Relaxin-3 regulates corticotropin-releasing factor gene expression in cultured rat hypothalamic 4B cells

（ラット視床下部 4B 細胞における relaxin-3 による *Crf* 遺伝子発現調節の機序についての検討）

申 請 者　　弘前大学大学院医学研究科
成育科学領域小児病態学教育研究分野

氏　　名　　八　木　弘　子

指導教授　　伊　藤　悦　朗

Abstract

The ancestral insulin/relaxin peptide superfamily member relaxin-3 is an important regulator of food intake and behaviors related to anxiety and motivation. Relaxin family peptide receptor 1 (RXFP1) and RXFP3 are expressed

in the rat hypothalamic paraventricular nucleus (PVN). Corticotropin-releasing factor (CRF) is produced in the PVN in response to stressors and promotes adrenocorticotropic hormone secretion from the anterior pituitary. We hypothesized that relaxin-3 directly regulates *Crf* expression in the hypothalamus and investigated its effect on *Crf* expression in cultured hypothalamic 4B cells. Relaxin-3 increased *Crf* mRNA levels and stimulated

Crf promoter activity. Both protein kinase A and C pathways contributed to relaxin-3-induced *Crf* promoter activity. *Rxfp1* and *Rxfp3* mRNA and their proteins were expressed in cultured hypothalamic 4B cells. Relaxin-3 decreased *Rxfp1* mRNA and protein levels and increased *Rxfp3* mRNA and protein levels. These results suggested that the action of relaxin-3 in cultured hypothalamic 4B cells may be regulated through both RXFP1 and RXFP3.

Keywords: Relaxin; corticotropin-releasing factor; hypothalamus; stress

1. Introduction

Relaxin-3 is a 5.5-kDa polypeptide hormone and an ancestral member of the relaxin/insulin superfamily. Recent studies have shown that relaxin-3 performs an important role in modulating feeding [1], stress responses [2, 3], arousal [4], and anxiety-related and motivation-related behaviors [5]. The expression of relaxin-3 is highly specific in the brain, particularly in the nucleus incertus, and has been primarily studied in rats and mice [3, 6]. The distribution of the nucleus incertus efferent projections largely parallels that of relaxin-3-containing fibers [7, 8]. Nucleus incertus neurons widely innervate the septum, hippocampus, thalamus, hypothalamus, and brainstem [7, 8].

Relaxin-3 acts via relaxin family peptide receptors (RXFPs), which belong to the G protein-coupled receptor superfamily. Relaxin-3 binds to RXFP3 with a high affinity and to RXFP1 with a lower affinity and activates both receptors [9, 10]. RXFP3 and RXFP1 induce different intracellular responses. Relaxin-3 activation of RXFP3 induces coupling to $G\alpha_{i2}$ and $G\alpha_{OB}$, resulting in the inhibition of adenylate cyclase [11]. In contrast, RXFP1 couples primarily to the three G proteins $G\alpha_{S}$, $G\alpha_{OB}$, and $G\alpha_{i3}$. Activation of RXFP1 induces cAMP accumulation as a result of the interaction with these G-proteins [12, 13]. Distribution of *Rxfp3* mRNA and binding sites of an RXFP3-selective agonist largely overlap with those of relaxin-3-containing fibers [14, 15]. One of the highly concentrated areas is the paraventricular nucleus (PVN),

which is the main center of stress response. RXFP1 is also highly expressed in the PVN as well as widely expressed in other distinct regions of the brain [16].

Corticotropin-releasing factor (CRF) is a major regulator of the hypothalamic-pituitary-adrenal (HPA) axis in stress response [17]. Intracerebroventricular (ICV) administration of relaxin-3 has been shown to increase *c-fos* and *Crf* mRNA expression in the rat PVN and to elevate plasma adrenocorticotropic hormone (ACTH) and corticosterone levels [18, 19]. Furthermore, relaxin-3-positive neurons in the nucleus incertus are activated by stress conditions in rats [2, 3]. Recently, de Avila et al. showed that relaxin-3, but not an RXFP3-selective agonist, increases *c-fos* mRNA levels in the PVN and supraoptic hypothalamic nuclei and elevates plasma corticosterone levels in rats [20].

Since the precise mechanism of regulation of the HPA axis by relaxin-3 is not known, the current study aimed to elucidate the genomic regulation of *Crf* expression by relaxin-3 in cultured rat hypothalamic 4B cells. We determined the direct effect of relaxin-3 on promoter activity and mRNA levels of *Crf* and evaluated RXFP1 and RXFP3 expression.

2. Materials and Methods

2.1. Materials

Recombinant relaxin-3 was purchased from Peprotech (Rocky Hill, NJ, USA) and inhibitor

H89 from Seikagaku Corporation (Tokyo, Japan). The protein kinase A (PKA) inhibitor 14-22 amide (PKAi) and protein kinase C (PKC) inhibitors Ro-32-0432 (RO) and bisindolylmaleimide I (BIM) were purchased from Calbiochem (San Diego, CA, USA).

2.2. Cell culture

Rat fetal hypothalamic 4B cells were kindly provided by Dr. John Kasckow [21]. The 4B cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 as previously described [22].

2.3. RNA isolation

Total cellular RNA was isolated using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Complementary DNA (cDNA) was synthesized using random hexamer primers and the SuperScript First-Strand Synthesis System (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions, using 0.5 μg of total RNA as the template.

2.4. Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR)

The cDNAs generated from the total cellular RNA samples were subjected to RT-qPCR.

