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RESEARCH ARTICLE

Novel CCK-dependent vasorelaxing dipeptide, Arg-Phe, decreases blood pressure and food intake in rodents

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Scope: We found that a dipeptide, Arg-Phe (RF), had vasorelaxing activity in mesenteric artery isolated from spontaneously hypertensive rats (SHRs) (EC_{50} = 580 nM). We then investigated its mechanism of action, and elucidated its physiological functions.
Methods and results: Vasorelaxing activities of RF-related peptides were tested. The retro-sequence dipeptide FR was inactive, suggesting that the RF sequence is important for a potent vasorelaxing effect. RA and AF were also inactive. RF-NH_2 had vasorelaxing activity, implying that the C-terminal amidation of RF is tolerated. Nitric oxide (NO) and prostaglandins (PGs) are known to be vasorelaxing factors; however, the vasorelaxing activity of RF was inhibited by neither N^G-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor, nor indomethacin, a COX inhibitor. Interestingly, the activity was blocked by lorglumide, an antagonist of the cholecystokinin $(CCK)_1$ receptor; however, RF had no affinity for CCK receptors, suggesting that RF stimulates CCK release. Orally administered RF decreased blood pressure in SHRs, and this antihypertensive activity was also blocked by a CCK_1 antagonist. RF had CCK-like suppressive effects on food intake and gastrointestinal transit. RF increased intracellular Ca^{2+} flux and CCK release in enteroendocrine STC-1 cells.
Conclusion: A novel CCK-dependent vasorelaxing RF decreases both blood pressure and food intake.

Keywords:
Blood pressure / Cholecystokinin / Dipeptide / Food intake / L-amino acid ligase / Vasorelaxation

1 Introduction

A number of low-molecular-weight bioactive peptides have been found from the protease hydrolysate of various food proteins [1–7]. It was reported that angiotensin I-converting enzyme (ACE)-inhibitory peptides derived from various dietary proteins showed antihypertensive activities. On the other hand, vasorelaxing peptides also often exhibit antihypertensive activity. Indeed, vasorelaxing three–six amino acid peptides derived from food proteins, including ovokinin(2–7), rapakinin, and rubimetide, decreased blood pressure after the oral administration in spontaneously hypertensive rats (SHRs) [7–9]. In the current study, we found that Arg-Phe (RF), a simple dipeptide consisting of two L-amino acids, exhibits vasorelaxing activity (EC_{50} = 580 nM). RF had no ACE-inhibitory activity. This vasorelaxing activity seems to be more potent than that of previously reported bioactive peptides derived from food proteins.

Next, we investigated the mechanism underlying the vasorelaxing activity of RF. Nitric oxide (NO) and prostaglandins (PGs) are the candidates of vasorelaxing factors [10]; however, inhibitors of NO synthase and cyclooxygenase (COX) did not block the vasorelaxing activity of RF. Interestingly, this vasorelaxing activity was blocked by an antagonist of cholecystokinin (CCK), a well-known peptide as an endogenous satiety signalling molecule. It has been reported that the CCK system also is involved in the relaxation of the mesenteric artery in SHRs [8]. We tested whether the antihypertensive activity

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Abbreviations: **ACE**, angiotensin I-converting enzyme; **CaR**, calcium-sensing receptor; **L-NAME**, N^G-nitro-L-arginine methyl ester; **NO**, nitric oxide; **PG**, prostaglandin; **RF**, Arg-Phe; **SHRs**, spontaneously hypertensive rats

of RF is mediated by the CCK system. We also investigated whether RF decreased food intake and gastrointestinal transit similarly to CCK.

2 Materials and methods

2.1 Reagents

RF synthesized by the chemosynthetic method was obtained from Kokusan Chemical Co., Ltd. (Tokyo, Japan). RF-related peptides were synthesized by the Fmoc strategy. The amino acids Arg and Phe, and the COX inhibitor indomethacin were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N^G-nitro-L-arginine methyl ester (L-NAME), an NO synthase inhibitor, was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). AH6809 and lorglumide, antagonists of EP1/EP2/EP4 for PGE_2 and of CCK_1 receptor, respectively, were from Sigma-Aldrich Co. (St. Louis, MO). CAY10441 and BWA868C, antagonists of IP for PGI_2 and DP for PGD_2, respectively, were obtained from Cayman Chemical Company (Ann Arbor, MI). ONO-AE3-208, an antagonist of EP4 for PGE_2, was kindly provided by Ono Pharmaceutical Co. Ltd. (Osaka, Japan).

