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Increased luminescence of the GloSensor cAMP assay in L β T2 cells does not correlate with cAMP accumulation under low pH conditions

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Abstract. Cyclic adenosine monophosphate (cAMP) plays a pivotal role in gonadotrope responses in the pituitary. Gonadotropin-releasing hormone (GnRH) mediated synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are regulated by both the G_s/cAMP and G_q/Ca²⁺ signaling pathways. Pituitary adenylate cyclase-activating polypeptide (PACAP) also regulates GnRH responsiveness in gonadotropes through the PACAP receptor, which activates the G_s/cAMP signaling pathway. Therefore, measuring intracellular cAMP levels is important for elucidating the molecular mechanisms of FSH and LH synthesis and secretion in gonadotropes. The GloSensor cAMP assay is useful for detecting cAMP levels in intact, living cells. In this study, we found that increased GloSensor luminescence intensity did not correlate with cAMP accumulation in L β T2 cells under low pH conditions. This result indicates that cell type and condition must be considered when using GloSensor cAMP.

Key words: Cyclic adenosine monophosphate (cAMP), GloSensor, Low pH, Luminescence, L β T2

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Cyclic adenosine monophosphate (cAMP) is a second messenger that plays important roles in numerous biological processes [1]. In the pituitary, adrenocorticotropin (ACTH) is synthesized and secreted from corticotropes following stimulation by corticotropin-releasing hormone (CRH) acting through the G_s/cAMP signaling pathway [2]. In gonadotropes, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and gonadotropin-releasing hormone (GnRH)-mediated secretion are regulated by both the G_s/cAMP and G_q/Ca²⁺ signaling pathways. The cAMP and Ca²⁺ signaling pathways are influenced by the pulse frequencies of GnRH stimulation [3]. Pituitary adenylate cyclase-activating polypeptide (PACAP) is a hypothalamic pituitary-releasing factor, as well as an autocrine/paracrine factor within the pituitary, and regulates GnRH responsiveness through the PACAP receptor by increasing GnRH receptor expression and modulating GnRH intracellular signaling pathways [4]. The PACAP receptor is also mainly coupled to, and activates, the G_s/cAMP signaling pathway [5]. This indicates that cAMP plays a pivotal role in corticotrope and gonadotrope responses in the pituitary; consequently, measuring intracellular cAMP levels in real time becomes especially important for elucidating the molecular mechanisms of FSH and LH synthesis and secretion in gonadotropes.

The cellular levels of cAMP are typically quantified by radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA).

These methods show high specificity and sensitivity for the quantification of cAMP concentrations. However, a cAMP-specific antibody is needed with both methods, and additionally, cells must be lysed before quantification, with the result that changes in intracellular cAMP levels cannot be followed in real time using RIA and/or ELISA. The GloSensor cAMP assay uses genetically encoded biosensor variants with cAMP-binding domains fused to mutant forms of *Photinus pyralis* luciferase. On binding to cAMP, this enzyme undergoes conformational changes, leading to increased bioluminescence [6]. Thus, cells expressing the GloSensor plasmid can be used to evaluate cAMP levels in living cells in real time and without having to lyse the cells. Indeed, the GloSensor reporter has been utilized in numerous studies, including for the characterization of adenosine receptor antagonists expressed in native cells [7], the cAMP inducer-free screening of G_i-coupled receptor agonists [8], and the testing of selective agonists for the κ -opioid receptor [9]. Although the cAMP GloSensor has not been used in corticotropes and gonadotropes, its application is expected to provide useful information for elucidating the role of changes in cAMP levels in these hormone-producing cells in real time.

The OGR1, GPR4, and TDAG8 proteins are recognized as proton-sensing G protein-coupled receptors (GPCRs) that sense extracellular protons and activate intracellular signaling pathways through trimeric G proteins [10, 11]. These GPCRs are inactive at alkaline pH (pH 7.8), partially activated at physiological pH (pH 7.4), and fully activated at approximately pH 6.8 [10, 11]. The OGR1 receptor is mainly coupled to, and activates, the G_{q/11}/phospholipase C/Ca²⁺ signaling pathway when stimulated by extracellular protons. In contrast, GPR4 and TDAG8 are mainly coupled to, and activate, the G_s/cAMP signaling pathway when stimulated by extracellular protons [10, 11]. Expression of *TDAG8* is detected mainly in immune cells [12],

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while that of *GPR4* is widely detected in various tissues [13]. Under conditions of low pH, GPR4 has been shown to ameliorate intestinal inflammation [13], mediate central respiratory sensitivity to CO₂ [14, 15], and inhibit osteogenesis [16]. Moreover, GPR4 antagonists are reported to protect against myocardial infarction [17, 18] and exhibit modulatory effects in models of arthritis, hyperalgesia, and angiogenesis [19]. In relation to hormonal function, GPR4 increases insulin sensitivity [20].

