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ORIGINAL

Regulation of the expression of corticotropin-releasing factor gene by pyroglutamylated RFamide peptide in rat hypothalamic 4B cells

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Abstract. Pyroglutamylated RFamide peptide (QRFP), an important regulator of metabolism and energy homeostasis, has orexigenic effects. QRFP acts *via* a specific receptor, Gpr103. Gpr103 mRNA is expressed in the rat hypothalamic paraventricular nucleus (PVN). In the PVN, corticotropin-releasing factor (CRF), which plays a central role in regulating the stress response and is produced in response to stress, stimulates the release of adrenocorticotropic hormone from the anterior pituitary. We hypothesized that QRFP regulates CRF gene expression directly in the hypothalamus, and thus examined the direct effect of QRFP on the promoter activity and mRNA levels of CRF in hypothalamic cells. To examine these pathways, we used hypothalamic 4B cells, a homologous PVN neuronal cell line. Gpr103a and Gpr103b mRNA, and Gpr103 (a and b) proteins were expressed in the hypothalamic cells. The Gpr103 mRNA and protein levels were increased by QRFP. QRFP also stimulated CRF mRNA levels and CRF promoter activity directly in 4B cells following their transfection with the CRF promoter. The protein kinase A (PKA) and protein kinase C (PKC) pathways were involved in the QRFP-induced increases in CRF promoter activity. QRFP stimulated cAMP response element-binding protein (CREB) phosphorylation. CREB phosphorylation was inhibited by a PKC inhibitor. PKC-dependent signaling would be upstream of the CREB phosphorylation. Thus, QRFP-dependent pathways are involved in the regulation of CRF gene expression in the hypothalamus.

Key words: QRFP, Corticotropin-releasing factor, Hypothalamus, Stress

PYROGLUTAMYLATED RFAMIDE PEPTIDES (QRFP), including a 26-aa (QRFP26) and a 43-aa (QRFP43) peptides, are important regulators of metabolism or energy homeostasis. QRFP has orexigenic effects in rodents [1-5]. QRFP also regulates behavioral arousal, blood pressure, locomotor activity, and aldosterone and insulin secretion [2, 6, 7]. QRFP acts *via* a specific receptor, Gpr103, which belongs to the G protein-coupled receptor superfamily [2, 8]. QRFP43 exhibits more potent agonistic activity than QRFP26 [9].

Gpr103 mRNA is expressed in the hypothalamus and pituitary of humans and mice [2, 8]. Gpr103 mRNA is expressed in the rat hypothalamic paraventricular nucleus (PVN) [10]. There are two murine homologues of human Gpr103, termed Gpr103a and Gpr103b [2]. QRFP binds and activates both Gpr103a and Gpr103b [2]. QRFP is an endogenous ligand for the Gpr103 in pancreatic β-cells and skeletal muscle cells [11, 12].

Corticotropin-releasing factor (CRF), a major regulatory peptide in the hypothalamic–pituitary–adrenal (HPA) axis [13, 14], mediates a variety of physiological functions, including regulation of the HPA axis activity during periods of stress [15]. CRF, produced in the PVN in response to stress [16], is secreted into the pituitary portal circulation and triggers the synthesis of adrenocorticotropic hormone (ACTH) *via* CRF receptor type 1 in the anterior pituitary. ACTH then stimulates glucocorticoid release from the adrenal glands [16]. CRF is a potent anorexic peptide that

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plays an important role in inhibiting the central regulation of feeding behavior [17]. Because the HPA axis is an important regulator of metabolism through the actions of corticosterone, it is of interest to clarify in detail the actions of CRF on the HPA axis.

We hypothesized that QRFP regulates CRF gene expression directly in hypothalamus, and thus examined the direct effect of QRFP on the promoter activity and mRNA levels of CRF in hypothalamic cells. To explore this process, we used a homologous PVN neuronal cell line, hypothalamic 4B, because these cells express CRF, arginine vasopressin, CRF receptor type 1, and glucocorticoid receptors and show characteristics of the parvocellular neurons of the PVN [18]. We also explored the involvement of protein kinase pathways by using various kinase inhibitors in the 4B cells.

