Effects of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) 27 and PACAP 38 on the Release of Luteinizing Hormone (LH) from Cultured Bovine Anterior Pituitary Cells

Shinichiro KOYAMA, Tsutomu HASHIZUME,
Shinichi OHASHI¹⁾ and Shigeto KANEMATSU

*Laboratory of Animal Reproduction, Faculty of
Agriculture, Iwate University, Morioka, Iwate 020, and*

*¹⁾National Institute of Bioscience and Human
Technology, Tsukuba, Ibaraki 305, Japan*

Abstract. The effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on the release of luteinizing hormone (LH) from cultured bovine anterior pituitary cells was determined and compared with that of luteinizing hormone-releasing hormone (LHRH). PACAP 27 significantly increased the release of bovine LH at a dose as low as 10^{-14} M. PACAP 38 also significantly increased the release of LH and the minimal effective dose was 10^{-12} M. Maximal LH release induced by PACAP 27 (10^{-7} M) and PACAP 38 (10^{-7} M) were 40.5% and 71.6%, respectively of control levels. LHRH significantly increased the release of LH at a dose as low as 10^{-14} M. The increase in LH reached a maximum level which was 3-fold above the basal level at the highest concentration of LHRH tested (10^{-7} M, $P < 0.001$). LH release induced by PACAP 27, PACAP 38, and LHRH was in a dose-dependent manner. The results suggest that PACAP 27, PACAP 38, and LHRH stimulate the release of LH in the cattle by acting directly at anterior pituitary cells.

Key words: PACAP, LHRH, LH, Cattle.

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Pituitary adenylate cyclase-activating polypeptide with 38 residues (PACAP 38) and a shorter form of the peptide corresponding to the N-terminal 27 residues (PACAP 27) were recently isolated from the ovine hypothalamus and characterized [1, 2]. PACAP is localized to nerve fibers in both external and internal zones of the median eminence and the pituitary stalk that are in close contact with the hypophyseal portal capillaries [3]. PACAP specific receptors are found in the anterior pituitary cell membrane [4]. The physiological role of PAC-

AP in regulating the anterior pituitary gland has not been fully elucidated. As PACAP 27 and PACAP 38 stimulate adenylate cyclase activity in rat pituitary cells [1, 2, 5–7], it was suggested that the peptides might act as a hypophysiotrophic hormone. PACAP 38 was reported to induce the release of anterior pituitary hormones in rats but the results of the reports were at variance [1, 8, 9]. In a static pituitary cell culture system, PACAP 38 did not increase the release of any pituitary hormone despite considerable intracellular cyclic AMP (cAMP) accumulation in the rat [1]. Other studies demonstrated that PACAP 38 weakly stimulates LH and FSH release while it has no effect on GH,

ACTH or TSH release from rat pituitary cells in static culture system [8, 10]. On the other hand, the *in vivo* effect of PACAP 38 is also inconsistent. It has recently been reported that infusion of PACAP 38 at a rate of 3.2 pmol/kg per min has no effect on the release of gonadotrophin in human [11]. In contrast, it has been demonstrated that either intracerebroventricular or intraatrial injection of PACAP 38 stimulated the release of LH in male rats [9]. However, there are no reports concerning the physiological role of PACAP in LH release in ruminant animals. The effect of PACAP 27 on the release of LH remains to be investigated. Therefore, in the present study, we attempted to determine the direct effects of PACAP 27 and PACAP 38 on the LH release from cultured bovine anterior pituitary cells.

Materials and Methods

Peptides

PACAP 27 was synthesized by Dr. Shinichi Ohashi of National Institute of Bioscience and Human Technology, Tsukuba, Japan. PACAP 38 and LHRH were obtained from the Peptide Institute Inc. (Osaka, Japan).

Bovine pituitary cell dispersion

Pituitary glands were removed from eleven Holstein steers aged 17–18 months at a local slaughterhouse within 1 h after death, and were brought to our laboratory within 1 h in ice cold Krebs-Ringer bicarbonate buffer to which was added 1.8 mg/ml glucose, 0.5 mg/ml bacitracin (Wako Pure Chemical Industries LTD., Osaka, Japan), 1.0 mg/ml bovine serum albumin (BSA; Boehringer Mannheim, Germany), 0.1 mg/ml streptomycin, and 100 IU/ml penicillin (modified KRB buffer). The pituitary glands were cut sagittally into halves before immersion into the buffer.