2.2 Preparation of enzymatically synthesized Arg-Phe by L-amino acid ligase RizA

Recombinant L-amino acid ligase RizA was prepared in accordance with a previous report by Kino et al. [11, 12]. The reaction mixture (total volume, 1 mL) contained 0.1 mg/mL of the recombinant RizA, 12.5 mM ATP, 12.5 mM $MgSO_4 \cdot 7H_2O$, 12.5 mM Arg, and 12.5 mM Phe in 100 mM Tris-HCl buffer (pH 9.0). The reaction was performed at 30°C for 20 h, and was then stopped by boiling for 10 min.

2.3 Animals

Male SHRs/Izm (15–27 weeks old) and ddY mice (4 weeks old) were obtained from SLC (Shizuoka, Japan). The animals were kept in a temperature-controlled room (23 ± 1°C) on a daily 12-h light: 12-h dark cycle. SHRs and ddY mice were fed SP pellets (Funabashi Farm, Chiba, Japan) and CE-2 pellets (CLEA Japan, Inc., Tokyo Japan), respectively, with free access to water. All experiments were approved by the Kyoto University Ethics Committee for Animal Research Use.

2.4 Vasorelaxing assay

The vascular relaxation response was determined as follows [4, 5, 8]: the small mesenteric artery isolated from SHRs, 150–200 μm in diameter, was cut into helical strips. The strips were suspended in a bathing medium (Krebs-Henseleit solution containing 120 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose) maintained at 37°C, and continuously bubbled with O_2/CO_2 (95:5). The resting tension was adjusted to 0.3 g. The artery was preconstricted with 0.3 μM phenylephrine. Samples were applied 10 min after the application of phenylephrine. Relaxing activity was assayed in the absence and presence of inhibitors and antagonists applied 10 min before the addition of phenylephrine (only L-NAME was applied 50 min before phenylephrine). To determine the complete relaxation point, 100 μM papaverine was added at the end of each experiment.

2.5 Blood pressure measurement

Systolic blood pressure after the oral administration of RF to conscious SHRs was measured by the tail-cuff method using a MK-2000 (Muromachi Kikai Ltd., Tokyo, Japan) [4–6, 13]. The animals were trained to undergo measurements by the tail-cuff method for 3 weeks. RF dissolved in physiological saline was administered with a metal sonde after measuring the basal systolic blood pressure. CCK_1 receptor antagonists were administered intraperitoneally (i.p.) just before the administration of RF. Following the administration of RF, systolic blood pressure was measured every 2 h.

2.6 Food intake

The food intake experiment was performed as described previously [14–16]. Briefly, individually housed male ddY mice were deprived of food pellets for 18 h with free access to water after acclimation for more than 3 days. RF (3–10 mg/kg) in saline was administered i.p. Preweighed food pellets in each cage were measured 20 min and 1, 2 and 4 h after the administration, and the cumulative food intake was calculated. The food intake experiment started at 11 a.m.

2.7 Small intestinal transit

Small intestinal transit was measured according to the previous reports [13, 17, 18]. Male ddY mice were also deprived of food pellets for 18 h with free access to water. Peptides with or without antagonists and dissolved in saline were administered orally. A test meal (0.5 mL of 5% (w/v) Evans blue suspended in water containing 1% carboxymethylcellulose) was orally administered 30 min after the oral administration of RF. Five minutes after the test meal was given, the mice were sacrificed by cervical dislocation. The abdomen was opened, the small

intestine from the pylorus to the ileocecal junction was dissected, and the point to which the test meal had traveled was secured with thread to avoid changing the length of transit due to handling. The distance traveled by the test meal and the total length of the small intestine were measured. Small intestinal transit was calculated as the ratio of the distance traveled by the test meal to the total length of the small intestine, and expressed as a percentage. The small intestinal transit experiment started at 11 a.m.

