1 cells. LLC-PK_1 cells were harvested, seeded at a density of 2×10^6 cells on 6-well plates, and cultured for 2 days. The cells were washed twice with a calcium-free Hanks' solution, and replaced with the calcium-free Hanks' solution containing $^{45}Ca^{2+}$ (37 kBq/ml), in the absence or presence of CRSP-1 at a concentration of 1×10^{-6} M. Following incubation at 37 °C for 10 min, the cells were washed three times with ice-cold washing buffer (140 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.4), 80 mM sucrose, and 1 mM EGTA), and the radioactivity incorporated into the cells was measured using a Topcount scintillation counter (Packard, Meriden, CT, USA).

Measurement of $^{22}Na^+$ uptake into OK cells and LLC-PK_1 cells. OK cells expressing recombinant CT receptor or LLC-PK_1 cells were harvested, seeded at a density of 2×10^6 cells/well on 6-well plates, and cultured for 2 days. The cells were washed twice with a Hanks'-choline chloride solution (137 mM choline chloride, 5.4 mM KCl, 4.2 mM $NaHCO_3$, 3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.8 mM $MgSO_4$, 10 mM glucose, and 5 mM Hepes, pH 7.4). Then, the Hanks'-choline chloride-$^{22}Na^+$ (37 kBq/ml) solution containing CRSP-1 (1×10^{-8} -1×10^{-6} M) alone, one of ion transporter inhibitors (1×10^{-6} M) alone, CRSP-1 (1×10^{-6} M) and one of ion transporter inhibitors (1×10^{-6} M), or CRSP-1 (1×10^{-6} M) and EIPA (1×10^8 -1×10^{-6} M) was administered. Following incubation at 37 °C for 10 min, the cells were washed three times with ice-cold saline, and the $^{22}Na^+$ uptake into the cells was measured using a γ-counter (Cobra 5003, Packard).

Statistical analysis. Statistical analysis was performed using a one-way analysis of variance with repeated measurements, combined with a multiple comparison (Scheffe's *F* test). These analyses were carried out using StatView 5.01 (SAS Institute, Cary, NC, USA). The data are expressed as means ± SEM. *P* values less than 0.05 were considered significant.

Results

Fig. 1A shows the dose-dependent elevation of cAMP levels in the LLC-PK_1 cells stimulated with porcine CRSP-1, salmon CT, and porcine CT. CRSP-1, as well

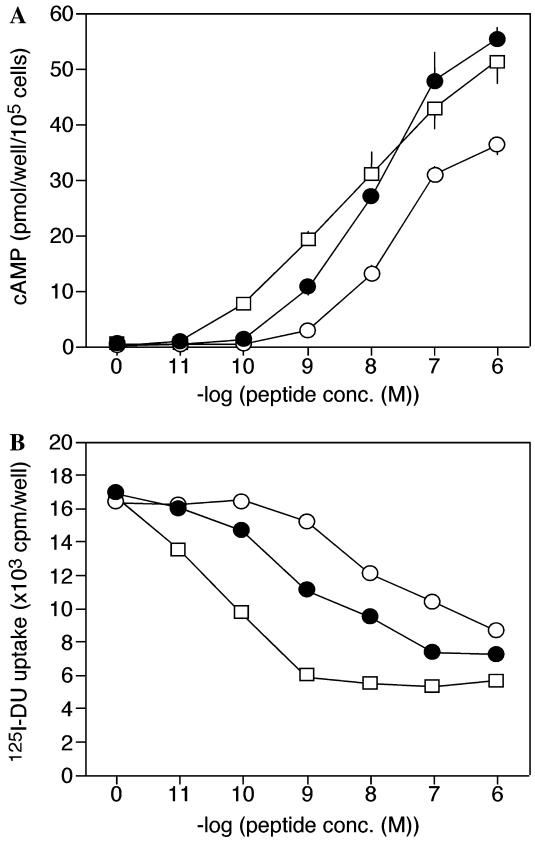

Fig. 1. Effects of CRSP-1, salmon CT, and porcine CT on cAMP production (A) and ^{125}I-DU uptake (B) into LLC-PK_1 cells. The cells were stimulated with porcine CRSP-1 (closed circle), salmon CT (open square) or porcine CT (open circle). (A) Dose-dependent increase of cAMP concentration in the culture medium of LLC-PK_1 cells. (B) Dose-dependent reduction of ^{125}I-DU uptake into LLC-PK_1 cells. Each point represents the mean ± SEM of three separate determinations.