In this study, we used the mouse L β T2 gonadotrope and the AtT20 corticotrope cell lines. L β T2 cells secrete LH following GnRH stimulation [21] and are also responsive to PACAP [22]. AtT20 cells, meanwhile, secrete ACTH when stimulated with CRH [23]. We found that the bioluminescence of the GloSensor reporter increased with decreasing extracellular pH in L β T2 cells, but not in AtT20 cells.

We first examined which proton-sensing GPCRs are expressed in L β T2 cells. As shown in Fig. 1A, expression of *OGR1* and *Gpr4* was observed in L β T2 cells, whereas that of *TDAG8* was barely detected. We next examined the expression level of *Gpr4* in L β T2 and AtT20 cells as GPR4 is mainly coupled to the Gs/adenylyl cyclase/cAMP signaling pathway. No difference in *Gpr4* expression was observed between the two cell lines, with levels approximately one-fifth of those observed in the mouse anterior pituitary lobe (Fig. 1B).

In L β T2 cells, when the extracellular pH decreased, GloSensor luminescence, which is assumed to reflect cAMP accumulation, increased in a time-dependent manner (Fig. 2A). The peak level of luminescence induced by low pH was more than that induced by PACAP; however, it takes longer to reach the peak induced by low pH than the peak induced by PACAP (Fig. 2A). This result suggests that low pH induces greater cAMP accumulation than PACAP in L β T2 cells. The intensity of the luminescence induced by PACAP was further increased under conditions of low pH (Fig. 2B), suggesting that low pH enhances PACAP-induced cAMP accumulation in L β T2 cells. In contrast, the luminescence intensity in AtT20 cells was only marginally increased under low extracellular pH (Fig. 2C). This was not due to a lack of a response to cAMP accumulation in AtT20 cells; indeed, luminescence was shown to increase following CRH stimulation, which is known to induce cAMP accumulation in AtT20 cells [24]. This result indicates that the increased luminescence observed under low pH is not consistent with the expression levels of *Gpr4* in both L β T2 and AtT20 cells.

We next used a GPR4 antagonist [17] to clarify whether the low pH-induced increase in luminescence in L β T2 cells was mediated by GPR4. The results showed that low pH- and PACAP-induced luminescence were only marginally affected by exposure to 10 μ M of the antagonist, a concentration that is normally sufficient to inhibit GPR4 activity [18] (Fig. 3A and 3B). Indeed, when we compared the peak level of luminescence induced by low pH in the presence or absence of the antagonist, the luminescence intensities were not attenuated by the antagonist (Fig. 3C).

Next, to examine whether the low pH-induced increase in luminescence in L β T2 cells reflected cAMP accumulation, we measured cAMP levels in both L β T2 and AtT20 cells using ELISA. As shown in Fig. 4A and 4C, a 30 min incubation at low pH did not elicit cAMP accumulation in either cell line. Conversely, cAMP accumulation was induced by both PACAP (Fig. 4A) and CRH (Fig. 4C), while low pH inhibited cAMP accumulation by both PACAP and CRH (Fig. 4A

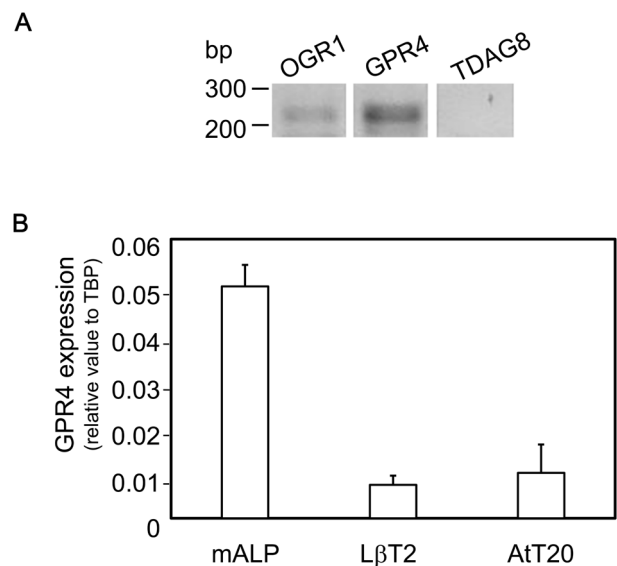


Fig. 1. Proton-sensing GPCR expression in L β T2 and AtT20 cells. (A) The mRNA expression levels of *Ogr1*, *Gpr4*, and *TDAG8* were assessed by RT-PCR. (B) qPCR was performed to estimate *Gpr4* mRNA levels in mouse anterior pituitary lobe (mALP) and in L β T2 and AtT20 cells. Data were calculated using the comparative CT method to estimate the copy number relative to that of *Tbp*. Data are expressed as the means \pm SE of triplicate measurements.

and 4C). As shown in Fig. 4B and 4D, similar results were obtained when we cAMP levels were measured 15 min after incubation, where GloSensor luminescence showed near peak values (Fig. 2). Thus, the ELISA result is different from the that of GloSensor in L β T2 cells.