Anterior pituitary (AP) tissues were diced into approximately 1 mm³ cubes and then placed into a dish with the buffer. The mean wet weights and standard errors of AP tissues in 11 steers were 1.37 ± 0.08 g. The AP tissues were immediately used for primary cell culture according to a modification of the procedure of Vale *et al.* [12]. The AP tissues were rinsed 4 times with HEPES buffer [NaCl, 137 mM; KCl, 5 mM; Na₂HPO₄·2H₂O, 0.7 mM; N-2-hydroxyethylpiperazine-N'-2-ethane-

sulfonic acid (HEPES; GIBCO, Gland Island, NY, USA), 25 mM; glucose, 10 mM; CaCl₂·2H₂O, 36 μM], placed into a siliconized, liquid scintillation vial containing 5 ml 0.4% collagenase (Wako Pure Chemical Industries LTD.) and 1% BSA in HEPES buffer, and then incubated in a water bath for 45 min at 37 C with agitation. The fragments were dispersed by pipetting 300 times every 15 min during the incubation. The tissues were then carefully decanted into a glass centrifuge tube and were centrifuged at 450 × g for 5 min. Approximately 4 ml of the buffer were discarded, and the pituitaries were poured into a scintillation vial containing 5 ml 0.25% pancreatin (pancreatin 4 ×; GIBCO) in Ca²⁺-free HEPES buffer, and incubated for 10 min at 37 C with agitation. After incubation, 5 ml of Dulbecco's modified Eagle medium (DMEM; GIBCO) which was supplemented with 10% horse serum (GIBCO), 2.5% new born calf serum (GIBCO), 1% non-essential amino acids (GIBCO), 100 IU/ml penicillin and 50 μg/ml streptomycin was suspended with a Pasteur pipette. The cells were decanted into centrifuge tubes and centrifuged at 450 × g for 5 min. Approximately 4 ml of the buffer were discarded, 5 ml fresh DMEM were added, and then the cells were dispersed by gently drawing the fragments into and expelling them out of a Pasteur pipette. This washing procedure was repeated 4 times.

Cell culture

After centrifugation, the media were discarded and the pelleted cells were resuspended in DMEM (10 ml) with a Pasteur pipette. The dispersed cells were then stained with trypan blue, and the non-stained viable cells were counted with a hemocytometer. Approximately 9 × 10⁶ cells were obtained from a pituitary gland. The cell suspension was randomly plated in 24-well dishes (CORNING; Houston, TX) at a density of 5 × 10⁴ cells per ml per well. The plated cells were maintained in the DMEM in a humidified atmosphere of 5% CO₂ at 37 C for 72 h.

After incubating for 3 days, the cells were washed 2 times with modified KRB to remove the medium and floating cells. Following washing, the media were replaced with fresh modified KRB buffer containing each dose of the test substance. After 2 h at 37 C, media (0.9 ml each) were collected for the LH assay. The medium was stored at -20 C until assayed for the hormone concentrations.

LH radioimmunoassay (RIA)

Measurement of bovine LH was performed in duplicate by a double antibody RIA as described previously [13]. The LH standard preparation and hormone for iodination were NIH-LH-B8 and LER-1056-C2, respectively. The hormones for the standard and iodination were supplied by Dr. L. E. Reichert Jr. (the Albany Medical College of Union University, Albany, NY, USA). An antiserum against LH (NIH-LH-S18) was generated in guinea pigs in our laboratory, and the crossreaction with other hormones and parallelism was determined as described in a previous report [14]. Goat anti-guinea pig IgG serum (2nd antibody) was supplied by Dr. K. Wakabayashi of the Institute of Endocrinology, Gunma University, Japan. The minimum detectable value of LH was 1.4 ng/ml. In this assay, the intra- and inter-assay coefficients of variation were 2.8% and 3.9%, respectively.