as salmon CT and porcine CT, stimulated the cAMP production, and the potency order of the three peptides in the stimulatory activity of cAMP production was salmon CT > CRSP-1 > porcine CT. Since the increase of the intracellular cAMP concentration induces a change in growth of a variety of cell types, we next evaluated the effect of these peptides on the growth of the LLC-PK_1 cells by measuring the ^{125}I-DU uptake into chromosomal DNA. Parallel to the dose-dependent elevation of the cAMP production, these three peptides reduced the ^{125}I-DU uptake into LLC-PK_1 cells with the order of potency being salmon CT > CRSP-1 > porcine CT (Fig. 1B).

We next investigated the effects of CRSP-1 on ion transport. The effect of CRSP-1 on the $^{45}Ca^{2+}$ uptake into LLC-PK_1 cells is shown in Fig. 2. CRSP-1 significantly but weakly reduced the $^{45}Ca^{2+}$ uptake at a concentration of 1×10^{-6} M, and the effect of CRSP-1 on the reduction was weaker than that of salmon CT. A significant reduction of $^{45}Ca^{2+}$ uptake was not observed when LLC-PK_1 cells were stimulated with CRSP-1 at a concentration of 1×10^{-7} M (data not shown).

Fig. 3A shows the time course of $^{22}Na^{+}$ uptake into LLC-PK_1 cells in the absence or presence of CRSP-1. The $^{22}Na^{+}$ uptake into LLC-PK_1 cells was observed even at 0.1 min and reached a plateau at 60 min. When the cells were stimulated with CRSP-1 at a concentration of 1×10^{-7} M, the $^{22}Na^{+}$ uptake was significantly enhanced at 5 min and the enhancement reached a maximum at 10 min. Based on this result, we incubated the LLC-PK_1 cells with peptides and $^{22}Na^{+}$ for 10 min, and observed the enhancement of $^{22}Na^{+}$ uptake into the cells (Fig. 3B). CRSP-1 enhanced the $^{22}Na^{+}$ uptake into LLC-PK_1 cells from a concentration of 1×10^{-8} M. The potency order of the $^{22}Na^{+}$ uptake-increasing activity was salmon CT > CRSP-1 > porcine CT and was in agreement with that of cAMP production. Thus, we studied the effect of CRSP-1 on the $^{22}Na^{+}$ uptake in greater detail.

To verify that the activation of the CT receptor by CRSP-1 induces $^{22}Na^{+}$ uptake, porcine CT receptor

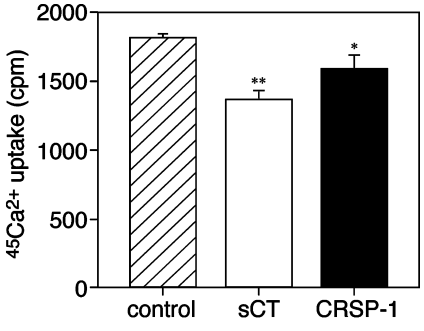

Fig. 2. Effects of CRSP-1, salmon CT, and porcine CT on $^{45}Ca^{2+}$ uptake. The cells were incubated with $^{45}Ca^{2+}$ only (control), with $^{45}Ca^{2+}$ and salmon CT (sCT, 1×10^{-6} M) or porcine CRSP-1 (CRSP-1, 1×10^{-6} M). Each bar represents the mean ± SEM of three separate determinations. *$P < 0.05$; **$P < 0.001$.