To compare the GloSensor and ELISA results, we further examined the effect of forskolin which is a diterpenoid isolated from *Coleus forskohlii*. Forskolin is used for activating adenylyl cyclase directly to produce cAMP in a cell. As shown in Fig. 5A–5C, forskolin application resulted in increased GloSensor luminescence in L β T2, AtT20, and HEK293T cells, with luminescence peaking approximately 10 min after addition of the reagent. Accumulation of cAMP in these cells after 10 min of incubation with or without forskolin was also evaluated by ELISA. The result indicated that cAMP accumulation increased in the presence, but not absence, of the reagent (Fig. 5D–5F). The result also indicated that the level of cAMP accumulation after 10 min without the reagent was almost equal to that at the beginning (0 min) of incubation in these cell lines (Fig. 5D–5F).

We also examined the possibility that the increase in luminescence in L β T2 cells resulting from a low pH may have reflected cGMP accumulation rather than that of cAMP. GloSensor variants can sense cGMP in addition to cAMP, although the sensitivity to cGMP is less than 100-fold that of cAMP [6]. The result is shown in Fig. 6. Neither low pH nor PACAP induced cGMP accumulation under the condition in which atrial natriuretic peptide (ANP) induced its accumulation in L β T2 cells [25].

The results indicate that the increase in GloSensor luminescence at low pH did not correlate with the quantity of cAMP or cGMP produced in L β T2 cells. To elucidate the mechanism by which low pH induces

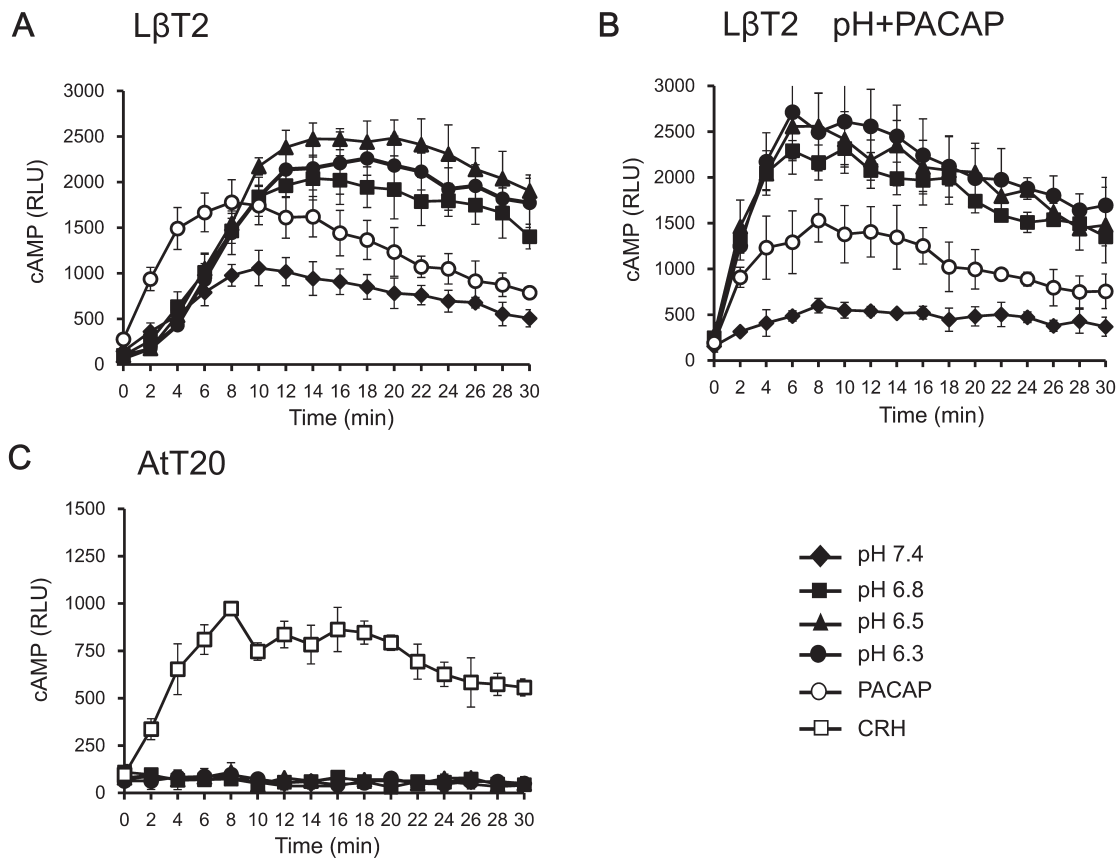


Fig. 2. Low pH, PACAP, and CRH induced GloSensor cAMP luminescence in LβT2 and AtT20 cells. (A) Time-dependent changes in GloSensor cAMP luminescence intensity in LβT2 cells following stimulation by the indicated pH or 100 nM PACAP dissolved in the culture medium at pH 7.4. (B) Time-dependent changes in GloSensor cAMP luminescence intensity in LβT2 cells following stimulation by the indicated pH in the presence of 100 nM PACAP. (C) Time-dependent changes in GloSensor cAMP luminescence intensity in AtT20 cells following stimulation by the indicated pH or 100 nM CRH (pH 7.4). Data are expressed as the means \pm SE of triplicate measurements obtained in a single representative experiment and expressed as relative luminescence units (RLU). Similar data were obtained in more than three separate experiments (A–C).