Statistical analysis

In each experiment with 10^{-14} to 10^{-7} M peptides, five to nine wells were used, and they were repeated two times. For an additional experiment, six to eight wells were used in each experiment with 10^{-16} to 10^{-7} M peptides. In each experiment hormone concentrations in the control wells were averaged and the mean was set as 100%. All other hormone values were expressed as percentages of these 100% values. The data were expressed as mean $\pm$ SE. Statistical analyses were performed with Student's *t* test after Bartlett's test for uniformity of the variances [15]. In order to examine the dose dependent response of the peptides to release LH, a linear regression analysis method was also employed [15].

Results

The effects of different concentrations of PACAP 27 (10^{-16} to 10^{-7} M) on the release of LH from cultured bovine anterior pituitary cells are shown in Fig. 1. PACAP 27 significantly increased the release of LH at the concentrations as low as 10^{-14} M ($P < 0.01$) compared with control media. The maximum effect of PACAP 27 on LH release was observed at 10^{-7} M (9.74 ± 0.75 ng/ml vs. 6.93 ± 0.06 ng/ml in controls). However, PACAP 27 failed to release LH at the concentrations lower than

10^{-15} M. Percent increases in LH at the 10^{-14} , 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} and 10^{-7} M PACAP 27 were 27.5, 30.6, 32.1, 35.0, 35.6, 36.2, 38.0 and 40.5% of the controls, respectively. As shown in Fig. 1, the LH responses to PACAP 27 were in a dose-dependent manner in a dose range of 10^{-16} to 10^{-7} M ($r = 0.86$; $P < 0.01$).

The effects of the different concentrations of PACAP 38 (10^{-16} to 10^{-7} M) on the release of LH from cultured bovine anterior pituitary cells are shown in Fig. 2. PACAP 38 significantly increased the release of LH at concentrations as low as 10^{-12} M ($P < 0.05$). The maximum effect was obtained at 10^{-7} M (21.31 ± 2.83 ng/ml vs. 12.42 ± 0.24 ng/ml in controls). However, PACAP 38 failed to stimulate the release LH at lower concentrations than 10^{-13} M. Percent increases in LH at the 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} and 10^{-7} M PACAP 38 were 23.5, 42.7, 47.9, 55.2, 56.0 and 71.6%, compared with control levels, respectively. As shown in Fig. 2, PACAP 38 at concentrations from 10^{-16} to 10^{-7} M linearly increased the LH concentrations in the media ($r = 0.97$; $P < 0.001$).

The effects of the different concentrations of LHRH (10^{-16} to 10^{-7} M) on the release of LH from cultured bovine anterior pituitary cells are shown

Fig. 1. Effects of PACAP 27 on the release of LH from cultured bovine anterior pituitary cells. Each value represents the mean $\pm$ SE of 8 to 23 determinations. Significant differences from control are indicated by asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig. 2. Effects of PACAP 38 on the release of LH from cultured bovine anterior pituitary cells. Each value represents the mean $\pm$ SE of 6 to 17 determinations. Significant differences from control are indicated by asterisks (* P <0.05, ** P <0.01, *** P <0.001).

Fig. 3. Effects of LHRH on the release of LH from cultured bovine anterior pituitary cells. Each value represents the mean $\pm$ SE of 7 to 24 determinations. Significant differences from control are indicated by asterisks (* P <0.05, ** P <0.01, *** P <0.001).

in Fig. 3. LHRH significantly increased the release of LH at concentrations as low as 10^{-14} M (P <0.05). The maximum effect of LHRH was obtained at 10^{-7} M (16.59 ± 1.32 ng/ml vs. 4.07 ± 0.12 ng/ml in controls). However, LHRH failed to stimulate the release of LH at lower concentrations than 10^{-15} M. Percent increases in LH at the 10^{-14} , 10^{-13} , 10^{-12} , 10^{-11} , 10^{-10} , 10^{-9} , 10^{-8} and 10^{-7} M LHRH were 14.0, 15.8, 18.6, 32.9, 44.1, 125.7, 238.6 and 307.7% of the controls, respectively. As shown in Fig. 3, the LH response to LHRH was linear at the dose ranged from 10^{-16} to 10^{-7} M ($r=0.84$; P <0.01).