Materials and Methods

Materials

Rat fetal hypothalamic 4B cells, developed from primary hypothalamic culture, were provided by Dr. John Kasckow [18]. QRFP43 was purchased from the Peptide Institute (Osaka, Japan). H89 was purchased from Seikagaku Corporation (Tokyo, Japan). The protein kinase A (PKA) inhibitor 14-22 amide (PKAi), and protein kinase C (PKC) inhibitors Ro-32-0432 and bisindolylmaleimide I (BIM) were purchased from Calbiochem (San Diego, CA).

Cell culture

4B cells were incubated in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were plated at 5×10^4 cells/well for 3 days before each experiment. One day before each experiment, the cells were washed and then serum-starved overnight in DMEM/F12 supplemented with 0.2% bovine serum albumin (BSA). On the experiment day, cells were incubated in medium with vehicle or QRFP. At the end of each experiment, total cellular RNA or protein was collected and stored at –80°C until the appropriate assay was performed.

RNA extraction

Total cellular RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Then, cDNA was synthesized from the total RNA (0.5 μg) using random hexamers as primers with the SuperScript First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions.

Reverse transcriptase-polymerase chain reaction

RT-PCR was carried out in a programmable thermal controller (Bio-Rad, Hercules, CA) with the following oligonucleotide primers [19]: Gpr103a-forward (F) (5′-GCACGGAAGCCTGGGAAT-3′), Gpr103a-reverse (R) (5′-ATGTGTCTCCTTTGGTTTCTTCCA-3′), Gpr103b-F (5′-TGTGATGCACAGAAGAGTGAAGTTAG-3′), and Gpr103b-R (5′-TGCTGCCCCCAAATGCT-3′). The cycling conditions for Gpr103a and Gpr103b were 1 × (95°C, 10 min), 35 × (95°C, 15 s; 60°C, 1 min; 72°C, 1 min), and 1 × (72°C, 5 min). A total RNA sample was used for negative control for RT-PCR. Products were separated by electrophoresis on a 1.2% and 4.5% agarose gel containing ethidium bromide for Gpr103a and Gpr103b, respectively. The expected sizes of the PCR products for Gpr103a and Gpr103b were 104 bp and 79 bp, respectively.

Real-time RT-PCR

Real-time RT-PCR was performed to examine the effect of QRFP (1 nM - 1 μM) on changes in mRNA levels. The resulting cDNAs were subjected to real-time RT-PCR as follows. The expression level of rat Gpr103a or Gpr103b was evaluated using quantitative real-time RT-PCR based on the previous mentioned specific sets of primers and following probes: Gpr103a Probe (5′-FAM-AGCAAAGTTATCTCGACCACAGCGTCCA-TAMRA-3′) or Gpr103b Probe (5′-FAM-TGTGGGAGAGAGAATCCTGTAGAGATCA-TAMRA-3′). The expression level of rat CRF mRNA was evaluated using quantitative real-time RT-PCR based on the following specific sets of primers and probes: CRF-F (5′-TGGATCTCACCTTCCACCTTCTG-3′); CRF-R (5′-CCGATAATCTCCATCAGTTTCCTG-3′); and Probe (5′-FAM-GCCAGGGCAGAGCAGTTAGC-TAMRA-3′). Beta2-microglobulin, which was not changed by any treatment, was used as a housekeeping gene to normalize the expression results (Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA). Each reaction consisted of 1 × TaqMan Universal PCR Master Mix (Applied Biosystems), 1 × Assays-on-Demand Gene Expression Product (Rn00560865

m1 for rat β2-microglobulin) or a set of CRF primers and probes, and 1 μL of cDNA in a total volume of 25 μL. The following parameters were used in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The above assays involved specific sets of primers and a TaqMan probe spanning the exon/exon junction and should not, therefore, have been influenced by DNA contamination. Data were collected and recorded with ABI PRISM 7000 SDS software (Applied Biosystems) and expressed as a function of the threshold cycle (C_T). The amplification efficacies for each gene of interest and the housekeeping gene amplimers were found to be identical when analyzed with diluted samples [20].

Relative quantitative gene expression was calculated by the $2^{-\Delta\Delta C_T}$ method. In brief, for each sample assayed, the C_T of the reactions amplifying the gene of interest and a reference gene were determined. The C_T of the gene of interest of each sample was corrected by subtracting the C_T for the housekeeping gene (ΔC_T). Untreated controls were chosen as reference samples, and the ΔC_T for all experimental samples was reduced by the average ΔC_T for the control samples ($\Delta\Delta C_T$). Finally, the abundance of the experimental mRNA relative to that of the control mRNA was calculated with use of the formula $2^{-\Delta\Delta C_T}$.