increased luminescence in LβT2 cells, we examined the possibility that the uptake of the GloSensor cAMP substrate into the cells may be enhanced by decreasing pH. When we removed the substrate from the assay medium before measuring the luminescence, the total luminescence in the cells decreased (Fig. 7A and 7B); however, the increased luminescence induced by low pH was still observed (Fig. 7B), similar to that seen in the presence of the substrate (Fig. 7A). This result indicates that changing substrate uptake is not the cause of the increased luminescence at low pH seen in LβT2 cells.

The GloSensor cAMP assay has been utilized in numerous studies [7–9] and is useful for monitoring cAMP levels in cells in real time. In the present study, we showed that GloSensor cAMP luminescence was increased in LβT2, but not AtT20 cells, under conditions of low pH. The degree of the increase in luminescence at low pH was greater than that induced by PACAP (Fig. 2A). In addition, low pH further increased the PACAP-induced luminescence (Fig. 2B), whereas when we measured the levels of cAMP or cGMP in LβT2 cells under conditions of low pH, we could not detect any increased accumulation of either cAMP or cGMP. One possible explanation for the increased luminescence at low pH was that the permeability

of LβT2 cells to the GloSensor cAMP substrate was enhanced. This possibility is low, however, as indicated in Fig. 7. Even though we removed the substrate from the assay medium before measuring the luminescence, low pH still induced increased luminescence in LβT2 cells (Fig. 7B). Therefore, the mechanism by which low pH increases luminescence in LβT2 cells remains unknown. It is possible that the GloSensor may detect undefined factors other than cAMP or cGMP in LβT2 cells, but not in AtT20 and HEK293T cells. Another possibility may be that the biosensor might detect a local increase in cAMP levels in LβT2 cells that cannot be detected at the whole-cell level. Although cAMP can easily diffuse in the cytosol, cAMP also confines in microdomains near the sites of its production in a cell. This confined cAMP plays a role in activating several effectors [26]. The increased GloSensor luminescence in LβT2 cells may also reflect localized cAMP accumulation in microdomains in the cell. This possibility could be clarified using different optical methods, such as the fluorescence resonance energy transfer (FRET), which can detect local cellular cAMP accumulation [26].

GPR4 is recognized as a proton-sensing GPCR that predominantly couples with G_s to induce cAMP accumulation. In this study, we