2.8 Intracellular Ca^{2+} flux and CCK release in enteroendocrine STC-1 cells

STC-1 cells (a gift from Dr. D. Hanahan, University of California, San Francisco, CA) were grown in Dulbecco's modified Eagle's medium (Sigma, Cat. No. D5796) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Cells were routinely subcultured by trypsinization upon reaching 80–90% confluence [19, 20].

For the measurement of intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$), cells were seeded at a density of 3.3×10^4 cells/well in 96-well plates, and cultured overnight. STC-1 $[Ca^{2+}]i$ was measured with a Calcium Kit II-Fluo 4 (Dojindo Laboratories, Kumamoto, Japan). Cells in 96-well plates were loaded with fluo-4-AM in the kit dissolved in quenching buffer containing Hank's HEPES buffer, 1.25 mM probenecid and 0.04% pluronic F-127 at 37°C for 1 h. After incubation, the plates were placed into a POLARstar OPTIMA (BMG LABTECH GmbH, Ortenberg, Germany) to monitor cell fluorescence ($\lambda_{ex} = 485$ nm, $\lambda_{em} = 520$ nm) before and after the addition of RF.

STC-1 cells were seeded in 96-well culture plates at a density of 3.3×10^4 cells/well, and cultured overnight, when reached 80–90% confluence. Cells were washed twice with PBS to remove the culture media, and exposed to test agent dissolved in PBS for 60 min at 37°C. After incubation for 60 min, supernatants were collected and centrifuged at 800 × *g* for 5 min at 4°C to remove the remaining cells, and then stored at −80°C until CCK concentrations were measured with a commercial EIA kit (Phoenix Pharmaceuticals Inc., Belmont, CA).

2.9 Statistical analysis

Values are expressed as the mean ± SEM. Analysis of variance (ANOVA) followed by Fisher's test or Student's *t*-test was used to assess differences among three and more or two groups, respectively. *P*-value less than 0.05 were considered significant.

3 Results

3.1 RF relaxes the mesenteric artery of SHRs in a CCK-dependent manner

Typical vasorelaxing activity in mesenteric artery isolated from SHRs after the application of RF is shown in Fig. 1A. RF dose-dependently relaxed the mesenteric artery (Fig. 1B), and the EC_{50} value of vasorelaxing activity induced by RF was 580 nM. The retro-sequence peptide Phe-Arg (FR) was inactive at 10 μM, indicating that the amino acid sequence of RF is important for potent vasorelaxing activity. Arg-Ala (RA) and Ala-Phe (AF) were also inactive (Fig. 1C). Neither Arg nor Phe, the constitutive amino acids of

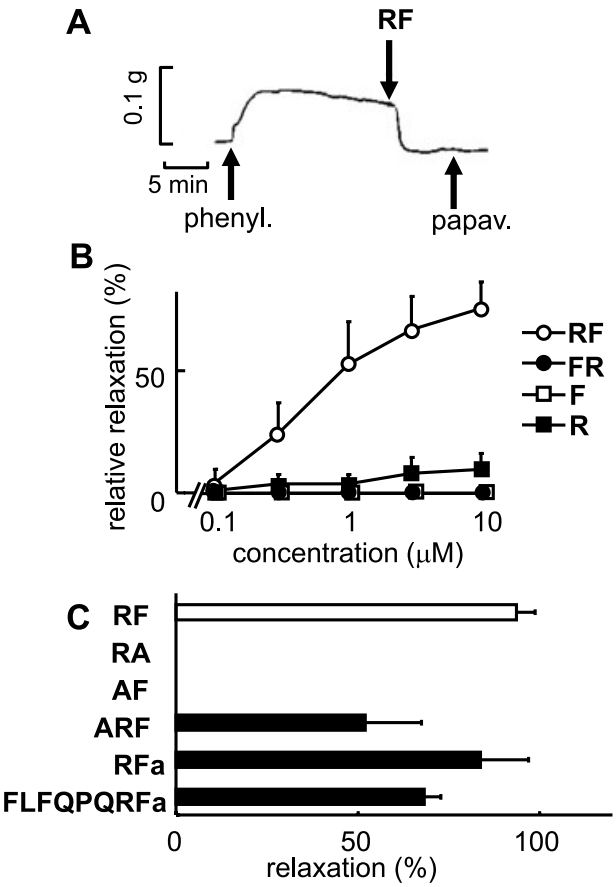

Figure 1. Vasorelaxing activities of RF and its analogues in the mesenteric artery isolated from spontaneously hypertensive rats. (A) Typical vasorelaxing activity of RF at the concentrations of 10 μM. (B) Dose-response curve of the vasorelaxing activities of RF, its retro-sequence peptide and constitutive amino acids. (C) Structure-activity relationship of vasorelaxing activities induced by RF analogues at 10 μM. (RFa: RFamide, FLFQPQRFa: FLFQPQRFamide). Each value is expressed as the mean ± SEM (B, $n = 3$–5; C, $n = 3$–7).