Specific primer and probe sets from the collection of Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA, USA) or specific Crf primers and a probe (5'–FAM-GCCAGGGCAGAGCAGTTAGC-TAMRA–3') were used to measure the expression levels of rat *Crf*, *Rxfp1*, and *Rxfp3* mRNA by RT-qPCR. The Crf primers included the forward primer (Crf-F) with the sequence 5'–TGGATCTCACCTTCCACCTTCTG–3' and the reverse primer (Crf-R) with the sequence 5'–CCGATAATCTCCATCAGTTTCCTG–3'. Expression levels were standardized to β2-microglobulin (*B2mg*), which was used as a reference gene since its expression levels were not significantly altered by the treatments (data not shown). A total volume of 25 μL was used for the PCRs, which contained TaqMan universal PCR master mix (Applied Biosystems), gene expression products (Rn01495351_m1 for rat *Rxfp1*, Rn00710992_s1 for rat *Rxfp3*, and Rn00560865 m1 for rat *B2mg*) or Crf primers and probes, and 500 ng cDNA. Thermal cycling conditions were set in accordance with the study by Ishigame et al [22].

2.5. Western blot analysis

Western blot analysis was performed to determine the effects of relaxin-3 (10 nM) on protein expression of cAMP response element-binding protein (CREB), phosphorylated CREB (pCREB), RXFP1, and RXFP3 as previously described [22]. The housekeeping protein β-actin

was used as an internal control protein. The samples (7.5 μg each) were boiled and proteins separated on 4–20% gradient polyacrylamide gels. The proteins were transferred from the gels to polyvinylidene fluoride membranes (Daiichi Kagaku, Tokyo, Japan). The membranes were blocked with Detector Block® buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and incubated for 1 h with each of the following rabbit anti-human antibodies: anti-pCREB (1:500; Rockland Antibodies & Assays, Limerick, PA, USA), anti-CREB (1:500; Cell Signaling Technology, Beverly, MA, USA), anti-RXFP1 (1:1,000; Biorbyt LLC, San Francisco, CA, USA), anti-RXFP3 (1:1,000; St John's Laboratory, London, UK), and anti-β-actin (ab8227, 1:10,000; Abcam, Cambridge, MA, USA).

2.6. Plasmid construction and transfection

To determine the promoter activity, 4B cells were transfected with luciferase reporter constructs driven by the *Crf* promoter [22]. The DNA fragment was used to produce the *Crf* promoter-driven luciferase reporter construct *Crf*-907luc as previously described [22]. Transfection efficiency was 70%.

2.7. Luciferase activity

Luciferase assays were performed according to the manufacturer's protocol (Toyo Inki, Tokyo,

Japan) to examine effects of relaxin-3 (0.1–10 nM) on the activity of each promoter.

2.8. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Each experiment (triplicate samples of cells) was performed three times. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's least-significant difference (LSD) protected post-hoc test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Relaxin-3 stimulated *Crf* mRNA levels

We treated 4B cells with relaxin-3 to examine the effects on *Crf* expression. The levels of *Crf* mRNA were significantly increased by treatment with 10 nM relaxin-3 to approximately 1.8-fold higher levels than the basal levels after 24 h of incubation (LSD test; $P < 0.005$, Fig. 1A). The increased *Crf* mRNA levels induced by relaxin-3 were dose-dependent, with doses from 1 nM to 10 nM demonstrating significant effects (LSD test; $P < 0.01$, Fig. 1B).

3.2. Relaxin-3 stimulated *Crf* 5'–promoter activity

We next tested the effects on *Crf* 5'-promoter activity by treating cells with 10 nM relaxin-3.

The *Crf* 5'-promoter activity was significantly increased by approximately 1.6-fold compared to that of the basal activity 2 h after relaxin-3 incubation (LSD test; $P < 0.05$, Fig. 2A). While the *Crf* 5'-promoter activity increased with relaxin-3 treatment in a dose-dependent manner (Fig. 2B), only the 10 nM concentration produced a significant effect (LSD test; $P < 0.005$).

The contributions of protein kinase pathways to the regulation of relaxin-3-induced *Crf* 5'-promoter activity were examined. The hypothalamic 4B cells were preincubated for 30 min with inhibitors H89, PKAi, RO, or BIM prior to the treatment with relaxin-3. The basal activities of the *Crf* 5'-promoter were not affected by any of the inhibitors alone. The relaxin-3-induced *Crf* promoter activity was significantly inhibited by 1 μM H89 with the dissociation constant of the inhibitor (Ki) being 48 nM and 31.7 μM for PKA and PKC, respectively (LSD test; $P < 0.05$, Fig. 2C). Similarly, the PKA pathway was also blocked by PKAi (100 nM) with a Ki of 36 nM for PKA. The relaxin-3-induced *Crf* promoter activity was significantly inhibited by 1 μM RO with the half maximal inhibitory concentration (IC50) being 28 nM and 9 nM for PKCβI and PKCα, respectively (LSD test; $P < 0.05$). The promoter activity was also significantly inhibited by 100 nM BIM with the Ki being 10 nM for PKC (LSD test; $P < 0.05$).

3.3. Relaxin-3 stimulated CREB phosphorylation

To determine the involvement of CREB phosphorylation in the relaxin-3-induced signaling,

4B cells were incubated with 10 nM relaxin-3. CREB phosphorylation was significantly increased by 5 min after the addition of 10 nM relaxin-3 to the 4B cells (LSD test; $P < 0.001$, Fig. 3).

3.4. Relaxin-3 modulated *Rxfp1* and *Rxfp3* mRNA levels

We next determined the effects of relaxin-3 on changes in *Rxfp1* and *Rxfp3* mRNA levels. The levels of *Rxfp1* mRNA were significantly decreased from 2 h to 6 h after the addition of 10 nM relaxin-3 (LSD test; $P < 0.05$, Fig. 4A). The *Rxfp1* mRNA levels were decreased in a dose-dependent manner (Fig. 4B) with significant effects being observed at 1 and 10 nM concentrations of relaxin-3 (LSD test; $P < 0.05$). On the other hand, the *Rxfp3* mRNA levels were increased at 24 h after the addition of relaxin-3 (Fig. 4C) with significant effects being observed with 10 nM relaxin-3 (LSD test; $P < 0.01$, Fig. 4D).