Western blot analysis

Western blot analysis was performed to examine the effect of QRFP (100 nM) on changes in the protein expression of Gpr103, phosphorylated cAMP response element-binding protein (pCREB), and CREB [21]. Beta-actin was used as a housekeeping protein. The cells were washed twice with phosphate-buffered saline (PBS) and lysed with Laemmli sample buffer. Cell debris was pelleted by centrifugation and the supernatant was recovered. The samples were boiled and subjected to electrophoresis on a 4–20% gradient polyacrylamide gel, and proteins were transferred to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking with Detector Block® buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the membrane was incubated for 1 h with each antibody (anti-Gpr103 (a and b) antibody [1:1,000 dilution], which detected both Gpr103 (a and b) proteins (Bioworld Technology Inc., Louis Park, MN); anti-pCREB [1:500 dilution]/CREB [1:1,000 dilution] antibodies (Cell Signaling Technology, Beverly, MA); and anti-β-actin [1:5,000 dilution] antibody, ab8227, Abcam, Cambridge, MA), washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (1:20,000 dilution; Daiichi Kagaku). The chemiluminescent substrate SuperSignal West Pico (Pierce Chemical Co., Rockford, IL) was used for detection, and the membrane was exposed to BioMax film (Eastman Kodak Co., Rochester, NY).

Construct and transfection

Cells were transfected with a CRF promoter-driven luciferase reporter construct to examine the activity of the promoter [21]. A 1,077-base pair (bp) restriction fragment containing the human CRF promoter (–907 to +170 relative to the proximal transcription start point) was obtained by PCR. The PCR products were then confirmed by sequence analysis. The DNA fragments were used to produce the CRF promoter-driven luciferase reporter construct, CRF-907luc, by a two-step cloning method. First, the DNA fragment was cloned into a pGEM-T Easy vector (Promega Corp., Madison, WI). The vector was then digested with Kpn I and Hind III and subcloned into the Kpn I and Hind III cloning sites of the pA3-Luc plasmid. This CRF promoter includes a functional cAMP-response element, negative glucocorticoid regulatory element, and serum response element [22].

For the luciferase activity assay, cells were plated in 12-well (22-mm diameter) culture trays at 60% confluency. The next day, cells were transfected using the X-tremeGENE 9 DNA Transfection Reagent Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions; 3 μL X-tremeGENE/1 μg DNA was used. Each well contained 0.5 μg of total DNA. The culture medium was then replaced with DMEM supplemented with 10% fetal bovine serum. One day before each experiment, cells were washed and starved overnight using DMEM supplemented with 0.2% BSA.

Luciferase activity

A luciferase assay was performed to examine the effect of QRFP (1 nM - 100 nM) on changes in the activity of each promoter according to the manufacturer's protocol. At the end of each experiment, cells were washed twice with PBS without Ca^{2+} or Mg^{2+}, harvested with PicaGene lysis buffer (Toyo Inki, Tokyo, Japan), and centrifuged at 9,660 *g* for 2 min.

We used 20 μL of each supernatant. The reactions were started by the injection of 100 μL of luciferin solution in PicaGene buffer. Light output was measured for 20 s at room temperature using a luminometer (Berthold Lumat LB9501, Postfach, Germany). Beta-galactosidase activity was used as an internal control. Cells were treated in triplicate in each experiment, and the average of three independent experiments is shown.

Statistical analysis

Each experiment was performed three times. Samples were provided in triplicate for each group of experiments. Each value is expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by Fisher's protected least-significant difference *post-hoc* test. The level of statistical significance was set at $P < 0.05$.

Results

Expression of Gpr103a in 4B cells

Expressions of both Gpr103a and Gpr103b mRNA were detected in rat 4B cells and hypothalamus (Fig. 1A). Both levels were significantly increased 24 h after addition of 100 nM QRFP in 4B cells (Fig. 1A). In order to examine physiological regulation of Gpr103, expression levels of Gpr103 (a and b) proteins were studied by western blot. We found that Gpr103 protein levels were increased 24 h after addition of 100 nM QRFP in 4B cells (Fig. 1B).