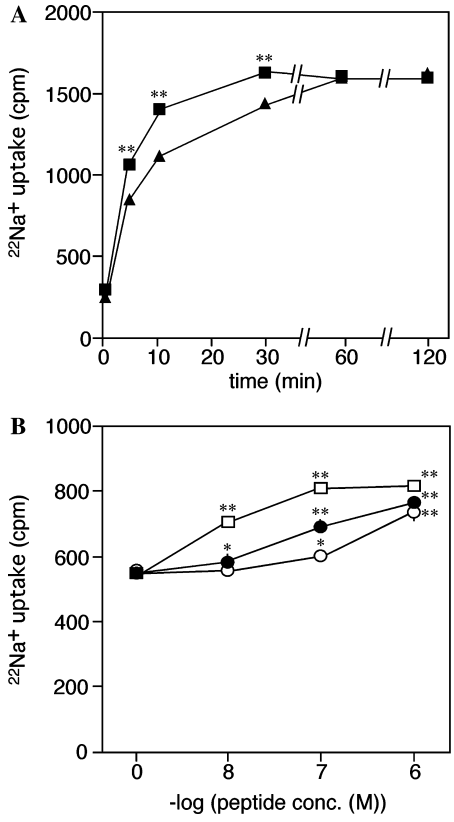

Fig. 3. Time course and dose-dependent enhancement of $^{22}Na^{+}$ uptake into LLC-PK_1 cells. (A) LLC-PK_1 cells were incubated with $^{22}Na^{+}$ in the absence (closed triangle) or presence of 1×10^{-7} M of CRSP-1 (closed square) for 0.1, 5, 10, 30, 60, and 120 min. (B) LLC-PK_1 cells were incubated with $^{22}Na^{+}$ only or with $^{22}Na^{+}$ and the indicated concentrations of porcine CRSP-1 (closed circle), salmon CT (open square), and porcine CT (open circle) for 10 min. Each point represents the mean ± SEM of three separate determinations. *$P < 0.05$; **$P < 0.001$.

cDNA inserted into mammalian expression vector (pcDNA-CTR) was transfected into OK cells, and the $^{22}Na^{+}$ uptake into the cells was measured in the presence of CRSP-1. CRSP-1 stimulated the cAMP production in OK cells, only when pcDNA-CTR was transfected into the cells (Fig. 4A). Parallel to the elevation of cAMP production, the $^{22}Na^{+}$ uptake into OK cells was enhanced with CRSP-1 (Fig. 4B). These results confirm that CRSP-1 actually enhanced the $^{22}Na^{+}$ uptake through the CT receptor-cAMP pathway.

To determine which Na^{+} transporter is activated with CRSP-1, we administered various transporter inhibitors, such as furosemide ($Na^{+}/K^{+}/Cl^{-}$ cotransporter inhibitor), benzthiazide (Na^{+}/Cl^{-} cotransporter inhibitor), EIPA (Na^{+}/H^{+} exchanger inhibitor), SITS (Cl^{-}/bicarbonate exchanger inhibitor), and bumetanide ($Na^{+}/K^{+}/Cl^{-}$ cotransporter inhibitor), into the culture medium of LLC-PK_1 cells at a concentration of 1×10^{-6} M, and measured their effects on the CRSP-1-induced $^{22}Na^{+}$ uptake. Although furosemide, bumetanide, benzthiazide or SITS did not alter the $^{22}Na^{+}$ uptake, EIPA abolished the