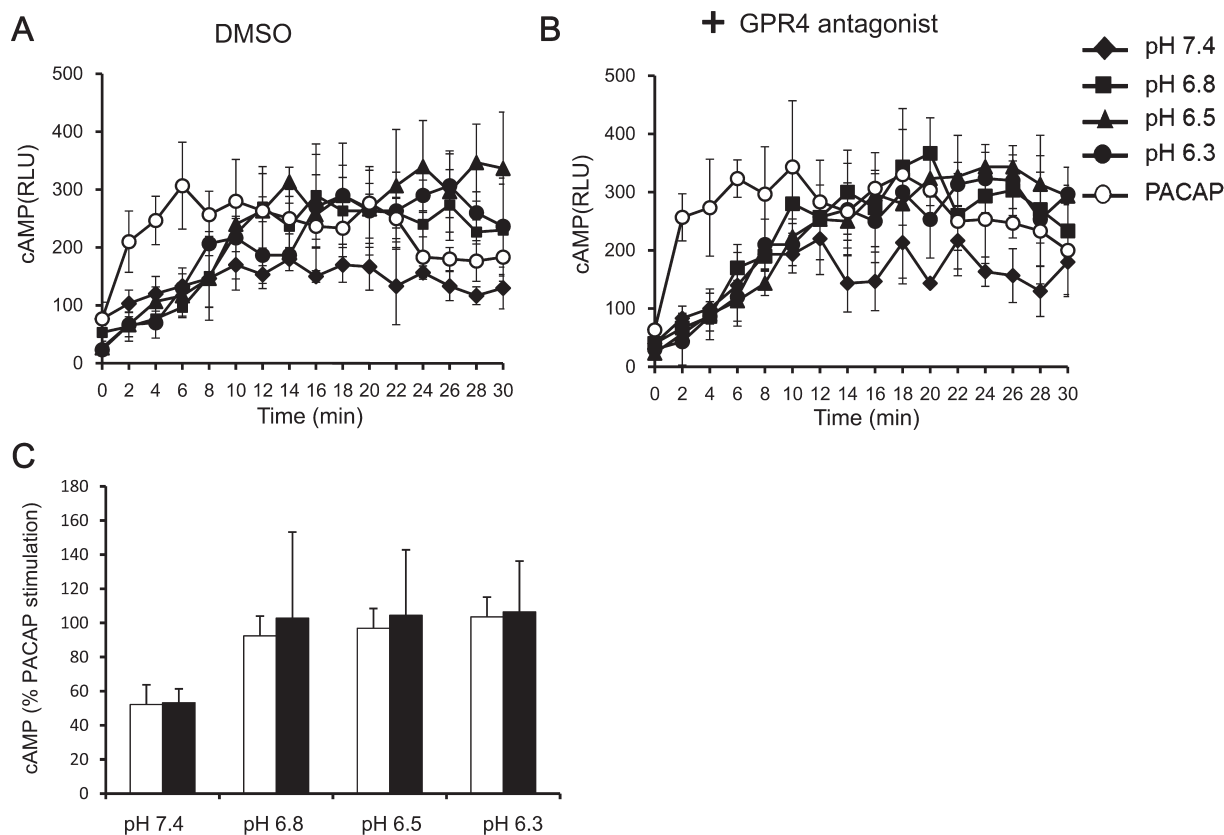


Fig. 3. Effect of a GPR4 antagonist on the increase of low pH-induced luminescence. (A) Time-dependent changes in the luminescence intensity of the GloSensor cAMP reporter in L β T2 cells following stimulation by the indicated pH or 100 nM PACAP (pH 7.4) in the presence of dimethyl sulfoxide (DMSO). DMSO was used to dissolve the GPR4 antagonist [17]. (B) Time-dependent changes in the luminescence intensity of GloSensor cAMP in L β T2 cells following stimulation by the indicated pH or 100 nM PACAP in the presence of 10 μ M GPR4 antagonist. Data are expressed as the means \pm SE of triplicate measurements obtained in a single representative experiment and expressed as relative luminescence units (RLU). Similar data were obtained in three separate experiments (A, B). (C) Influence of the GPR4 antagonist on the increase in GloSensor cAMP luminescence induced by low pH. Data are expressed as a percentage obtained by dividing the luminescence value indicating the maximum response under the indicated pH by the maximum luminescence value induced by PACAP measured in the same experiment. The black columns show the values in the presence of the GPR4 antagonist and the white columns show the values in the absence of the antagonist. Data are expressed as the means \pm SE of values obtained from three separate experiments, as described in (A) and (B).

could not detect any cAMP accumulation under low pH conditions using ELISA, despite *Gpr4* being expressed in both L β T2 and AtT20 cells (Fig. 1); however, this result does not necessarily indicate that GPR4 is not active in L β T2 or AtT20 cells. *Gpr4* expressed in human umbilical vein endothelial cells (HUVECs) is known to be involved in cell adhesion through the cAMP/Epac pathway [14, 27]. Although the HUVEC is known as a cell that strongly expresses endogenous *Gpr4* [27], cAMP accumulation due to low pH is only approximately 1.5-fold to its base level, lower than that in HEK293 cells overexpressing *Gpr4*. Low pH induces an accumulation of cAMP of approximately four fold in HEK293 cells [14]. Therefore, a small increase in cAMP levels is enough to induce the response observed for the HUVECs. It is possible that *Gpr4* expressed in L β T2 or AtT20 cells may induce local cAMP accumulation that cannot be detected by ELISA, as mentioned above. Local cAMP accumulation would play a role in activating some effectors in these cells. To elucidate the role of *Gpr4* expression in L β T2 or AtT20 cells, other methods

must be used, such as the FRET method mentioned above.

In conclusion, we have shown that, in L β T2 cells, the low pH-induced increase in luminescence using GloSensor cAMP does not correlate with cellular cAMP accumulation when measured by ELISA. The results in this study indicate that the cell type and condition must be considered when cAMP production is measured using GloSensor cAMP.

Materials and Methods

Materials

Human pituitary adenylate cyclase-activating polypeptide (PACAP38) and human atrial natriuretic peptide (human ANP) were purchased from Sigma-Aldrich (Tokyo, Japan), human corticotropin-releasing hormone (human CRH) from the PEPTIDE INSTITUTE (Osaka, Japan), forskolin (FSK) from FUJIFILM Wako (Osaka, Japan), the pGloSensor-22F-cAMP plasmid and GloSensor cAMP