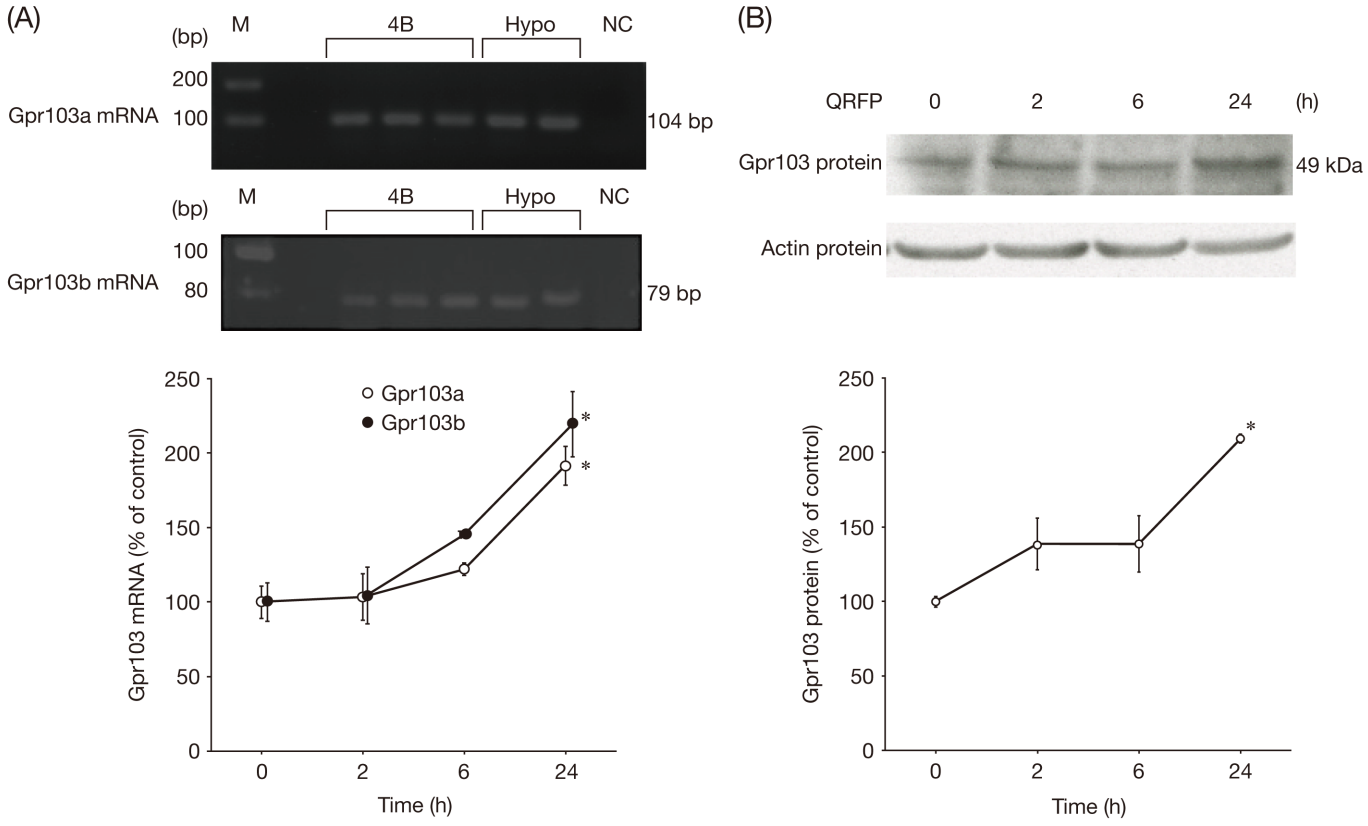

Fig. 1 Expression of Gpr103 mRNA and protein in 4B cells. Cells were treated in triplicate, and the average of three independent experiments and a representative blot are shown. Statistical analysis was performed using one-way ANOVA, followed by *post-hoc* tests. * $P < 0.05$ (compared with control). (**A**) Effects of QRFP on Gpr103a and Gpr103b mRNA levels in 4B cells. Cells were incubated with medium containing 100 nM QRFP for the durations shown. M, marker; 4B, 4B cells; Hypo, rat hypothalamus (positive control); NC, negative control for RT-PCR. Open circles, Gpr103a; closed circles, Gpr130b. (**B**) Effects of QRFP on Gpr103 protein levels in 4B cells. Cells were incubated with medium containing 100 nM QRFP for the durations shown. Beta-actin (actin) was used as a housekeeping protein.