dipeptide RF, had vasorelaxing activity under our experimental conditions. In addition, the vasorelaxing activity of RF was independent of ACE inhibitory activities (the IC_{50} for RF was more than 100 μM).

Next, we tested the vasorelaxing activities of RF analogues. There are a number of RF sequences in not only dietary proteins but also endogenous neuropeptides, including an RFamide group with the structure of RF-NH_2 in the C-terminus. We then synthesized peptides corresponding to the common C-terminus substructure of RFamide peptides, and found that RF-NH_2 itself has vasorelaxing activity (Fig. 1C). The neuropeptide FF (NPFF), an endogenous RFamide peptide, also had vasorelaxing activity in the isolated mesenteric artery of SHRs. The tripeptide Ala-Arg-Phe exhibited vasorelaxing activities. Taken together, the N-terminal elongation and C-terminal amidation of RF seem to be tolerated.

Recently, a novel method enabling cost-effective and large-scale production of dipeptides using L-amino acid ligase (Lal; EC 6.6.2.28), which catalyzes the formation of a peptide bond between unprotected amino acids in an ATP-dependent manner in aqueous solution, has also been established [11]. Because of the substrate specificity of Lal, not all combinations of dipeptides have been synthesized. Thus, we did indeed synthesize RF enzymatically, and confirm its biological activity. RF synthesized by the Lal-based method had vasorelaxing activity comparable to that obtained by conventional chemosynthesis (Supporting Information Fig. S1). This is the first evidence that a dipeptide synthesized by Lal relaxes the blood vessels.

3.2 The vasorelaxing activity of RF was mediated through the CCK system

NO and PGs are known to be vasorelaxing factors; however, the vasorelaxing activity of RF (10 μM) was inhibited by neither L-NAME (100 μM), an NO synthase inhibitor, nor indomethacin (3 μM), a COX inhibitor (Fig. 2). Antagonists of IP, DP and EP1/EP2/EP4 receptors for PGI_2, PGD_2 and PGE_2, respectively, did not block the vasodilation induced by RF. Interestingly, this activity of RF was blocked by lorglumide (30 μM), an antagonist of the CCK_1 receptor; however, the affinity of RF (100 μM) for CCK_1 and CCK_2 receptors was negligible (data not shown), suggesting that RF stimulates CCK release. Taken together, the dipeptide RF may relax the mesenteric artery of SHRs via the release of CCK and activation of the CCK_1 receptor.

3.3 Orally administered RF exhibits antihypertensive via the CCK system

We also investigated the effect of RF on blood pressure and the mechanism underlying its antihypertensive activity. Orally administered RF dose dependently decreased systolic blood pressure in SHRs (Fig. 3A). As shown in Fig. 3B, this