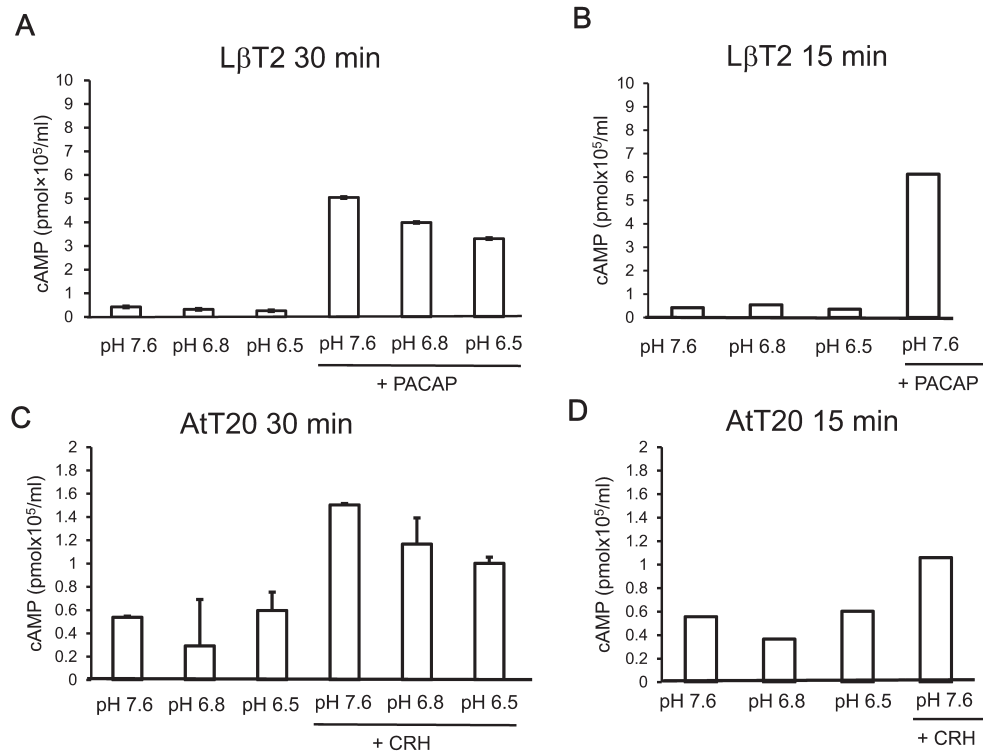


Fig. 4. Effect of low pH on cAMP accumulation in LβT2 (A, B) or AtT20 cells (C, D). LβT2 cells were stimulated with the indicated pH in the presence or absence of 100 nM PACAP for 30 min (A) or 15 min (B). AtT20 cells were stimulated with the indicated pH in the presence or absence of 100 nM CRH for 30 min (C) or 15 min (D). The levels of cAMP were measured by ELISA. Data represent the means ± SE of triplicate measurements obtained in a single representative experiment. Similar data were obtained in more than 2 independent experiments (A, C) or in a single representative experiment (B, D).

reagent from Promega (Tokyo, Japan), a dual luciferase kit from Promega (Tokyo, Japan), fatty acid-free Bovine serum albumin (BSA) from Calbiochem-Novabiochem Co. (San Diego, CA, USA), and Lipofectamine 2000 transfection reagent from Life Technologies (Tokyo, Japan). The sources of all the other reagents were as previously described [28, 29].

Quantitative real-time polymerase chain reaction

Quantitative real-time PCR was performed as described by Yoshida *et al.* [30]. Briefly, total RNA was prepared from LβT2 and AtT20 cells using ISOGEN II (Nippon Gene, Tokyo, Japan). cDNA was synthesized with PrimeScript Reverse Transcriptase (TaKaRa Bio, Otsu, Japan) using 1 μg of total RNA after DNase I treatment, and then subjected to quantitative PCR using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a SYBR Green Real-Time PCR Master Mix Plus (Toyobo, Osaka, Japan), including 0.5 μM of gene-specific primer sets. The sequences of the primers used in this study are as follows (5′–3′): OGR1 forward ACCGAGTCTGCTTTGAGCAT, reverse AAAGCAAGCCAGGAAGATGA; TDAG8 forward TGGGCTACGCAATACCCTTG, reverse CTAAAACGCAGC GGATGAGC; GPR4 forward GCAAGCTCTTTGGCTTCATC, reverse GTGTGGTTGTAGCGATCACG; and TBP forward GATCAAACCCAGAATTGTTCTCC, reverse

ATGTGGTCTTCCTGAATCCC. The expected sizes of the *OGR1*, *TDAG8*, and *GPR4* amplicons were 218, 189, and 229 bp, respectively. Quantification of the PCR products was performed using the comparative CT method (Δ CT method) to estimate the mRNA copy number relative to that of the TATA box binding protein gene (*Tbp*) used as an internal standard (Fig. 1B). Each PCR product was validated by sequencing (data not shown).

Cell culture

LβT2, AtT20, and HEK293T cells were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing 50 ng penicillin/ml, 50 ng streptomycin/ml, and 10% fetal bovine serum (FBS). All the cells were grown in 5% CO₂ at 37°C in a humidified environment.