3.5. Relaxin-3 modulated RXFP1 and RXFP3 protein levels

To determine whether RXFP1 and RXFP3 protein levels were also modulated by relaxin-3, 4B cells were incubated with 10 nM relaxin-3 and the protein levels analyzed by western blotting. After the addition of the relaxin-3, RXFP1 protein levels were significantly decreased between 2 h and 24 h (LSD test; $P < 0.05$, Fig. 5A), while the RXFP3 protein levels were significantly

increased between 2 h and 6 h (LSD test; $P < 0.05$, Fig. 5B).

4. Discussion

ICV injection of relaxin-3 increases the PVN *Crf* mRNA levels and plasma levels of both ACTH and corticosterone in rats [19]. Intra-PVN treatment of male rats with relaxin-3 also increases the plasma levels of ACTH and corticosterone [23]. Relaxin-3 regulates the HPA axis via the hypothalamic CRF and vasopressin neurons [18]. CRF protein or its secretion has not been detected in cultured hypothalamic 4B cells. In the current study, we found that relaxin-3 directly stimulated the promoter activity of *Crf* in the cultured hypothalamic 4B cells, resulting in increased mRNA levels. While relaxin-3 directly stimulates the HPA axis, it is unclear whether endogenous relaxin-3 is able to stimulate it under stress in vivo.

In our study, the potent PKA inhibitors blocked relaxin-3-induced *Crf* promoter activity. These findings suggest that relaxin-3 stimulated *Crf* gene expression via a PKA-related signaling pathway. In fact, our study also showed that relaxin-3 stimulated the phosphorylation of CREB. A functional cAMP-response element is involved in the 5'*Crf*-promoter region [24]. Activation of the PKA pathway results in the phosphorylation of CREB, which then acts on the *Crf* promoter [24, 25]. It has also been shown that relaxin-3 increases intracellular Ca^{2+} and PKC activity [26]. Our study also showed that the potent PKC inhibitors completely inhibited

the relaxin-3-induced *Crf* promoter activity. Therefore, both PKA and PKC pathways may contribute to relaxin-3-induced signaling through crosstalk in cultured hypothalamic 4B cells.

Both RXFP1 and RXFP3 have been detected in the rat hypothalamus [15, 16]. In our current study, cultured rat hypothalamic 4B cells expressed *Rxfp1* and *Rxfp3* mRNA as well as their respective proteins. Relaxin-3 activates the HPA and hypothalamic-pituitary-gonadotropin axes via RXFP1 [20] and may activate CRF neurons via RXFPs in the cultured hypothalamic 4B cells. The increased *Crf* promoter activity and mRNA levels induced by relaxin-3 may act through the action of RXFP1 since a selective RXFP3 agonist by itself fails to increase plasma corticosterone levels and the expression of PVN *c-fos* mRNA rats [20]. Therefore, relaxin-3 may stimulate the genomic expression of *Crf* in cultured hypothalamic 4B cells via RXFP1.

Our results showed that RXFP protein levels were directly modulated by relaxin-3. Thus, relaxin-3-dependent pathways likely contribute to the regulation of RXFP in cultured hypothalamic 4B cells. In fact, relaxin-3 decreased *Rxfp1* mRNA levels and increased *Rxfp3* mRNA levels in cultured hypothalamic 4B cells. It is possible that relaxin-3 activates CRF neurons under stress via RXFP1. As a form of homeostasis, both decreases in RXFP1 and increases in RXFP3 expression may produce limiting effects on relaxin-3. Thus, RXFP1 and RXFP3 may have an excitatory and inhibitory impact, respectively, on target cells [27]. Therefore, it appears that RXFP1 is primarily involved in a stress response, while RXFP3 may

mediate a “stress-coping” response.

In conclusion, relaxin-3 stimulated *Crf* mRNA levels and its promoter activity in cultured hypothalamic 4B cells. Hypothalamic 4B cells expressed both *Rxfp1* and *Rxfp3* mRNA and their respective proteins. Changes in RXFP expression suggest that the action of relaxin-3 may be regulated in cultured hypothalamic 4B cells through both RXFP1 and RXFP3.

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Disclosure of potential conflicts of interest

None of the authors have any potential conflicts of interest associated with this research.

Compliance with Ethical Standards

Human participants were not involved in this research.

Authors’ Contributions

All authors participated in writing the manuscript and approved the final manuscript.

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Figure Legends

Fig. 1. Relaxin-3 stimulated *Crf* mRNA levels. (A) Effects of relaxin-3 on *Crf* mRNA levels over time demonstrated a time-dependent response. Cells were incubated with 10 nM relaxin-3 for the indicated amount of time (h). (B) The stimulatory effects of relaxin-3 on *Crf* mRNA levels was dose-dependent. Cells were incubated with increasing concentrations of relaxin-3 (0.1 nM to 10 nM) for 24 h. *$P < 0.05$ compared with basal expression at time 0 or mock-treated controls (C).

