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Easy detection of hormone secretion from LβT2 cells by using Gaussia luciferase

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Abstract. Reproduction is regulated by gonadotropins secreted from gonadotrophs. The production and secretion of gonadotropins are mainly regulated by gonadotropin-releasing hormone (GnRH). Agonists or antagonists that influence GnRH action on gonadotrophs are important to regulate reproduction; however, these factors have not been fully characterized due to the lack of simple and easy-to-use techniques to detect gonadotropin secretion from gonadotropin-producing cells. In the present study, we found that Gaussia luciferase (Gluc), which was expressed in LβT2 cells, can be secreted like a luteinizing-hormone (LH) upon stimulation with GnRH. The Gluc secreted into the medium was easily monitored as luminescence signals. The detection range of the GnRH-induced Gluc activity was comparable to that of the enzyme-linked immunosorbent assay for LH. In addition, when the Gluc was expressed in AtT20 cells, which produce adrenocorticotropic hormone (ACTH), the Gluc activity in the medium increased in parallel with the ACTH secretion upon stimulation with corticotropin-releasing hormone. Thus, the Gluc assay in the present study can be easily used for high-throughput screening of factors that influence LH or ACTH secretion from LβT2 or AtT20 cells, respectively.
Key words: Gaussia luciferase, hormone secretion, LβT2

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Reproduction is regulated by gonadotropins secreted from gonadotrophs in the anterior pituitary gland [1]. Gonadotropins act on the gonads to stimulate sex hormone production [1], and gonadotropin deficiency results in hypogonadism, which can lead to infertility. Secretion of gonadotropins from the cells is mainly regulated by gonadotropin-releasing hormone (GnRH) from the hypothalamus [2]. Fertility drugs are utilized to treat infertility, and a target of these drugs is the hypothalamic release of GnRH, which alters the secretion of gonadotropins from gonadotrophs [3]. Other factors, such as a pituitary adenylate cyclase-activating polypeptide (PACAP), also stimulate gonadotropin secretion from gonadotrophs in association with GnRH [4]. Thus, identifying agonists or antagonists that influence GnRH action on gonadotrophs is important in order to regulate reproduction.

A gonadotropin-producing cell line, such as LβT2, provides a useful model to search for factors that regulate gonadotropin secretion and investigate the mechanisms of gonadotropin secretion regulation [5]. However, these factors and mechanisms have not yet been fully characterized. The main limitation is the lack of simple and easy-to-use techniques to detect hormone secretion from hormone-producing cell lines. Radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) have generally been used to detect and quantify hormones secreted into the medium by hormone-producing cell lines. Although these methods show high specificity and sensitivity to detect and quantify the target hormones, a specific antibody to the target hormone is necessary for these assays. In addition, these assays are generally expensive, are time-consuming for analysis, and, in the case of RIAs, require the use of radioactivity.

Gaussia luciferase (Gluc) is a protein naturally secreted by the copepod *Gaussia princeps*. This luciferase has been widely used in reporter assays, such as those monitoring promoter activity, endoplasmic reticulum stress, protein-protein interactions, and secretory pathways [6]. To monitor insulin secretion from Min6 cells, the insulin that is fused to Gluc is used for video rate bioluminescence imaging in the living cells [7]. The advantage of this assay is that only the secreted insulin-Gluc in the medium is reacted with a Gluc substrate, coelenterazine, to produce light. This assay enables the detection in real time of insulin-Gluc secretion as luminescence signals. The assay does not require any antibody to detect hormone