Effects of QRFP on CRF mRNA levels

We examined the effects of QRFP on the time- and dose-dependent changes in CRF mRNA levels. QRFP significantly stimulated CRF mRNA levels in a dose-dependent manner with significant effects observed at 100 nM and 1 μM (Fig. 2A). The time-course study showed that QRFP significantly increased CRF mRNA levels (Fig. 2B). The maximum effect of QRFP was observed at 24 h, with an approximately 2.1-fold increase in CRF mRNA levels compared with the basal level.

Effects of QRFP on CRF 5′-promoter activity

We next examined the effects of QRFP on the time- and dose-dependent changes in CRF 5′-promoter activity. QRFP significantly stimulated CRF 5′-promoter activity in a dose-dependent manner with significant effects observed at 100 nM (Fig. 3A). The time-course study showed that QRFP significantly increased CRF 5′-promoter activity (Fig. 3B). The maximum effect of QRFP was observed at 6 h, with an approximately 1.5-fold increase in CRF 5′-promoter activity compared with the basal level.

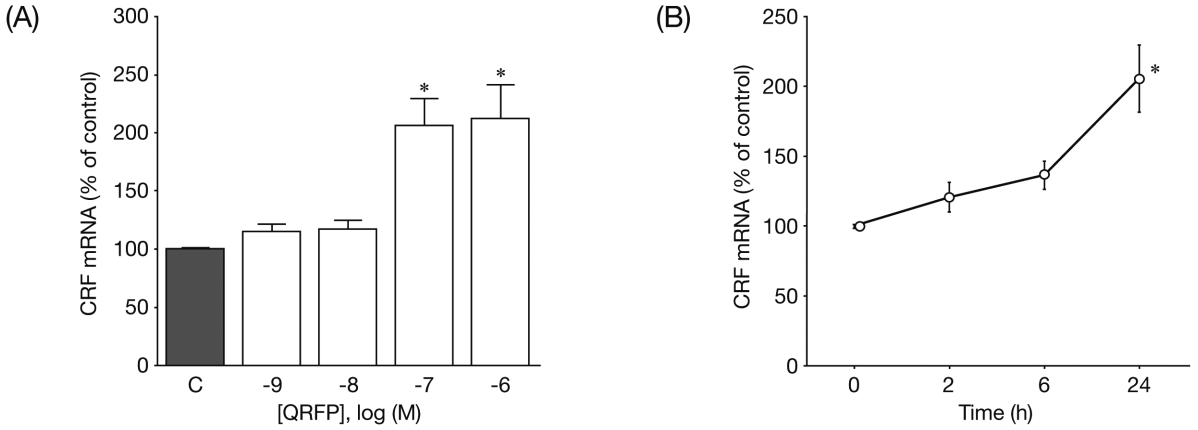

Fig. 2 Effects of QRFP on CRF mRNA levels in 4B cells. Control cells (C) were treated with medium alone. Cells were treated in triplicate and the average of three independent experiments is shown. Statistical analysis was performed using one-way ANOVA, followed by *post-hoc* tests. * $P < 0.05$ (compared with control). (**A**) Dose-dependent effects of QRFP on CRF mRNA levels: cells were incubated for 24 h with medium containing between 1 nM and 1 μM QRFP. (**B**) Time-dependent effects of QRFP on CRF mRNA levels: cells were incubated with medium containing 100 nM QRFP.