Fig. 2. Relaxin-3 stimulated *Crf* 5'-promoter activity. Data are presented as the relative activity normalized to basal luciferase activity in the absence of an inhibitor and relaxin-3, which was set at 100%. (A) Effects of relaxin-3 on *Crf* 5'-promoter activity over time demonstrated a time-dependent response. Cells were incubated with 10 nM relaxin-3 for the indicated amount of time (h). (B) The stimulatory effects of relaxin-3 on *Crf* 5'-promoter activity was dose-dependent. Cells were incubated with increasing concentrations of relaxin-3 (0.1 nM to 10 nM) for 2 h. (C) Effects of protein kinase inhibitors on relaxin-3-induced *Crf* 5'-promoter activity. After 30 min of incubating 4B cells in medium containing inhibitors H89, PKAi, Ro-32-0432 (RO), or BIM or containing vehicle (C), the cells were transfected with a *Crf* 5'-promoter plasmid construct and incubated with or without 10 nM relaxin-3 for 2 h. *$P < 0.05$ compared with basal expression at time 0 or mock-treated controls (C). +$P < 0.05$ compared with relaxin-3

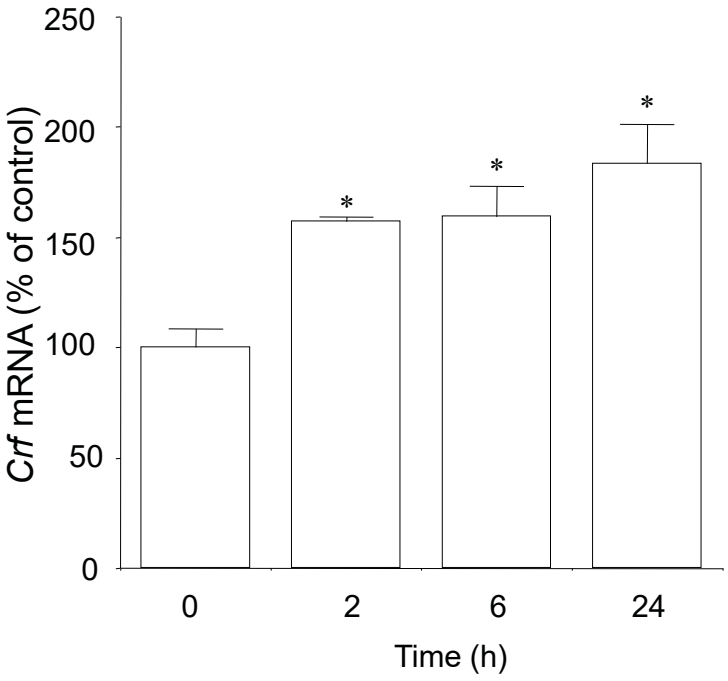
treatment alone.

Fig. 3. Relaxin-3 stimulated the phosphorylation of CREB (pCREB). Cells were incubated with 10 nM relaxin-3 for the indicated amount of time (min or h), and western blot analysis was performed to determine protein levels of CREB and pCREB. *P < 0.05 compared with basal expression at time 0. A representative blot is shown at the top of the figure.

Fig. 4. Relaxin-3 modulated *Rxfp1* and *Rxfp3* mRNA levels. (A and C) Effects of relaxin-3 on *Rxfp1* and *Rxfp3* mRNA levels over time demonstrated a time-dependent response. Cells were incubated with 10 nM relaxin-3 for the indicated amount of time (h). (B and D) The inhibitory and stimulatory effects of relaxin-3 on *Rxfp1* and *Rxfp3* mRNA levels, respectively, were dose-dependent. Cells were incubated with increasing concentrations of relaxin-3 (0.1 nM to 10 nM) for 24 h. *P < 0.05 compared with basal expression at time 0 or mock-treated controls (C).

Fig. 5. Relaxin-3 modulated RXFP1 and RXFP3 protein levels. Cells were incubated with 10 nM relaxin-3 for the indicated amount of time (h), and western blot analysis was performed to determine protein levels of RXFP1 (A) and RXFP3 (B). *P < 0.05 compared with basal expression at time 0. Representative blots are shown at the top of the figure.

(A)


250
200
150
100
50
0
Crf mRNA (% of control)
*
*
*
0
2
6
24
Time (h)

(B)

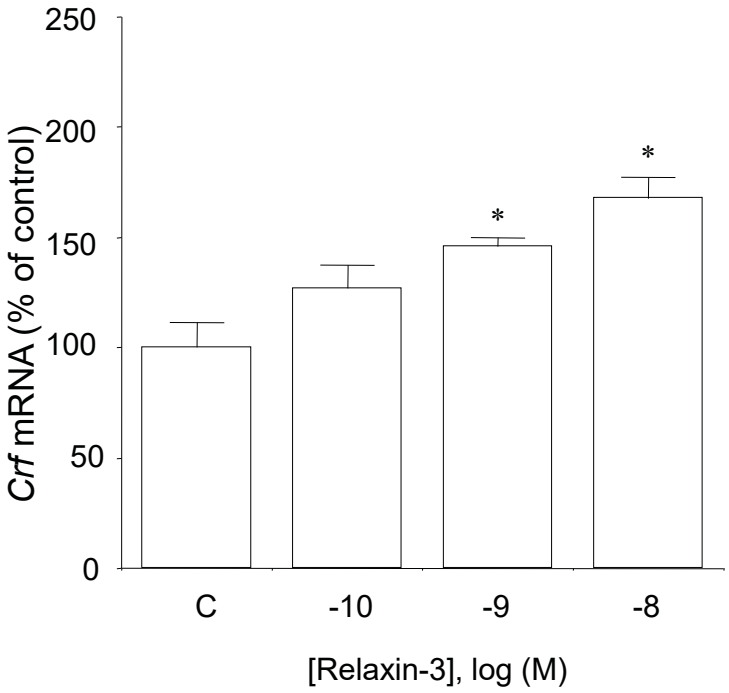
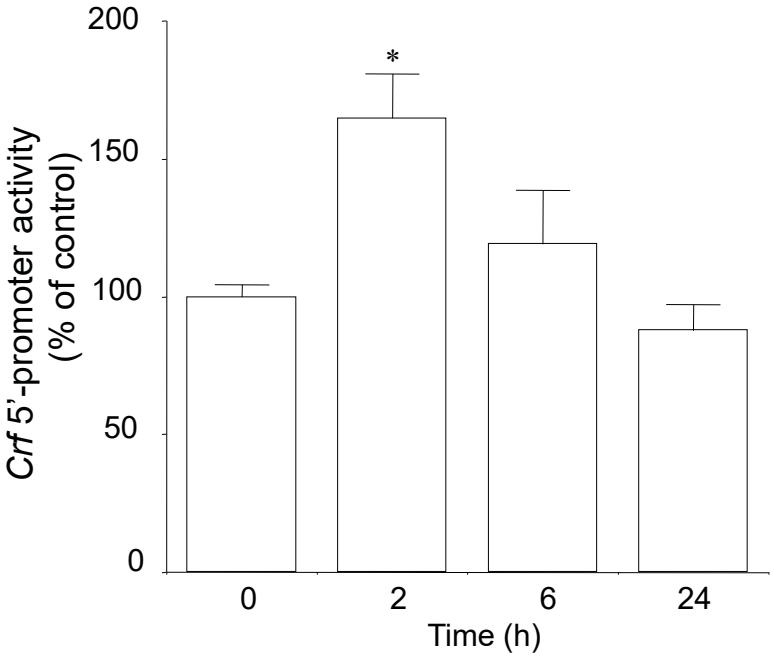