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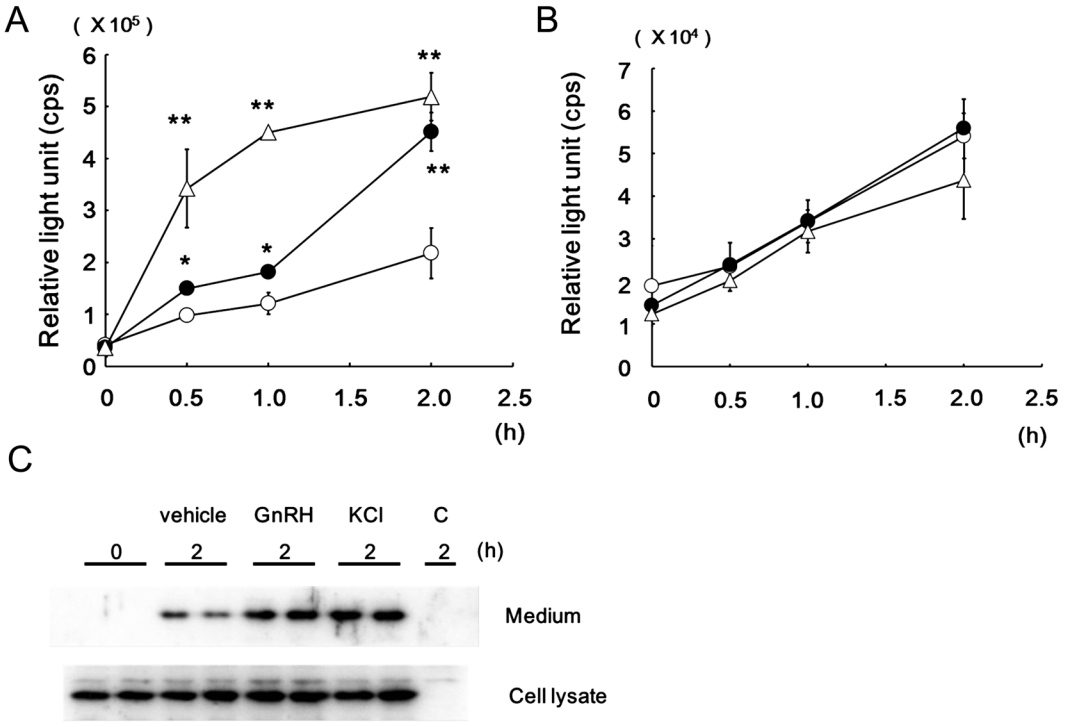

Fig. 1. Time-dependent increment of Gluc activity in the LβT2-cultured medium (A) and NIH3T3-cultured medium (B), and Gluc protein secretion in LβT2-cultured medium (C). The cells were transfected with pCMV-Gluc2. (A and B) Gluc-expressing cells were incubated for the indicated times without treatment (open circle), and in the presence of 10 nM GnRH (closed circle), or 50 mM KCl (open triangle). Gluc activity in the medium at each time point was measured as described in "Materials and Methods". The asterisks indicate that the Gluc activities are significantly different from that in the absence of agents, * $P < 0.05$, ** $P < 0.01$. (C) Gluc-expressing LβT2 cells or control LβT2 cells (C) were incubated for 2 h in the absence (vehicle) or presence of GnRH or KCl. Gluc protein in the medium and cells was detected by a Gluc antibody as described in "Materials and Methods".

secretion in real time.

In the present study, we found that Gluc that was not fused to gonadotropins can be secreted into the medium in a GnRH receptor-dependent manner from Gluc-expressing LβT2 cells. We also showed that the receptor-dependent Gluc secretion was not restricted to LβT2 cells, but could also be detected in AtT20 cells, which produce and secrete ACTH [8]. On the other hand, GnRH-dependent Gluc secretion was not detected even from the GnRH receptor-expressing HEK293 cells, which are non-excitable cells. These results suggest that Gluc can be used to detect hormone secretion easily and in real time from LβT2 or AtT20 cells. This feature is suitable for high-throughput screening of the factors influencing hormone secretion from these cell lines.

When we expressed Gluc in LβT2 cells, the luciferase activity in HEPES-buffered medium increased time-dependently for 2 h without any stimulation (open circles in Fig. 1A). The increment of the activity was enhanced when the cells were stimulated with GnRH or KCl (closed circles and open triangles, respectively, in Fig. 1A). In contrast, GnRH- or KCl-induced activity was not detected in the medium of Gluc-expressing NIH3T3 cells, which are not secretory cells, although the Gluc activity in the medium increased time-dependently, as had that of the LβT2 cells (Fig. 1B). We examined whether the GnRH- or KCl-induced enhancement of Gluc activity reflects the increased secretion of Gluc protein into the medium. As shown in Fig. 1C, Gluc-protein secretion into the medium was enhanced when the cells were stimulated by GnRH or KCl for 2 h.