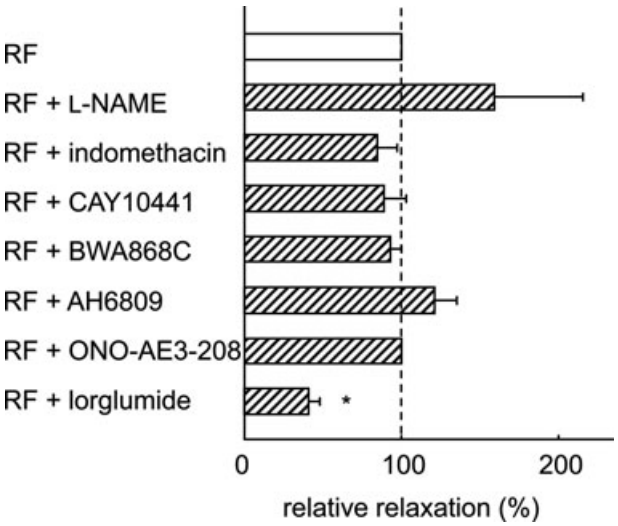

Figure 2. Involvement of the CCK_1 receptor in the vasorelaxing activities of RF in SHRs. L-NAME (NOS inhibitor, 100 μM), indomethacin (COX inhibitor 3 μM), CAY10441 (IP antagonist, 100 nM), BWA868C (DP antagonist, 100 nM), AH6809 (antagonist for EP1 and EP2, 1 μM), ONO-AE3-208, EP4 antagonist, 10 nM) and lorglumide (CCK_1 receptor antagonist, 30 μM) were applied before the addition of RF (10 μM). Values are expressed as the mean ± SEM ($n = 3$–5). $^*P < 0.05$ compared with RF alone.

antihypertensive effect of RF (15 mg/kg, orally) was completely blocked by pretreatment with an antagonist of the CCK_1 receptor, lorglumide (0.3 mg/kg, i.p.), which did not change blood pressure under our experimental conditions [8]. These results suggest that the RF-induced antihypertensive effect as well as vasorelaxing activity is mediated by activation of the CCK_1 receptors.

3.4 RF decreases food intake and small intestinal transit

CCK is known to be a satiety factor, decreasing food intake and gut motility; thus, we investigated whether RF has CCK-like activities. RF suppressed food intake after i.p. administration at a dose of 10 mg/kg in a dose-dependent manner in male ddY mice fasted for 18 h (Fig. 4A). Orally administered RF delayed small intestinal transit, as shown in Fig. 4B.

3.5 RF increases CCK release by enteroendocrine STC-1 cells

To investigate whether RF stimulates CCK release, the enteroendocrine cell line STC-1 was used. RF dose dependently increased intracellular Ca^{2+} flux (Fig. 5A). RF also increased the CCK concentration in the supernatant in response to RF (Fig. 5B). These results suggest that RF activates enteroendocrine cells to release CCK.

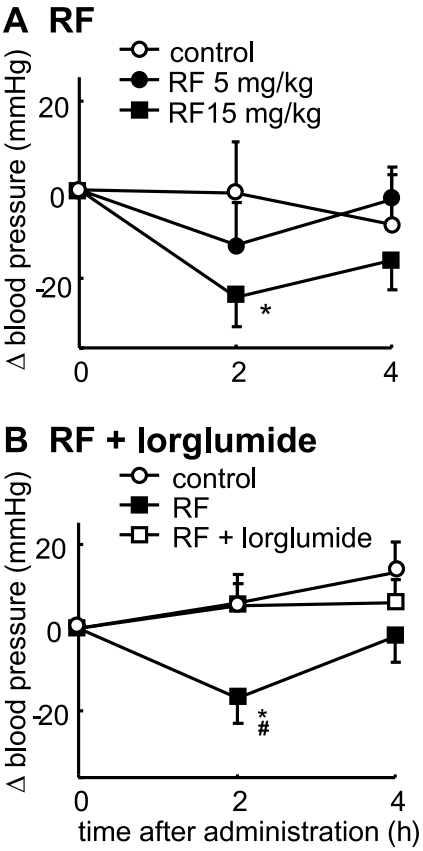

Figure 3. Antihypertensive activity of RF after oral administration. (A) RF at a dose of 5–15 mg/kg was administered to SHRs, and blood pressure was determined by the tail-cuff method before and 2 and 4 h after administration. (B) Effect of lorglumide on RF-induced antihypertensive activity. Lorlgumide (0.3 mg/kg, i.p.) was administered 30 min before the oral administration of RF (15 mg/kg). Each value is expressed as the mean ± SEM (A, n = 8–12; B, n = 10–11). $^{*}P < 0.05$ compared with saline-control group. $^{\#}P < 0.05$ compared with RF-treated group.