250
200
150
100
50
0
Crf mRNA (% of control)
*
*
*
C
-10
-9
-8
[Relaxin-3], log (M)

Fig. 1

(A)


200
150
100
50
0
*
Crf 5'-promoter activity
(% of control)
0
2
6
24
Time (h)

(B)

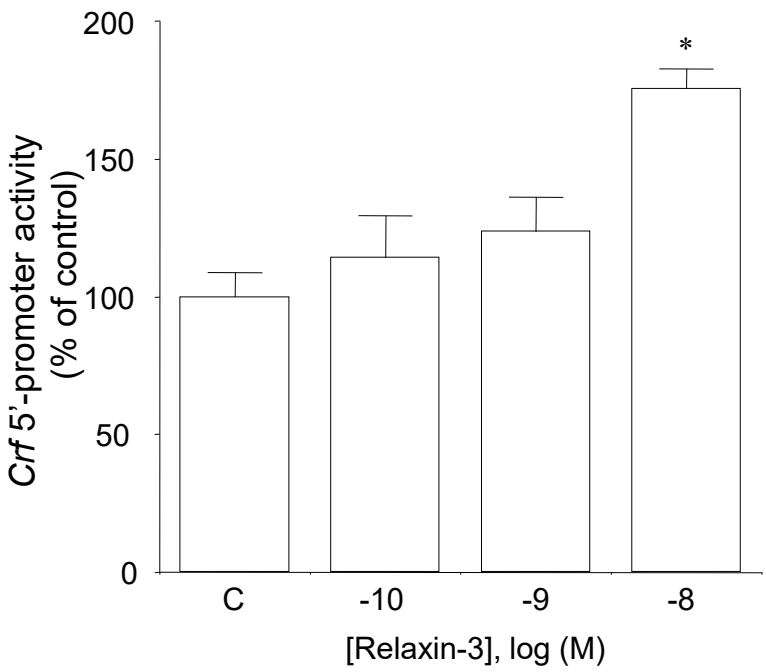

200
150
100
50
0
*
Crf 5'-promoter activity
(% of control)
C
-10
-9
-8
[Relaxin-3], log (M)

Fig. 2

(C)