We next examined whether the GnRH-induced Gluc activity was due to the activation of GnRH receptors expressed in LβT2 cells. Antide, a GnRH receptor antagonist, inhibited GnRH-induced Gluc activity, indicating that the luciferase activity was mediated through GnRH receptors (Fig. 2A). To investigate whether this result reflects GnRH-induced gonadotropin secretion through GnRH receptors, we measured the amount of luteinizing hormone (LH) in the medium using an ELISA. As shown in Fig. 2B, antide inhibited GnRH-induced LH secretion. On the other hand, the antagonist did not attenuate KCl-induced LH secretion. KCl directly activates voltage-dependent Ca^{2+} channels and stimulates LH secretion without GnRH receptor activation. As shown in Fig. 2A, antide did not attenuate KCl-induced Gluc activity. Thus, the GnRH-induced Gluc activity reflects LH secretion from LβT2 cells. GnRH-induced Gluc activity was detected from 0.01 nM GnRH and increased dose-dependently to 10 nM (Fig. 2C). This detectable range is comparable to that of the ELISA used in the present study (0.01–3 nM).

AtT20 is a mouse ACTH-producing cell line, which we then utilized to examine whether Gluc can be used to detect hormone secretion from other hormone-producing cell lines. When Gluc was

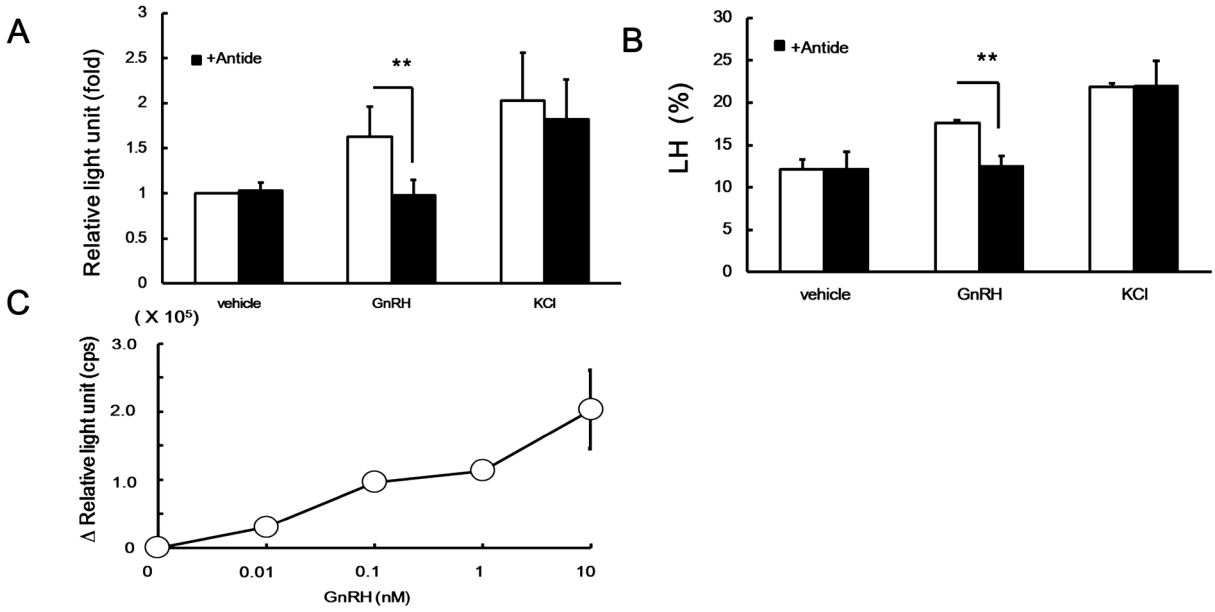

Fig. 2. Effect of antide on GnRH-induced Gluc activity (A), on GnRH-induced LH secretion (B), and the difference in Gluc activity in the absence and presence of antide (C) in LβT2 cells. (A) LβT2 cells were transfected with pCMV-Gluc2. Gluc-expressing cells were stimulated with 10 nM GnRH or 50 mM KCl for 2 h in the presence (closed column) or absence (open column) of 100 nM antide. Results show the relative value, where the relative light unit of the vehicle without antide is expressed as 1. Asterisks indicate that the Gluc activities are significantly different from those in the absence of antide. ** $P < 0.01$. (B) LβT2 cells were stimulated with 10 nM GnRH or 50 mM KCl for 4 h in the presence (closed column) or absence (open column) of 100 nM antide. Results indicate the amount of LH secreted in the medium as the percentage of the total amount of LH contained in the medium and in the cells. Asterisks indicate that the secreted LH amount is significantly different from that in the absence of antide. ** $P < 0.01$. (C) The Δ relative light unit indicates the difference between the absence and presence of antide under the indicated GnRH concentrations.

expressed in AtT20 cells, Gluc activity in the medium also increased when the cells were stimulated with CRH (Fig. 3A), which stimulates the secretion of ACTH from AtT20 cells (Fig. 3B).