GloSensor cAMP assay

The pGloSensor-22F-cAMP plasmid (1 μg) was transfected into LβT2, HEK293T, or AtT20 cells (1.5×10^6 cells) using the Lipofectamine 2000 reagent as previously described [28]. Two days after transfection, cells expressing the GloSensor were seeded in culture medium (100 μl/well) in a poly-D-lysine pre-coated 96-well, clear-bottomed white wall microplate and incubated overnight in 5% CO₂ at 37°C in a humidified environment. On the day of the cAMP assay, the culture medium was changed to a culture medium

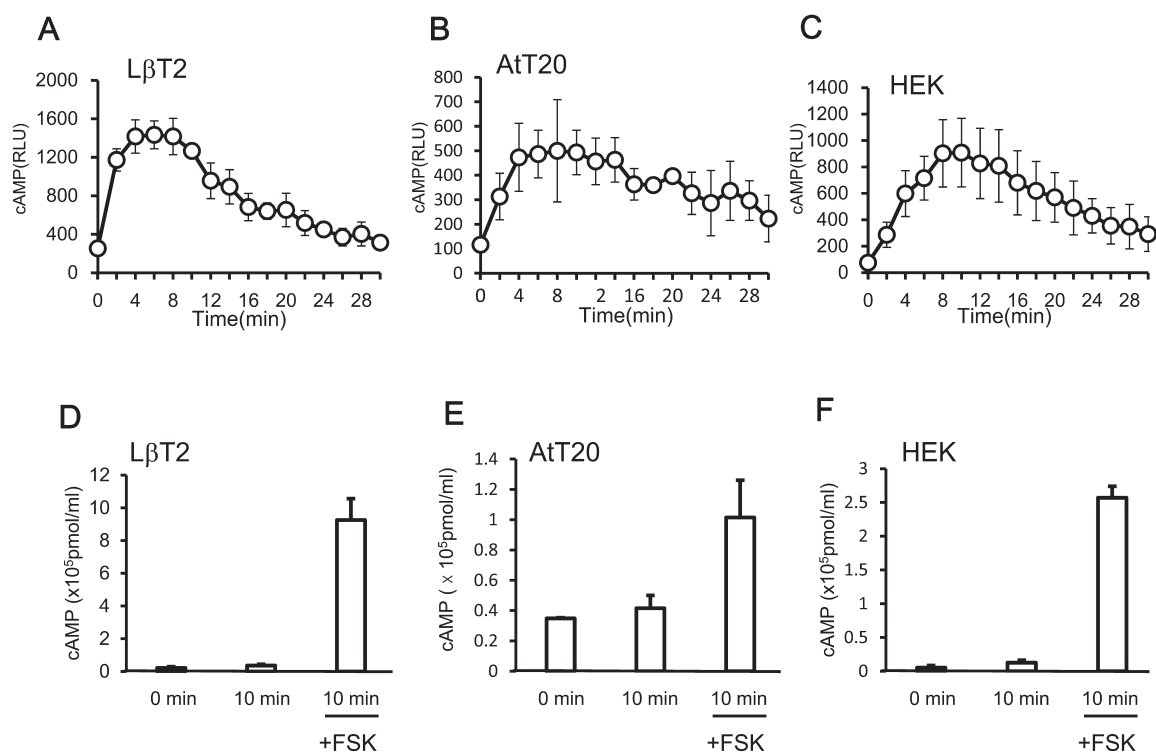


Fig. 5. Effect of forskolin (FSK) on cAMP accumulation in LβT2 (A, D), AtT20 (B, E), and HEK293T (C, F) cells. FSK-induced cAMP accumulation was measured using GloSensor cAMP (A–C). The cells were stimulated with 10 μM FSK. Data are the means ± SE of triplicate measurements obtained in a single representative experiment. The quantity of cAMP at the beginning (0 min) or after 10 min of incubation either with or without 10 μM FSK was measured by ELISA (D–F). Data are the means ± SE of 3 separate experiments.

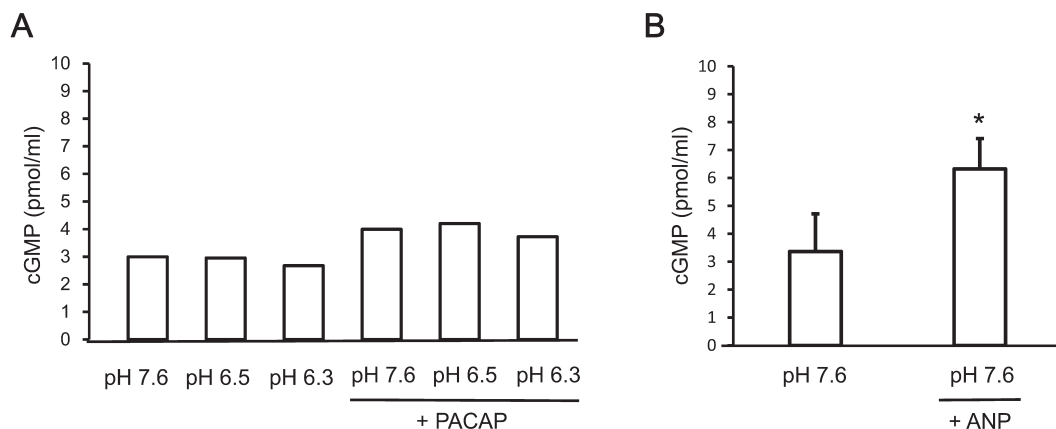


Fig. 6. Effect of low pH on cGMP accumulation in LβT2 cells. The cells were stimulated with the indicated pH in the presence or absence of 100 nM PACAP (A) or 1 μM ANP for 30 min. Data are the means of duplicate measurements obtained in a single representative experiment (A) or the means ± SE of triplicate measurements (B). Asterisks (*) indicate that the level of cGMP was significantly different (B). * P < 0.05