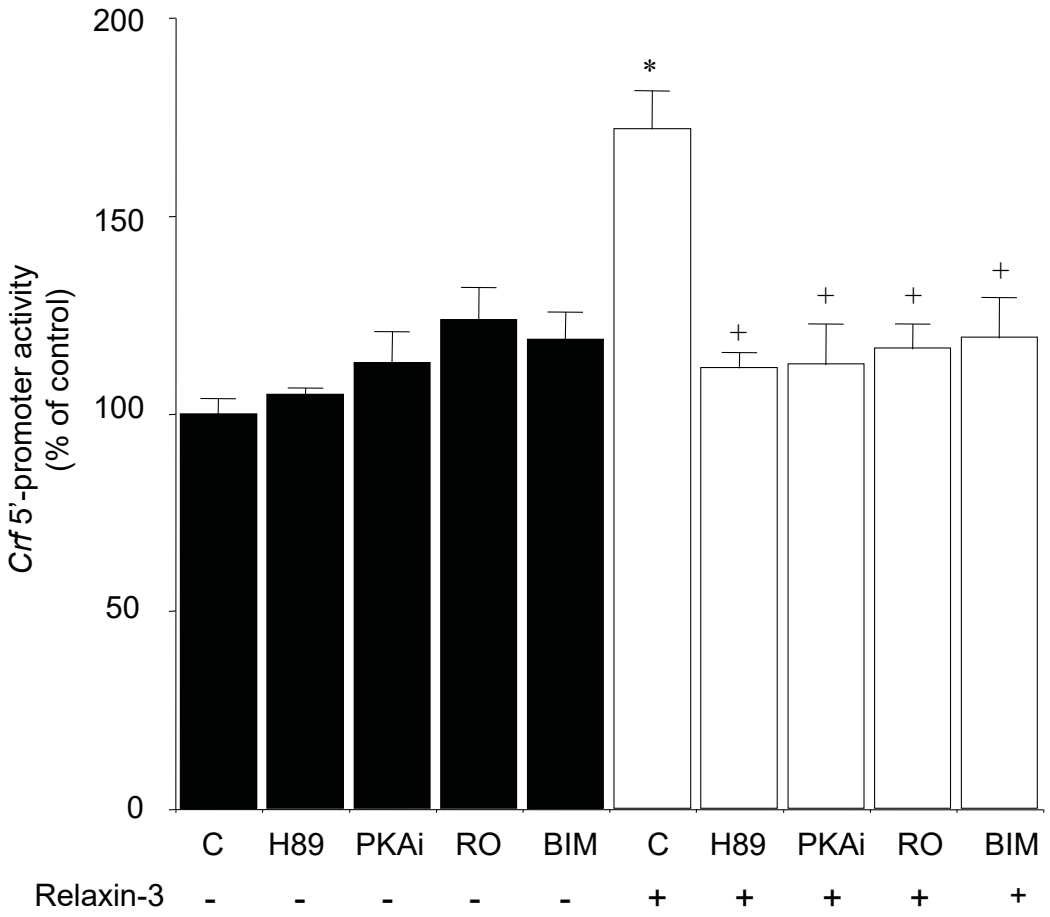

200
150
100
50
0
*
+
+
+
+
Crf 5'-promoter activity
(% of control)
C
H89
PKAi
RO
BIM
C
H89
PKAi
RO
BIM
Relaxin-3
-
-
-
-
-
+
+
+
+
+

Fig. 2

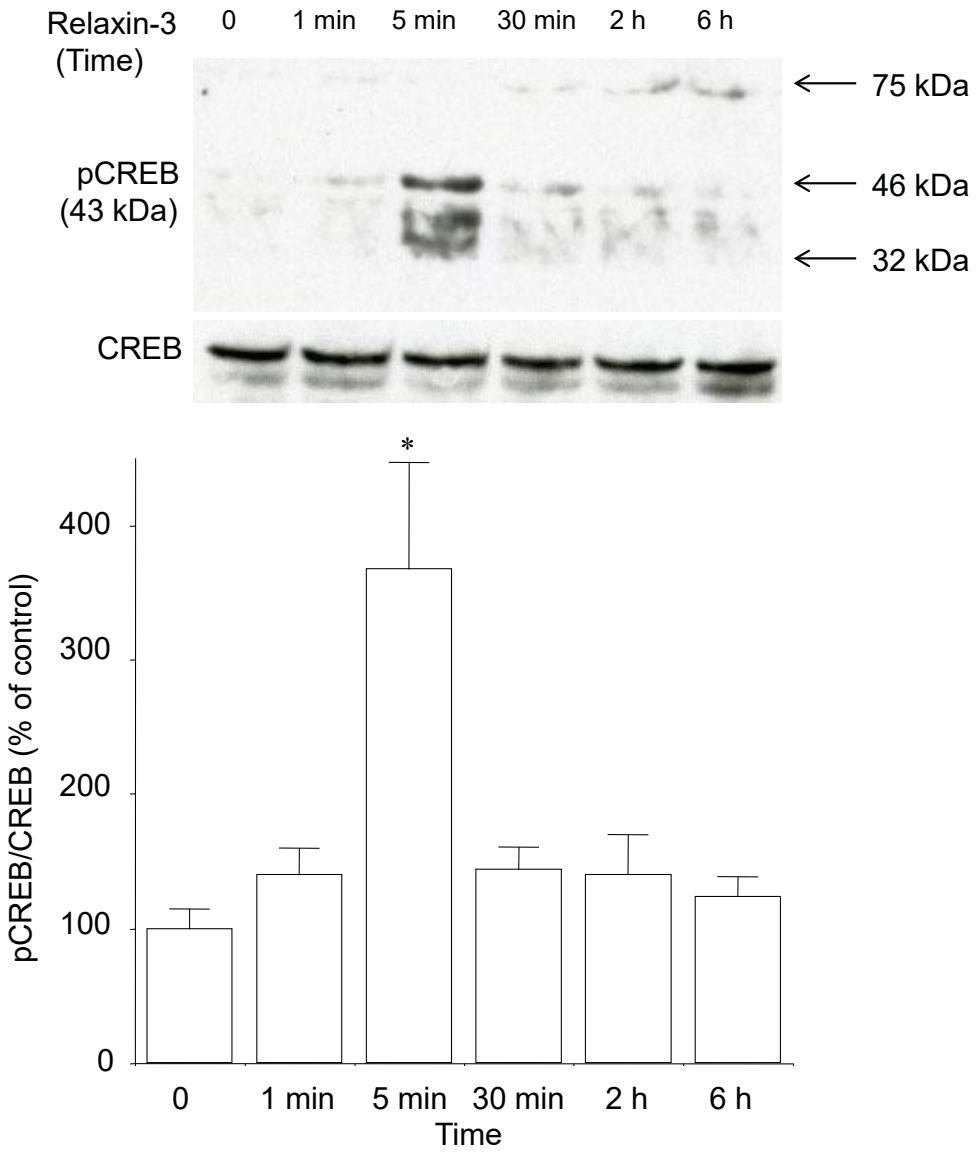
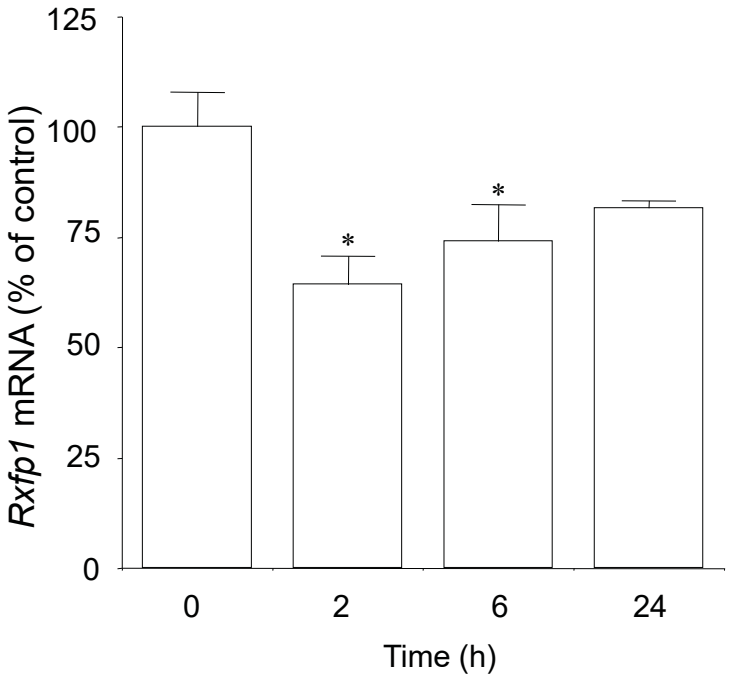