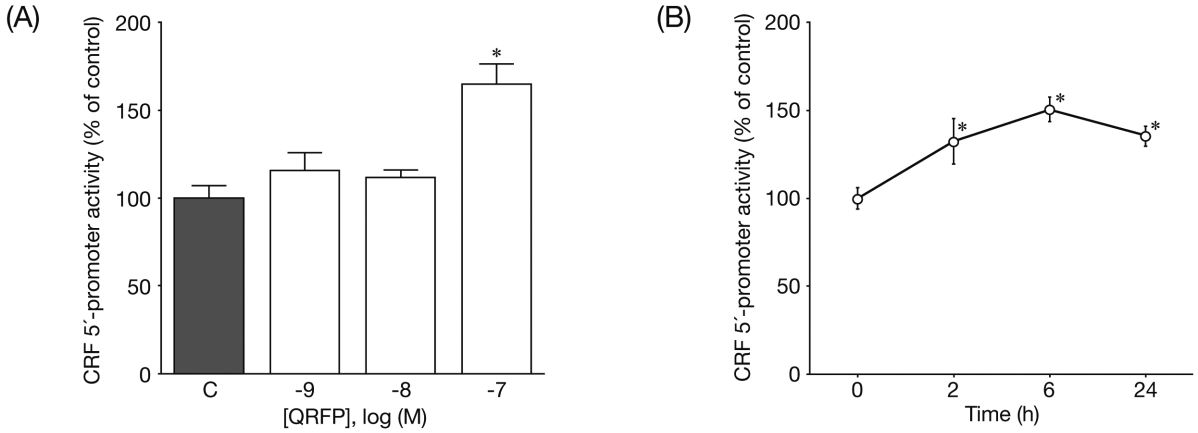

Fig. 3 Effects of QRFP on CRF 5′-promoter activity in 4B cells. Control cells (C) were treated with medium alone. Cells were treated in triplicate and the average of three independent experiments is shown. Statistical analysis was performed using one-way ANOVA, followed by *post-hoc* tests. * $P < 0.05$ (compared with control). (**A**) Dose-dependent effects of QRFP on CRF 5′-promoter activity: cells were incubated for 6 h with medium containing between 1 and 100 nM QRFP. (**B**) Time-dependent effects of QRFP on CRF 5′-promoter activity: cells were incubated with medium containing 100 nM QRFP.

Effects of protein kinase inhibitors on QRFP-induced CRF 5′-promoter activity

We next investigated which protein kinase pathway was involved in the regulation of QRFP-induced CRF 5′-promoter activity. To determine whether a protein kinase pathway might be involved in the regulation of CRF 5′-promoter activity, 4B cells were pre-incubated with H89, PKAi, Ro-32-0432, or BIM for 30 min before incubation with 100 nM QRFP. Incubation of each inhibitor alone did not affect the basal activity of the CRF 5′-promoter for the indicated time.

To determine whether PKA was involved in QRFP-induced CRF 5′-promoter activity, 4B cells were pre-incubated for 30 min with H89 or PKAi, prior to incubation with QRFP. As shown in Fig. 4, 1 μM H89 (Ki for PKA and PKC = 48 nM and 31.7 μM, respectively) significantly blocked QRFP-induced CRF promoter activity. PKAi (100 nM; Ki for PKA = 36 nM) also significantly blocked QRFP-induced CRF promoter activity. These results suggested that PKA was one of the signaling pathways activated by QRFP.

To determine whether the PKC pathway was involved in QRFP-induced CRF 5′-promoter activity, 4B cells were pre-incubated for 30 min with Ro-32-0432 or BIM prior to incubation with QRFP. Both Ro-32-0432 (1 μM; IC_{50} for PKCβI and PKCα = 28 and 9 nM, respectively) and BIM (1 μM; Ki for PKC = 10 nM), selective PKC inhibitors, significantly inhibited QRFP-induced CRF 5′-promoter activity. Therefore, the PKC pathway was also involved in QRFP-induced signaling.