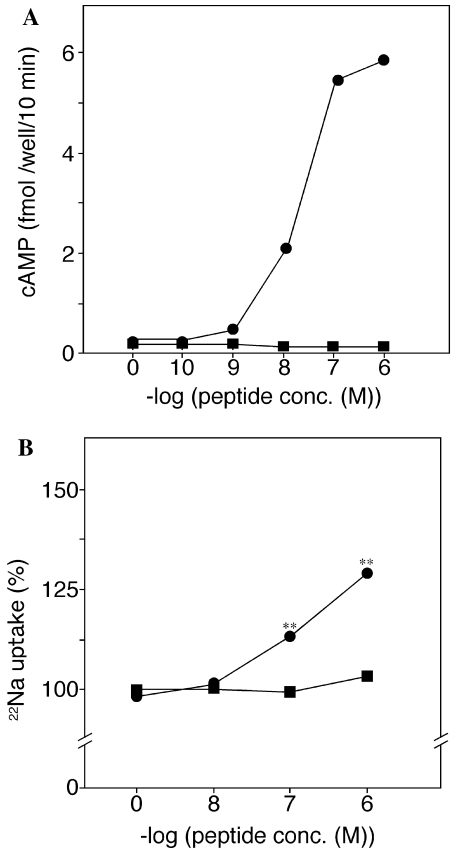

Fig. 4. Effects of CRSP-1 on the intracellular cAMP level (A) and the $^{22}Na^{+}$ uptake (B) into intact OK cells and OK cells transfected with porcine CT receptor. (A) OK cells transfected with porcine CT receptor cDNA (closed circle) and blank vector (closed square) were stimulated with the indicated concentrations of CRSP-1 for 10 min. (B) OK cells transfected with porcine CT receptor cDNA (closed circle) and blank vector (closed square) were incubated with $^{22}Na^{+}$ and the indicated concentrations of CRSP-1 for 10 min. Each point represents the mean ± SEM of three separate determinations. $^{**}P < 0.001$.

effect of CRSP-1 on the $^{22}Na^{+}$ uptake into LLC-PK$_1$ cells (Fig. 5C). These results indicate that the CRSP-1-induced enhancement of $^{22}Na^{+}$ uptake is mediated by a Na^{+}/H^{+} exchanger (NHE).

To identify which isoform of NHEs is responsible for the $^{22}Na^{+}$ uptake that is enhanced by CRSP-1, we treated the cells with different concentrations of EIPA from 1×10^{-8} to 1×10^{-6} M (Fig. 6). The inhibitory effect of EIPA was observed even at a concentration of 1×10^{-8} M. When CRSP-1 and EIPA were administered together at concentrations of 1×10^{-6} M, the effect of CRSP-1 on the $^{22}Na^{+}$ uptake into LLC-PK$_1$ cells was abolished, and the $^{22}Na^{+}$ uptake was reduced to that level when only EIPA had been added to the medium.

Discussion

The effects of CT on renal epithelial cells are known to be mediated by the CT receptor-cAMP pathway [4]. In preceding studies, we demonstrated that porcine CRSP-1 is a more potent and specific agonist for the CT receptor. In the present study, therefore, we investigated the effect of CRSP-1 on the cell physiological events that were induced by the elevation of the cAMP production in the renal epithelial cell line, LLC-PK$_1$.

Fig. 1 shows the anti-proliferative effect of CRSP-1, as well as its stimulatory effect on cAMP production in LLC-PK$_1$ cells, and these effects were 10-fold stronger than those of porcine CT. CT inhibits the growth of LLC-PK$_1$ cells by increasing the intracellular cAMP concentration [6,7]. Jans et al. [6] reported that the growth of LLC-PK$_1$ cells having a mutation in the cAMP-dependent protein kinase (A-kinase) was not inhibited with salmon CT or a vasopressin analogue. Taking these results together, CRSP-1 was deduced to suppress the proliferation of LLC-PK$_1$ through the cAMP-A-kinase pathway.

CRSP-1 and salmon CT reduced $^{45}Ca^{2+}$ uptake into the LLC-PK$_1$ cells (Fig. 2). Several in vivo studies have revealed that urinary Ca^{2+} reabsorption is regulated by the balance between the passive Ca^{2+} influx across the apical membrane via an electrochemical gradient and the active Ca^{2+} extrusion across the basolateral membrane induced with Ca^{2+} pumps and Na^{+}/Ca^{2+} exchangers [8]. Although we cannot specify the target of the CRSP-1-induced suppression of Ca^{2+} uptake, this result raises the possibility that CRSP-1 reduces the plasma Ca^{2+} concentration by inhibiting urinary Ca^{2+} reabsorption into the renal epithelial cells in the kidney.