Relaxin-3
(Time)
0
1 min
5 min
30 min
2 h
6 h
75 kDa
pCREB
(43 kDa)
46 kDa
32 kDa
CREB
*
pCREB/CREB (% of control)
400
300
200
100
0
0
1 min
5 min
30 min
2 h
6 h
Time

Fig. 3

(A)


125
100
75
50
25
0
Rxfp1 mRNA (% of control)
*
*
0
2
6
24
Time (h)

(B)

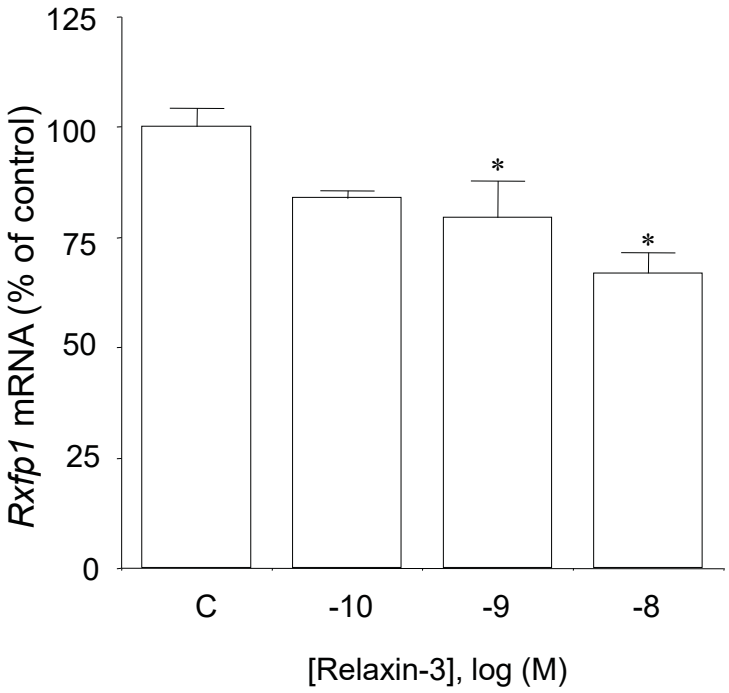
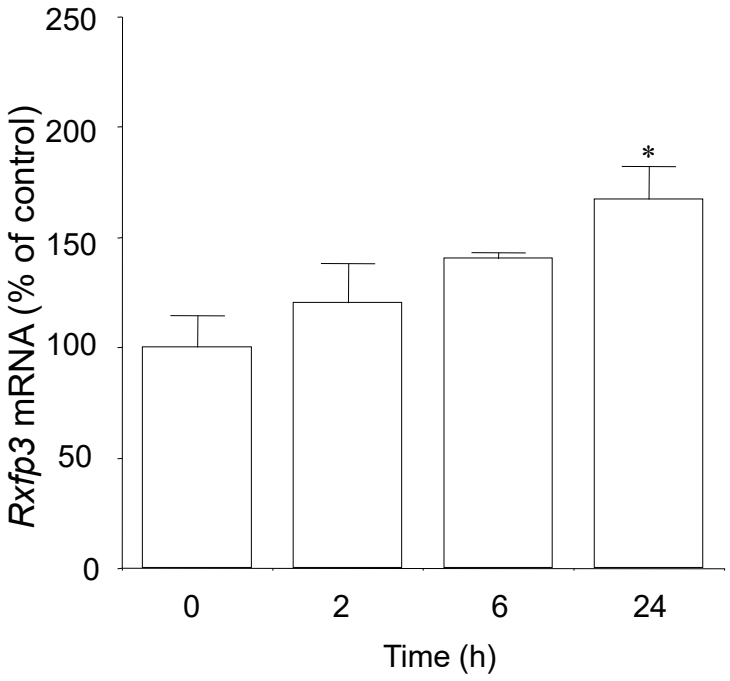

125
100
75
50
25
0
Rxfp1 mRNA (% of control)
*
*
C
-10
-9
-8
[Relaxin-3], log (M)

Fig. 4

(C)


250
200
150
100
50
0
Rxfp3 mRNA (% of control)
*
0
2
6
24
Time (h)

(D)