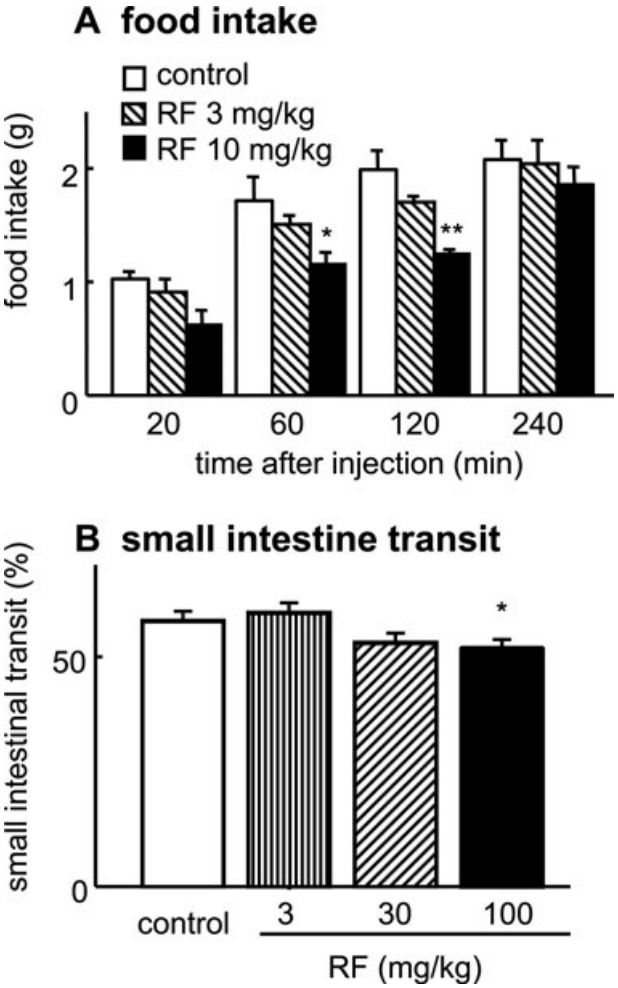

Figure 4. RF suppressed food intake and delayed small intestinal transit. Mice fasted for 18 h were injected with RF (3–10 mg/kg, i.p. or 3–100 mg/kg, orally), and food intake (A) or small intestine transit (B), respectively, was measured. Each column is the mean ± SEM (A, $n = 4$; B, $n = 10$–18). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with each group by ANOVA followed by Fisher's test.

4 Discussion

We found that the dipeptide RF relaxes the mesenteric artery and lowered blood pressure after oral administration to SHRs. The vasorelaxing and antihypertensive activities of RF were blocked by a CCK_1 receptor antagonist; however, RF had no affinity for CCK receptors suggesting that these activities are mediated by CCK release and activation of the CCK_1 receptor. CCK, a brain-gut hormone, is a well-known satiety signal [21, 22]; however, it was also reported to be present in the mesenteric artery [23, 24]. It has been reported that CCK relaxes the mesenteric artery and decreases blood pressure via the CCK_1 receptor [25–30], which was consistent with our findings that the vasorelaxing and hypotensive activity of RF is mediated by the CCK system. In the arteries without endothelium-dependent response to acetylcholine, the vasorelaxing activity of RF at 10 μM (59 ± 4%) might be lower, but still present. On the other hand, RF increased CCK secretion from enteroendocrine cells, STC-1, which raised the possibility that RF might stimulate nerve cells including the mesenteric artery. It was previously reported that rapakinin, a hypotensive tripeptide derived from rapeseed, has vasorelaxing activity via activation of the PGI_2–IP system followed by the CCK–CCK_1 system [8]; however, the mechanism of RF-induced vasodilation via the CCK system without activation of the PGI_2 system may be different. It was reported that novokinin, an orally active angiotensin AT_2 agonist peptide designed based on ovokinin(2–7), more potently relaxed the mesenteric artery isolated from SHRs than that from normotensive Wistar-Kyoto (WKY) rats. RF-induced vasorelaxing activity in normotensive rodents should be investigated further.

Arg and Phe, the constitutive amino acids of RF, and FR, a retrosequence dipeptide of RF, were also inactive, suggesting