containing 2% GloSensor cAMP substrate and pre-incubated for 2 h at 37°C. The pre-incubated medium was then changed to the culture medium (25°C) at the indicated pH, containing 500 μM 3-isobutyl-1-methylxanthine (IBMX) and 2% GloSensor cAMP substrate in the presence or absence of PACAP (100 nM), CRH

(100 nM), forskolin (10 μM), or the GPR4 antagonist (10 μM) at 25°C. To evaluate the effect of the GloSensor cAMP substrate on the increase in luminescence at low pH, the GloSensor cAMP substrate was removed to eliminate the changes in substrate uptake occurring at low pH (Fig. 7). To adjust the pH of the culture medium, an ap-

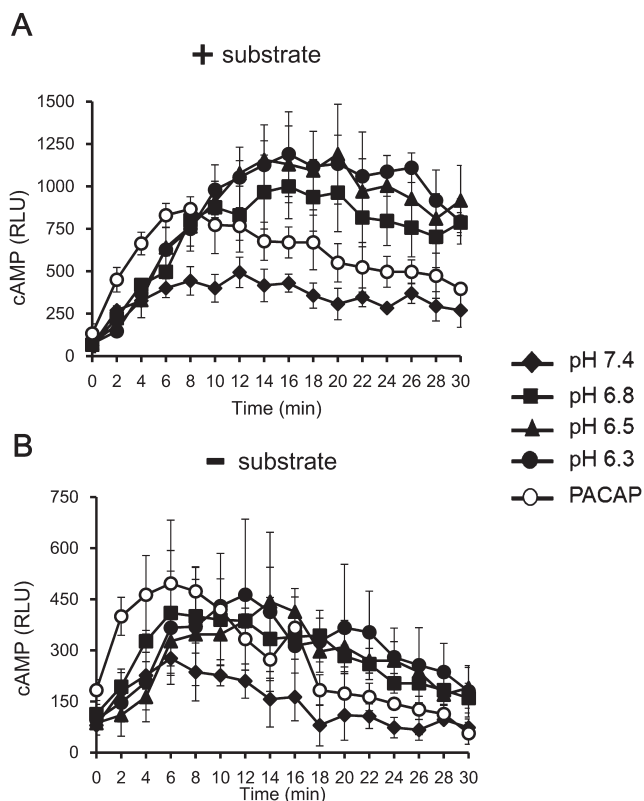


Fig. 7. Effect of the GloSensor cAMP substrate on the increase in luminescence at low pH. The low pH-induced increase in luminescence was measured in the presence (A) or absence (B) of the GloSensor cAMP substrate. L β T2 cells were pre-loaded with the substrate during the pre-incubation period. The substrate was then removed in (B), but not in (A), before the luminescence was measured. Data represent the means \pm SE of triplicate measurements obtained in a single representative experiment and expressed as relative luminescence units (RLU). Similar data were obtained in two separate experiments (A, B).

appropriate amount of 1 M HCl was added to the medium immediately prior to luminescence measurement. Luminescence was measured continuously over 30 min at 25°C, averaging 1 read per well every 2 min, using an Envision Multilabel plate reader (Perkin Elmer, Waltham, MA, USA).

ELISA

L β T2 cells were pre-incubated in HEPES-Regular at pH 7.4 (500 μ l/well) for 15 min in 12-well multiplates. The HEPES-Regular was composed of 25 mM HEPES, 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 2.5 mM NaHCO₃, 5 mM glucose, and 0.1% (w/v) BSA. Then, the HEPES-Regular was replaced with 200 μ l of HEPES-Regular at the indicated pH and containing 500 μ M IBMX, in the presence or absence of 100 nM PACAP or 1 μ M ANP, and incubated for a further 15 or 30 min. After stimulation, the cells were lysed in 1 ml of 1 M HCl, and the lysed samples were used for cAMP or cGMP determination. The quantity of cAMP or cGMP in the cells was estimated using direct cAMP or cGMP ELISA

kits (Enzo Life Science, Farmingdale, NY, USA).

Data presentation

The results of multiple observations are presented either as the means \pm SE or as a representative result, as indicated in the figure legends. Statistical significance was assessed by the Student's *t*-test (Fig. 6B). The values were considered significant at $P < 0.05$ (*).

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