The minimal effective concentration of PACAP 27 was 10^{-14} M which was comparable to that of LHRH. In contrast to the stimulating effects of LHRH and PACAP 27 on the release of LH, PACAP 38 failed to stimulate the release of LH at the concentration of 10^{-14} M. Percent increases in LH induced by 10^{-7} M PACAP 27 and 10^{-7} M PACAP 38 were about one tenth and one fifth of that of 10^{-7} M LHRH, respectively.

Discussion

The present study showed that PACAP 27, PACAP 38 and LHRH stimulated LH release from

bovine anterior pituitary cells *in vitro*. These findings may be important for speculating the physiological roles of PACAP 27 and PACAP 38 in ruminant animals.

In the present system with dispersed and cultured bovine anterior pituitary cells, LH release was significantly stimulated by LHRH (10^{-14} to 10^{-7} M). The minimum effective concentration of LHRH was 10^{-14} M, which was much lower than 10^{-11} M– 10^{-9} M reported in other studies using bovine pituitary cell cultures [16–20]. In other animals such as the rat, sheep, and pig, minimum effective concentrations of LHRH to stimulate LH release from anterior pituitary cells were between 10^{-12} and 10^{-8} M [8, 12, 21–28]. Therefore, our results indicate that the present system is very sensitive for studying the release of LH from bovine anterior pituitary cells.

Miyata *et al.* [1] initially reported that PACAP 38 had no effect on LH secretion in spite of increasing cAMP levels in static cell cultures in rats. PACAP 38 was reported, however, to stimulate the release of LH from cultured rat anterior pituitary cells [8]. In the present study, a dose-dependent increase of LH secretion was observed in response to PACAP 27 and PACAP 38 in cultured bovine pituitary cells. The present results were in agreement with those

of Culler and Paschall in rats [8].

On the other hand, the *in vivo* effect of PACAP 38 was also observed on LH secretion, and the increased LH levels by intraatrial injection of the peptide were much higher than that by intracerebroventricular injection [9]. Therefore, it was suggested that the LH releasing effect of PACAP 38 would be more potent via the anterior pituitary than via the hypothalamus [9]. The results of the present study, in which the PACAP 27 and PACAP 38 induced the release of LH from cultured bovine anterior pituitary cells, agreed with those of Osuga *et al.* [9] in rats.

Miyata *et al.* [2] reported that the content of PACAP 27 in ovine hypothalamic tissues appeared to be about one tenth of that of PACAP 38. Furthermore, Arimura *et al.* [29] reported that the hypothalamus contains the highest concentration of immunoreactive PACAP 38 and PACAP 27 among other tissues in the rat, and that the amount of PACAP 38 is nearly 15 times greater than PACAP 27. In the present study, the minimal effective concentration of PACAP 27 on LH release from cultured bovine anterior pituitary cells was 100 times lower than that of PACAP 38.

Similar distribution of immunoreactive PACAP neurons and fibers was found in the hypothalamus of sheep [3], monkey, and human [30]. Immunoreactive PACAP cell bodies were present in the supraoptic nuclei and the paraventricular nuclei. Immunoreactive nerve fiber networks were also observed throughout the hypothalamus, including both external and internal zones of the tuber cinereum, close to the transition of the pituitary stalk (median eminence) [3, 30].

In addition, the fibers run in parallel within the paraventriculo-infundibular tract toward the posterior pituitary, and a dense network of PACAP fibers was observed in both the internal and external zones of the tuber cinereum in the brain of human and monkey [30]. PACAP 38 content in the posterior pituitary was comparable to that of extrahypothalamic areas in the brain, while the anterior pituitary contained only a small amount of the peptide [29]. Specific high affinity binding sites for PACAP 27 and 38 were observed in rat pituitary cell membrane [4]. PACAP may be released either in the long hypophysial portal vessels or the short portal vessels from the posterior pituitary acting on the adenohypophysis [31].

In conclusion, our results suggest that PACAP 27 and PACAP 38 stimulate LH release in cattle via a direct action on anterior pituitary cells.

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