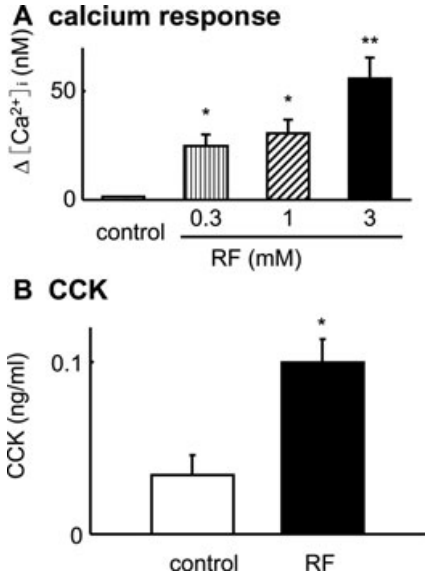

Figure 5. RF increased $[Ca^{2+}]i$ and CCK release in enteroendocrine STC-1 cells. (A) Dose-dependency of the RF-induced increase in $[Ca^{2+}]_i$. (B) Increase in CCK released into the culture medium after addition of RF (3 mM). Values are expressed as the mean ± SEM (A, $n = 3$–5; B, $n = 5$–6). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control group.

that the amino acid sequence of RF is important for potent vasorelaxing activity. Furthermore, the C-terminal amidation and the N-terminal elongation of RF were tolerated. Indeed, RFamide and NPFF had vasorelaxing activity. The first recognized the member of the RFamide neuropeptide family was a cardio-excitatory peptide isolated from ganglia of the clam [31]. Subsequently, a large number of RFamide peptides, which are endogenously released from precursor proteins, have been identified in the nervous system of animals within all major phyla [32]. It is reported that a number of RFamide peptides increase blood pressure [33–36], while the C-terminal structure of RFamide peptides may contribute to vasorelaxing activity to counteract their hypertensive effect. It has been reported that RFamide also affects food intake [32, 34, 37, 38]. Almost all physiological activities of RFamide peptides might be explained by specific receptors already identified; however, the contribution of vasorelaxing and anorexigenic activities associated with the C-terminal structure of RFamide should be clarified.

RF sequences are present in the primary structure of natural food proteins such as rice and soybeans as well as endogenous neuropeptides. Peptides having an RF sequence in the C-terminus maintain vasorelaxing and antihypertensive activities. For example, Ile-His-Arg-Phe (IHRF), a rice albumin-derived tetrapeptide having an RF sequence at the C-terminus, also exhibited vasorelaxing and antihypertensive activity (unpublished observation). These results do not rule out the possibility that food-protein-derived peptides having an RF sequence after digestion by enzymes present in the gastrointestinal tract potentially contribute to vasorelaxing and antihypertensive activity in the postprandial state.

We also demonstrated that RF synthesized by the Lal-based method, which has recently been established, shows potent vasorelaxing activity. So far, bioactive peptides have been commercially produced by enzymatic digestion of food proteins. If dipeptide, cost-effectively synthesized by the Lal-based method on an industrial scale, can be used, the dipeptide market might be expanded.

Metabolic syndrome is characterized by visceral obesity, hyperlipidemia, hypertension, and hyperglycemia. It was reported that the CCK signal is also decreased in metabolic syndrome [39, 40]. RF and its analogues, peptides stimulating CCK release, have blood pressure lowering and anorexigenic activities, which may contribute to the prevention of both hypertension and obesity among the four symptoms of metabolic syndrome.

The calcium-sensing receptor (CaR) was originally identified as a G-protein-coupled receptor (GPCR) for calcium, but CaR also functions as an L-amino acid receptor [41]. Aromatic amino acids (Phe, Trp) are potent ligands that are known to activate CaR. CaR mRNA is expressed in STC-1 cells as well as in organs such as the kidney, thyroid, stomach and intestine. Phe was also reported to stimulate CCK release via CaR [20]; however, RF-induced intracellular Ca^{2+} flux in STC-1 cells was not inhibited by an antagonist of CaR, NPS2143, suggesting that RF may activate STC-1 independently of CaR. Thus, CaR was ruled out as an RF receptor candidate. GPRC6A, a GPCR classified in the CaR family, has been identified as a receptor of basic L-amino acids such as Arg and Lys [42]; however, Lys-Phe lost all vasorelaxing activity (unpublished observation). Further investigation is needed to elucidate the receptor to which RF binds directly.

In conclusion, a novel CCK-releasing dipeptide, Arg-Phe (RF), has vasorelaxing activity. RF synthesized by Lal also mimicked this activity. Orally administered RF decreased blood pressure via activation of the CCK_1 receptor. RF also had CCK-like activities to suppress food intake and gastrointestinal transit.

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The authors have declared no conflict of interest.

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