Thus, Gluc can generally be used to detect hormone secretion from hormone-producing cells. We next examined whether Gluc activity could be enhanced when we used HEK293, a non-hormone-producing cell line. We did not detect any increment of GnRH-induced Gluc activity in the medium, even when human GnRH receptors (GnRHr) were expressed in Gluc-expressing HEK293 cells (Fig. 3C). The GnRH receptor activation was confirmed that the cells responded to GnRH to stimulate SRE-promoter activity (Fig. 3D). As expected, a KCl-induced increase in Gluc activity was not detected. This is consistent with the fact that HEK293 cells are non-excitable.

The result showing that the hormone-induced enhancement of Gluc activity in the medium reflects hormone secretion from the hormone-producing cell lines prompted us to examine whether Gluc could be partially colocalized with the hormone in these cells. To examine this possibility, we performed immunocytochemistry analyses of LH or ACTH, in addition to Gluc, in Gluc-expressing LβT2 cells or AtT20 cells, respectively (Fig. 4). The merged images of the hormone and Gluc in the cells show that Gluc was partially colocalized with LH in the case of LβT2 cells and with ACTH in the case of AtT20 cells.

In the present study, we found that Gluc could detect real-time hormone secretion from hormone-producing cell lines, namely LβT2 and AtT20. To detect hormone secretion from cells, an RIA or ELISA has generally been used. These assays can detect the secretion of target hormones with high specificity and a low limit of detection; however, these assays require the preparation of specific antibodies to the target hormones, and it takes time to get the results. On the other hand, the Gluc assay in the present study does not need the preparation of specific antibodies to the hormone and can easily detect GnRH-induced or KCl-induced increased activity, even 30 min after stimulation (Fig. 1A). The results also indicated that the detection limit and the detection range of LH were almost identical to those of ELISA (Fig. 2C).

The reason Gluc can be secreted as a hormone is currently unknown. Some of the Gluc might be colocalized with LH or ACTH within secretory granules in LβT2 or AtT20 cells, respectively (Fig. 4). This suggests that the Gluc in the secretory granules may be released with the hormones upon stimulation by a secretagogue such as GnRH, CRH, or KCl. In agreement with this assumption, Gluc activity was not enhanced in the culture medium of NIH3T3 or HEK293 cells, because these are not excitable cells (Figs. 1B and 3C). In the case of HEK293 cells, GnRH-induced Gluc activity was not detected, even when we used GnRH receptor-expressing HEK293 cells (Fig. 3C). Thus, Gluc can specifically detect hormone secretion when it is expressed in hormone-producing, i.e., excitable, cells.

Gluc has been used to detect real-time insulin secretion from the Min6 cell line [7]. In this study, Gluc was fused to the C terminus of