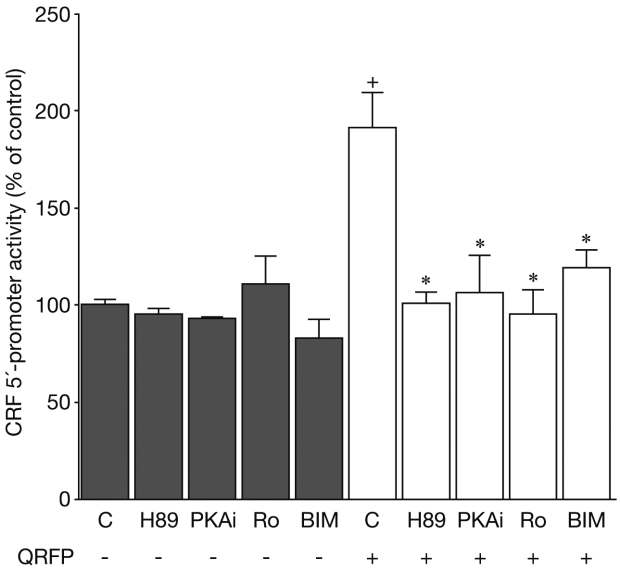

Fig. 4 Effects of protein kinase inhibitors on QRFP-induced CRF 5′-promoter activity in 4B cells. Data are presented as relative activity, and basal luciferase activity without QRFP was set at 100%. Cells were treated in triplicate and the average of three independent experiments is shown. Statistical analyses were performed using one-way ANOVA, followed by *post-hoc* test. $^{+}$ $P < 0.05$ (compared with control). * $P < 0.05$ (compared with QRFP alone). Cells transfected with a full-length (CRF-907luc) promoter construct were pre-incubated for 30 min with medium containing H89, PKAi, Ro-32-0432 (Ro), BIM, or vehicle, and then incubated for 6 h with/without medium containing 100 nM QRFP. Control cells (C) were incubated with the inhibitor vehicle with/without QRFP.

Effects of QRFP on CREB phosphorylation

4B cells were incubated with 100 nM QRFP to determine its effects on CREB phosphorylation. A time-course study showed that CREB phosphorylation was significantly increased 1 h after addition of 100 nM QRFP in 4B cells (Fig. 5A). Ro-32-0432 (1 μM) significantly inhibited QRFP-induced CREB phosphorylation (Fig. 5B).

Discussion

In the present study, rat hypothalamic 4B cells expressed both Gpr103a and Gpr103b mRNA, and Gpr103 (a and b) proteins. There was no Gpr103 antibody to discriminate between two subtypes for commercial basis. Gpr103b mRNA has been reported to be found in the mouse hypothalamus and the PVN [2], while Gpr103a and Gpr103b mRNA has been found in the rat hypothalamus [19]. These results suggest that QRFP is able to activate CRF neurons *via* the Gpr103 in rat hypothalamus. Our results also showed that Gpr103 protein levels were directly increased by QRFP. Thus, the results of our study have shown that QRFP-dependent pathways are involved in hypothalamic cells. Increases in Gpr103 expression may suggest that QRFP produces a permissive effect on Gpr103.

CRF protein and secretion have failed to be detected in 4B cells. Instead, we found that QRFP stimulated the promoter activity and mRNA levels of CRF in hypothalamic 4B cells. The potent PKA inhibitors H89 and PKAi blocked QRFP-induced CRF promoter activity. The potent PKC inhibitors Ro-32-0432 and BIM also inhibited QRFP-induced CRF promoter activity. Therefore, both PKA and PKC pathways were involved in QRFP-induced signaling. QRFP acts on