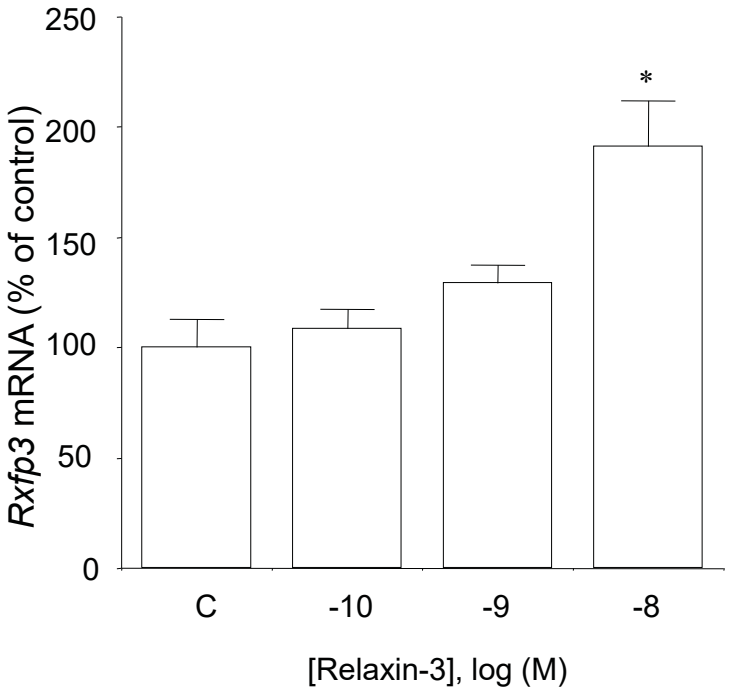

250
200
150
100
50
0
Rxfp3 mRNA (% of control)
*
C
-10
-9
-8
[Relaxin-3], log (M)

Fig. 4

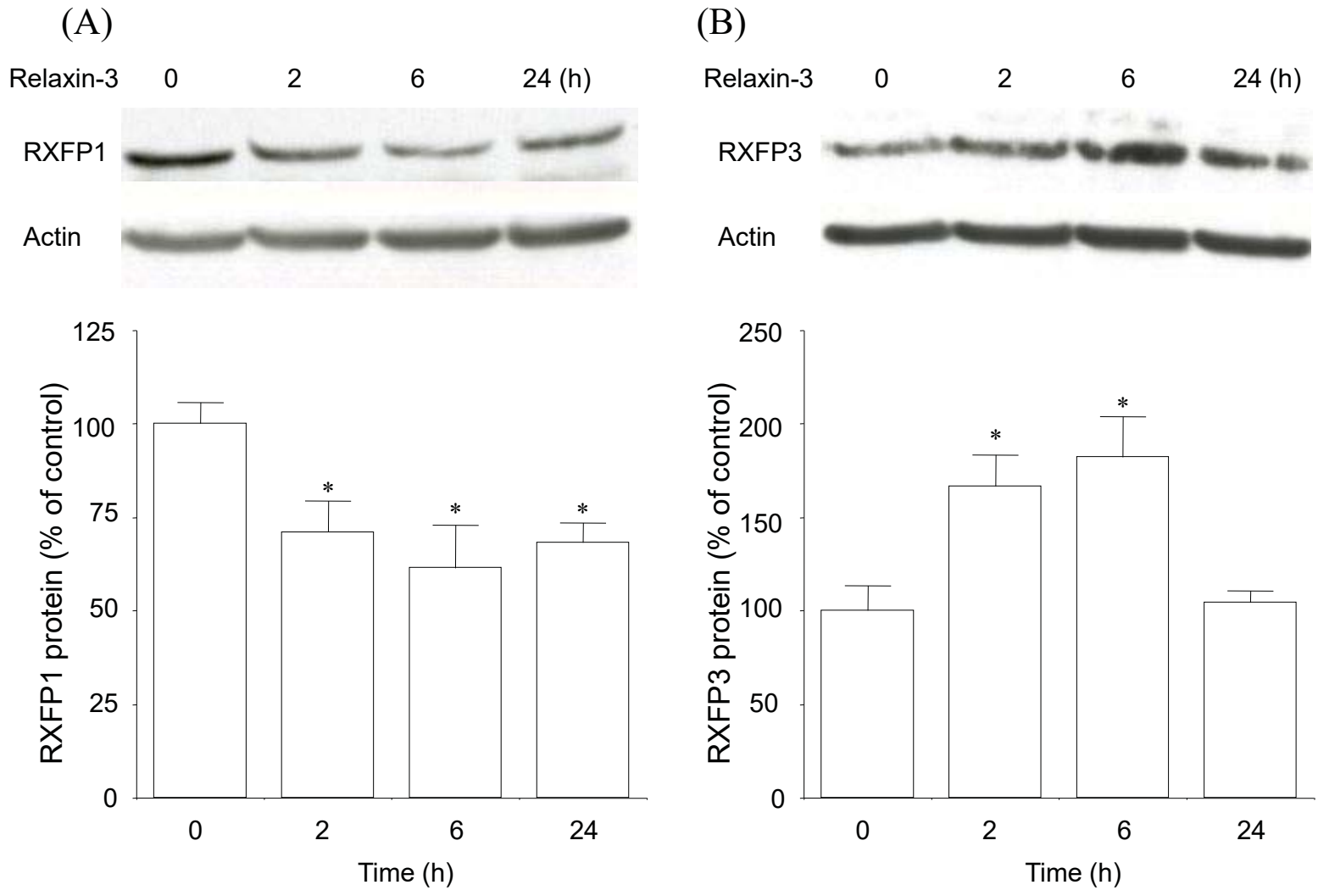

(A)
Relaxin-3
0
2
6
24 (h)
RXFP1
Actin
RXFP1 protein (% of control)
125
100
75
50
25
0
*
*
*
0
2
6
24
Time (h)
(B)
Relaxin-3
0
2
6
24 (h)
RXFP3
Actin
RXFP3 protein (% of control)
250
200
150
100
50
0
*
*
0
2
6
24
Time (h)

Fig. 5