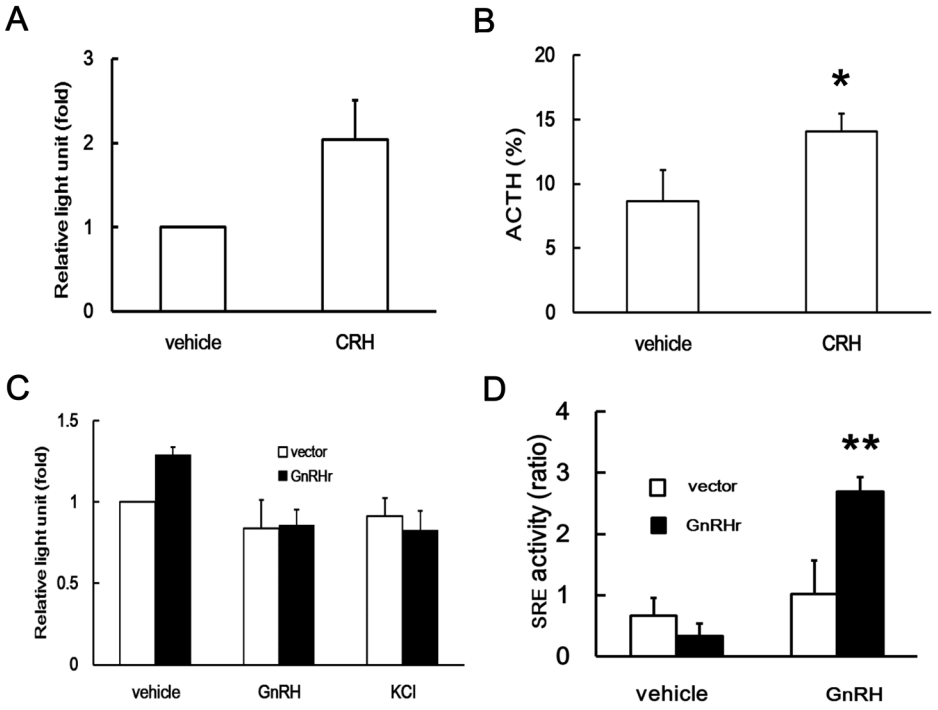

Fig. 3. Secretagogue-induced Gluc activity in AtT20-cultured medium (A) and ACTH secretion from AtT20 cells (B); secretagogue-induced Gluc activity in GnRH receptor-expressing HEK293-cultured medium (C), and GnRH-induced SRE activity of GnRH receptor-expressing HEK293 cells (D). (A) AtT20 cells were transfected with pCMV-Gluc2. Gluc-expressing cells were stimulated with 10 nM CRH or a vehicle for 4 h. Results are indicated as shown in Fig. 2. The relative light unit of the vehicle is expressed as 1. (B) AtT20 cells were stimulated with 10 nM CRH for 4 h. Results show the amount of ACTH secreted in the medium as the percentage of the total amount of ACTH contained in the medium and in the cells. Asterisks indicate that the secreted ACTH amount is significant. ** $P < 0.05$. (C) HEK293 cells were transfected with pCMV-Gluc2 together with human GnRH-receptor expression plasmid (GnRHr) or pcDNA3.1 (vector). Gluc-expressing cells were stimulated with 10 nM GnRH, 50 mM KCl, or vehicle for 2 h. Results are indicated as shown in Fig. 2. The relative light unit of the vehicle-treated vector-transfected cells is expressed as 1. (C) HEK293 cells were transfected with pSRE-luc and pTK-renilla plasmids, together with GnRHr or the vector, and incubated for 6 h in the presence or absence (vehicle) of 10 nM GnRH. Results are expressed as the ratio of firefly (pSRE-luc) to renilla (pTK-renilla) luciferase activity.

insulin protein to localize Gluc within the insulin secretory granules. This method is useful, but it cannot be generally applied to other hormone-producing cell lines. ACTH is produced and secreted from AtT20 cells in response to CRH (Fig. 3A). ACTH, however, cannot be directly fused to Gluc because it must be synthesized by proteolytic cleavage from pre-proopiomelanocortin (pre-POMC) before its secretion. In the present study, we could detect CRH-induced Gluc activity in the culture medium of AtT20 cells (Fig. 3A). In the present study, Gluc was not fused to ACTH. This suggests that the Gluc assay in the present study can be easily applied to detect the secretion of hormones that cannot be directly fused to the tagged protein.

The GnRH- or KCl-induced Gluc activity accounted for a small part of the total Gluc activity (Fig. 1A). This reflects the differences in cellular secretory pathways; there are two distinct secretory pathways, namely the constitutive and regulated pathways, in a variety of cells [9]. LβT2 and AtT20 cells have both pathways. It is known that the increased passage number of hormone-producing cell lines causes decreased hormone secretion through the regulated pathway and increased secretion through the constitutive pathway [10]. LH and ACTH are usually secreted mainly through the regulated pathway. On the other hand, follicle-stimulating hormone (FSH), another