The $^{22}Na^{+}$ uptake into LLC-PK$_1$ cells was enhanced with CRSP-1 (Fig. 3A). The $^{22}Na^{+}$ uptake into OK cells was enhanced only when the cells expressed recombinant CT receptors (Fig. 4), confirming that CRSP-1 enhances the $^{22}Na^{+}$ uptake into the renal epithelial cells through the CT receptor. As for the mechanism of the CRSP-1-induced enhancement of $^{22}Na^{+}$ uptake into LLC-PK$_1$ cells, this effect was abolished by treating with EIPA, an inhibitor of NHE (Fig. 5) [9]. Two isoforms, NHE1 and NHE3, are expressed in LLC-PK$_1$ cells [10,11], and A-kinase is reported to enhance the NHE1-dependent Na^{+} uptake and to suppress the NHE3-dependent Na^{+} uptake [12,13]. Based on these reports, we at first assumed that the effect of CRSP-1 on the Na^{+} uptake shown in Fig. 3 was the summation of the enhancement of the NHE1-dependent Na^{+} uptake and the suppression of the NHE3-dependent Na^{+} uptake. However, Fig. 6 indicates that CRSP-1 selectively activates NHE1 and enhances Na^{+} uptake, which can be explained by the different 50% inhibitory concentrations of EIPA on NHE1 (approximately 1×10^{-8} M) and NHE3 (more than 1×10^{-6} M) [14–17]. At a concentration of 1×10^{-8} M, EIPA can inhibit 30–40% of the $^{22}Na^{+}$ uptake through NHE1 and cannot inhibit it through NHE3, while at a concentration of 1×10^{-6} M EIPA can inhibit more than 95% of the $^{22}Na^{+}$ uptake

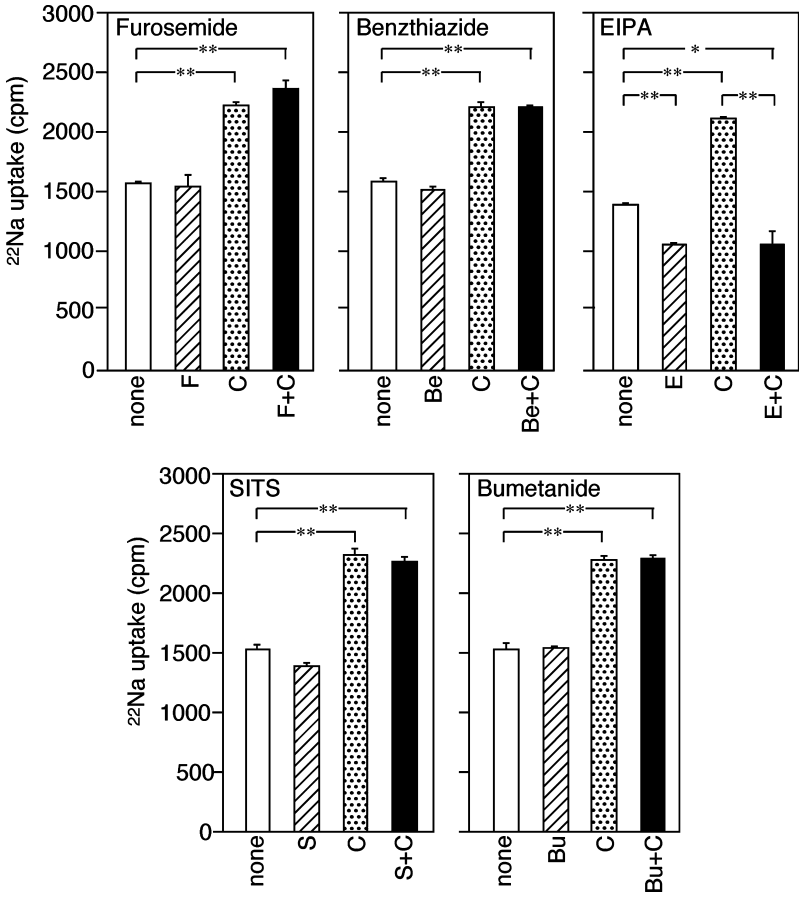

Fig. 5. Effects of ion transporter inhibitors on CRSP-1-induced $^{22}Na^{+}$ uptake into LLC-PK$_1$ cells. LLC-PK$_1$ cells were incubated for 10 min with none (open bar), 1×10^{-6} M of each ion transporter inhibitor (hatched bar), 1×10^{-6} M CRSP-1 (dotted bar), or 1×10^{-6} M CRSP-1 and 1×10^{-6} M of each ion transporter inhibitor (filled bar), in the presence of $^{22}Na^{+}$. C, CRSP-1; F, furosemide; Be, benzthiazide; E, EIPA; S, SITS; and Bu, bumetanide. Each bar represents the mean ± SEM of three separate determinations. $^{**}P < 0.001$.