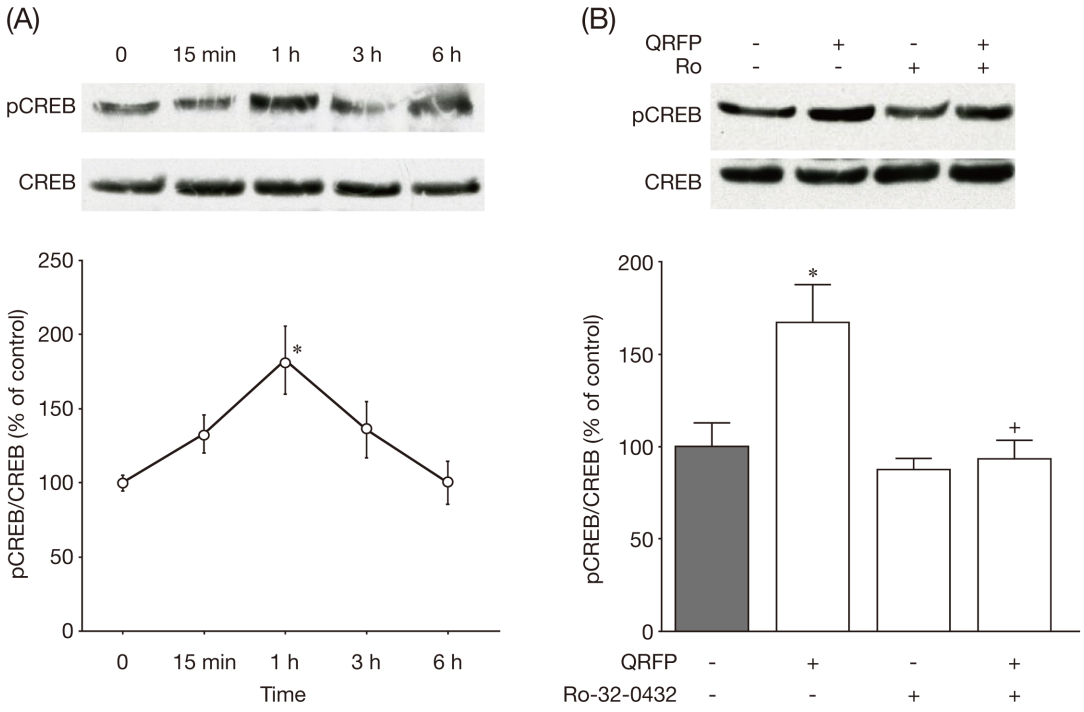

Fig. 5 Effects of QRFP on CREB phosphorylation in 4B cells. Control cells (C) were treated with medium alone. Cells were treated in duplicate and the average of three independent experiments is shown. Statistical analyses were performed using one-way ANOVA, followed by *post-hoc* test. (**A**) Time-dependent changes in QRFP-induced CREB phosphorylation in 4B cells. * $P < 0.05$ (compared with control). Cells were incubated with medium containing 100 nM QRFP for the durations shown. Western blot analysis was performed to examine the protein levels of phosphorylated (p) CREB/CREB, and a representative blot is shown. (**B**) Effects of Ro-32-0432 on QRFP-induced CREB phosphorylation in 4B cells. * $P < 0.05$ (compared with control). $^{+}$ $P < 0.05$ (compared with QRFP). Cells were pre-incubated for 30 min with medium containing Ro or vehicle, and then incubated for 1 h with medium containing 100 nM QRFP or vehicle.

Gpr103 which is Gαq protein-coupled receptor, resulting in increase in intracellular Ca^{2+} and PKC activity [2]. QRFP can also stimulate CRF gene expression through PKA-related signaling pathways. In fact, in the present study, QRFP stimulated CREB phosphorylation. This CREB phosphorylation was inhibited by a PKC inhibitor. Therefore, PKC-dependent signaling would be upstream of the CREB phosphorylation. Activation of the PKA pathway, which phosphorylates CREB, acts on the CRF promoter [23-25]. A functional cAMP-response element in the 5′ promoter region is involved in regulating CRF gene expression [21, 23, 24].

Intracerebroventricular injection of QRFP stimulates food intake and general locomotor activity in mice *in vivo* [2]. QRFP is an orexigenic hormone, whereas CRF would be an anorexigenic or anxiogenic factor in the hypothalamus. QRFP-stimulated CRF in the hypothalamus would cause ACTH and glucocorticoids secretion. However, it is unclear whether endogenous QRFP stimulates the HPA axis *in vivo*. Both Gpr103 and QRFP are primarily localized in human and rat adrenal cortex [7, 9]. QRFP stimulates cortisol secretion as well as induction of key steroidogenic enzymes in human adrenocortical cells [7]. Glucocorticoids may suppress hypothalamic CRF. On the other hand, it is possible that QRFP stimulates the HPA axis *in vivo*, and the release of glucocorticoids, representative orexigenic hormone, which helps to increase appetite. In fact, glucocorticoid-induced hyperphagia is observed in CRF over-expressing transgenic mice [26]. Further study is required to determine the effects of QRFP-induced CRF regulation on stress *in vivo*.

In conclusion, the results of the present study show that Gpr103 mRNA and protein are expressed in hypothalamic cells. QRFP increases Gpr103 mRNA and protein levels, and stimulates CRF mRNA levels and CRF promoter activity in 4B cells transfected with

the CRF promoter. The PKA and PKC pathways are involved in the QRFP-induced increases in CRF and promoter activity. QRFP stimulates CREB phosphorylation. CREB phosphorylation is inhibited by a PKC inhibitor. PKC-dependent signaling would be upstream of the CREB phosphorylation. QRFP-dependent pathways appear, therefore, to be involved in the regulation of CRF gene expression in hypothalamic cells. Further studies are required to determine whether this situation also applies *in vivo*.

Conflict of Interests

None of the authors have any potential conflicts of interest.

Acknowledgments

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