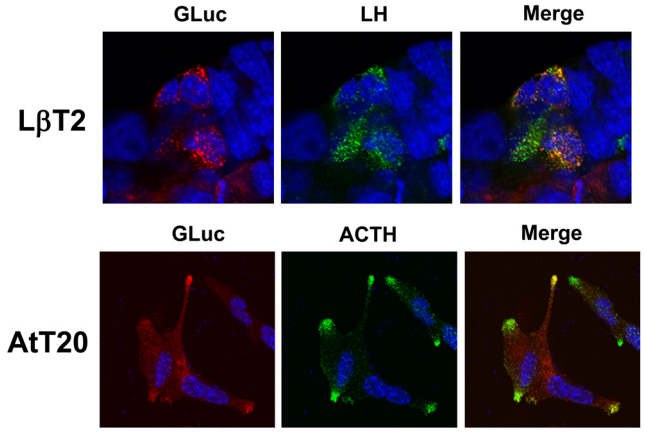

Fig. 4. Immunofluorescence of Gluc-expressing LβT2 cells (LβT2) or Gluc-expressing AtT20 cells (AtT20) using Gluc, LHβ, or ACTH antibodies. Merged images show that the expressed Gluc protein (Gluc) was partially colocalized with LHβ (LH) in LβT2 cells or ACTH (ACTH) in AtT20 cells. The cell nuclei were stained with DAPI (blue).

gonadotropin produced in LβT2 cells, is reported to be secreted through the constitutive pathway [11]. Therefore, we investigated the amount of LH secreted through the regulated pathway of LβT2 cells using antide. As shown in Fig. 2A and B, the extent of secretion by the regulatory pathway, corresponding to the antide-sensitive results, was similar between the Gluc assay and the LH ELISA. This indicates that the GnRH-induced Gluc activity reflects a regulated pathway.

In conclusion, we found that Gluc can detect hormone secretion from LβT2 and AtT20 cell lines easily, and in real time. The Gluc assay in the present study could be generally applied to other hormone-producing cells for high-throughput screening to identify factors with agonistic or antagonistic activity for hormone secretion.

Materials and Methods

Materials

Human GnRH and antide were purchased from Sigma-Aldrich Japan (Tokyo, Japan), and the human corticotropin-releasing hormone (human CRH) was from Peptide Institute (Osaka, Japan). The anti-Gluc antibody, pCMV-Gluc2, and BioLux Gluc assay kit were all from New England BioLabs (Tokyo, Japan). The dual luciferase kit was from Promega (Tokyo, Japan), fatty-acid-free BSA was from Calbiochem-Novabiochem (San Diego, CA, USA), and Lipofectamine 2000 Reagent was from Life Technologies (Tokyo, Japan). The sources of all other reagents were as described previously [12, 13].

Cell culture

LβT2 cells were a generous gift from Dr. Pamela Mellon, AtT20 and NIH3T3 cells were from ATCC, and HEK293 cells were from the RIKEN BRC. These cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 100 U penicillin/ml, 100 μg streptomycin/ml, and 10% FBS. All cells were grown in 5% CO_2 at 37°C in a humidified environment.

Preparation of receptor cDNA plasmids and expression

Human GnRHr (GnRHr) [14] (0.4 μg), pCMV-Gluc2 (0.4 μg), pcDNA3.1 (0.4 μg), pSRE-luc (0.6 μg), and/or pTK-renilla (0.12 μg) plasmids were transfected into the cells (10^6 cells) using Lipofectamine 2000 Reagent as described previously [12].

Immunocytochemistry

Immunofluorescence methods were performed as described previously [13, 15]. Briefly, LβT2 cells and AtT20 cells were transfected with pCMV-Gluc 2 or pcDNA3.1 as described above and cultured on poly-D-lysine-coated cover glass. They were fixed in 10% formalin in phosphate-buffered saline (PBS) for 15 min at room temperature (RT), washed three times with PBS, and treated twice with 0.1% Triton in PBS for 10 min at RT. The cells were incubated for 2 h at RT with rabbit anti-Gluc antibody (1:100), mouse monoclonal antibody against ovine LHβ (1:1000) [16], and guinea pig anti-amidated joining peptide (JP), a marker for ACTH cells (ST-3: 1:2000) [17, 18], in PBS containing 1% bovine serum albumin (BSA). After the incubation with each primary antibody, the cells were washed three times with PBS, and stained with Cy3-labeled affinity-purified donkey anti-rabbit IgG (1:600; Jackson Immunoresearch, PA, USA), Alexa 488-labeled affinity-purified donkey anti-mouse IgG (1:400; Jackson Immunoresearch), and Alexa 488-labeled affinity-purified donkey anti-guinea pig IgG (1:400; Jackson Immunoresearch) for 1 h at RT. The secondary antibody solution included 4',6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) to counterstain the nuclei. The cells were washed in PBS, mounted in PermaFluor (Thermo Fisher Scientific, Yokohama, Japan), and examined using a confocal laser scanning microscope system (A1, Nikon, Tokyo, Japan).