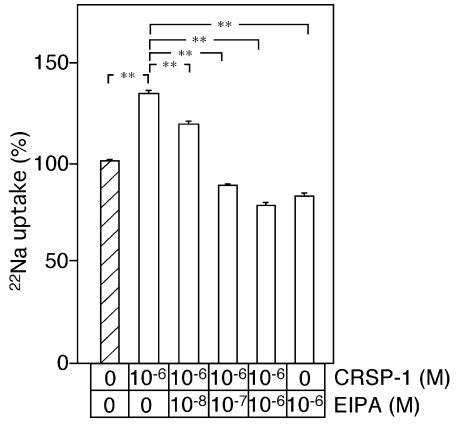

Fig. 6. Dose-dependent inhibitory effect of EIPA on CRSP-1-induced $^{22}Na^{+}$ uptake into LLC-PK$_1$ cells. LLC-PK$_1$ cells were incubated for 10 min with $^{22}Na^{+}$ only (hatched bar), or with $^{22}Na^{+}$ and the indicated concentrations of CRSP-1 and/or EIPA (open bars). Each bar represents the mean ± SEM of three separate determinations. $^{**}P < 0.001$.

through NHE1 and approximately 10% of it through NHE3 [14]. EIPA actually inhibited the CRSP-1-induced enhancement of the $^{22}Na^{+}$ uptake into the LLC-PK$_1$ cells by about 30% at a concentration of 1×10^{-8} M and abolished it at a concentration of 1×10^{-6} M (Fig. 6). This evidence indicates that CRSP-1 enhanced the $^{22}Na^{+}$ uptake into LLC-PK$_1$ cells by activating NHE1 and not by suppressing NHE3 under the present experimental conditions.

NHE1 is localized at the basolateral membrane of the epithelial cells [18,19], and elicits many kinds of cell physiological activities, including the regulation of intracellular pH, cell growth, differentiation, cell migration, and cytoskeletal organization [20]. The data obtained in this study confirmed that CRSP-1 regulates NHE1 activity via the CT receptor-A-kinase pathway in the LLC-PK$_1$ cells, suggesting that this peptide can induce NHE1-mediated cell physiological events in the renal epithelial cells.

On the other hand, the NHE3-dependent Na^{+} uptake into LLC-PK$_1$ (clone 4) cells was reported to be reduced with salmon CT [21], which indicates that CRSP-1 can reduce NHE3 activity by stimulating the CT receptor. In the LLC-PK$_1$ cells used in this study, however, we observed only the stimulatory effect of CRSP-1 on the NHE1 activity instead of observing its inhibitory effect

on the NHE3 activity, probably due to their much lower NHE3 activity than that of NHE1. As NHE3 is localized at the apical membrane of the epithelial cells and is mainly involved in natriuresis, CRSP-1 may be able to regulate the urinary sodium concentration via NHE3 being expressed in the renal epithelial cells.

In conclusion, we investigated the effects of CRSP-1 on the renal epithelial cell line LLC-PK_1, and showed that CRSP-1 enhances the Na^+ uptake mainly through NHE1, reduces the Ca^{2+} uptake, and inhibits the growth of this cell line. Furthermore, these effects of CRSP-1 are stronger than those of porcine CT, which is in agreement with their potencies toward the CT receptor. While the tissue concentration of CRSP-1 in the thyroid gland is estimated to be about 1/10 that of CT [1,22], CRSP-1 is present at a high concentration in the pituitary gland where CT was not detected. These data indicate that the CRSP-1 secreted from these glands can regulate renal epithelial cells in vivo.

Acknowledgments

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