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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

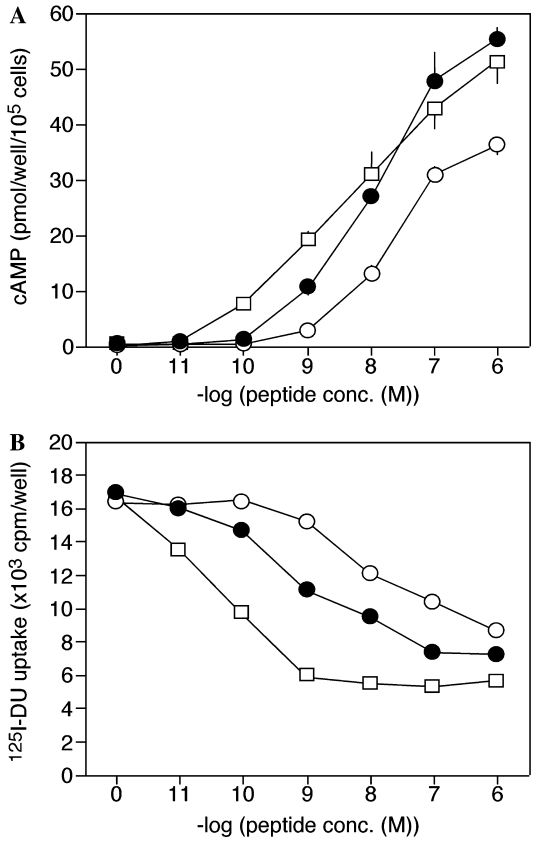

Fig. 1. Effects of CRSP-1, salmon CT, and porcine CT on cAMP production (A) and ^{125}I-DU uptake (B) into LLC-PK_1 cells. The cells were stimulated with porcine CRSP-1 (closed circle), salmon CT (open square) or porcine CT (open circle). (A) Dose-dependent increase of cAMP concentration in the culture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