Immunoblot analysis

The cells in a 6 cm-diameter culture plate were incubated in 3 ml of HEPES-buffered medium in the presence of the indicated agents for the indicated periods. HEPES-buffered medium was composed of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3.6 mM $NaHCO_3$, 5 mM glucose, 140 mM NaCl, 1.2 mM KH_2PO_4, 2.4 mM $MgSO_4$, and 4.8 mM KCl. The buffer was then collected and transferred into an Amicon Ultra-15 10K filter to concentrate the secreted Gluc to 150 μl. A concentrated sample (25 μl) was applied to 10% SDS-polyacrylamide gel electrophoresis. The cell lysate was prepared as follows: cells were washed in PBS and lysed in a lysis buffer at pH 7.5 containing 50 mM Tris, 30 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% v/v sodium dodecyl sulfate (SDS), 1% v/v Triton X-100, 1 mM NaF, 10% v/v glycerol, 0.5% aprotinin, 1% leupeptin, 0.5% pepstatin, 0.2% PMSF, 1% PNPD, and 5% Na_3VO_4. The amount of total protein was estimated using a Takara BCA protein assay kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. The supernatant and the cell lysate (5 μg of protein) were applied to 10% SDS-polyacrylamide gel electrophoresis followed by electrophoretic transfer to an Immobilon-P transfer membrane (Merck Millipore, Darmstadt, Germany). The membrane was blocked with 4% (w/v) nonfat dried milk in TBST (20 mM Tris, 150 mM NaCl, and 0.1% v/v Tween 20; pH 7.4) for 1 h. The membrane was then incubated overnight with a rabbit polyclonal antibody for Gluc (1:5000; New England BioLabs, Tokyo, Japan). After washing with TBST, the membrane was incubated with a horseradish peroxide (HRP)-labeled secondary antibody (donkey anti-rabbit IgG, Amersham, Tokyo, Japan) for 3 h. After washing with TBST, specific immunoreactivity was visualized using an ECL Plus System (Perkin Elmer Japan, Tokyo, Japan) with a Fujifilm LAS4000 Imager (GE Healthcare Japan, Tokyo, Japan).

Gluc assay

The cells in which pCMV-Gluc2 was expressed were stimulated by the indicated agents for the indicated time in HEPES-buffered medium (1 ml/well) in 12-well multiplates. Gluc activities were measured according to the instructions from the manufacturer (BioLux Gluc assay kit). Briefly, after stimulation by the indicated agents, 50 μl of the stimulated HEPES-buffered medium was diluted with 950 μl of PBS. Then 20 μl of the diluted sample was mixed with 50 μl of the Gluc assay solution. Gluc activities were measured for 10 seconds using a luminometer (GLOMAX, Promega, Tokyo, Japan).

Dual luciferase reporter assay

The serum response element (SRE)-driven promoter activity was measured using PathDetect signal transduction pathway cis-reporting systems (Agilent Technologies, Santa Clara, CA, USA) as described

previously [12].

ELISA

LβT2 cells were preincubated with or without antide in DMEM containing 0.1% BSA (300 µl/well) in 24-well multiplates for 4 h. The indicated amount of GnRH or KCl in DMEM (200 µl) was then added to each precultured well, and the cells were incubated for another 4 h. In the case of AtT20 cells, 10 nM of CRH in DMEM was added and incubated for 4 h. After stimulation, 150 µl of the medium was taken and microfuged at 12,000 rpm for 5 min to remove the cell debris. The supernatant was used in an ELISA. For LH or ACTH measurement in the LβT2 or AtT20 cells, the cells were lysed in 100 µl of PBS containing 1% Triton X-100, and the lysed sample was used in an ELISA. The amount of LH or ACTH in the medium and cells was estimated using a rat LH ELISA kit (S-type) (Shibayagi, Gunma, Japan), or an ACTH (Rat, Mouse) EIA kit (Phoenix Pharmaceuticals, CA, USA), respectively.

Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean ± SE from more than three different batches of cells unless otherwise stated. Statistical significance was assessed using an ANOVA; values were considered significant at $P < 0.05$ (*) or $P < 0.01$ (**).

Acknowledgments

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