Effects of Cholecystokinin on Luteinizing Hormone Release from Perfused Hypothalamus-Pituitary in the Castrated Bulls

Shigeto KANEMATSU, Narichika SATO, and Tsutomu HASHIZUME

*Department of Animal Husbandry, Faculty of Agriculture,
Iwate University, Ueda 3, Morioka-shi 020, Japan*

(Accepted for publication December 14, 1989)

Summary. The effect of cholecystokinin (CCK)-8 was studied on the release of luteinizing hormone (LH) from perfused medial basal hypothalamus (MBH) and/or adenohypophyses of castrated bulls in a sequential double chamber perfusion system. LH concentrations in the perfusion efflux from the pituitary in series with the hypothalamus decreased significantly ($P<0.05$) after perfusion of 10^{-8} M CCK as compared with control levels. In the similar perfusion LH concentrations in the efflux from the pituitary alone decreased significantly ($P<0.01$) after perfusion of 10^{-8} M CCK. 10^{-7} M CCK induced neither inhibitory nor stimulatory effects in perfusions of the pituitary in series with the hypothalamus except one fraction. Similarly, 10^{-7} M CCK failed to induce a significant change in LH release in perfusion of the pituitary alone. LH concentrations in the perfusion efflux from pituitary in series with the hypothalamus increased significantly at 50 and 60 min after the initiation of 10^{-6} M CCK perfusion as compared with the control level ($P<0.05$). LH concentrations in the efflux from the pituitary alone increased significantly 40, 50, 60 and 80 min after the initiation of perfusion with 10^{-6} M CCK.

The results indicate that CCK acts directly on the adenohypophysis to modulate release of LH in cattle.

KEY WORDS: CHOLECYSTOKININ, LH, HYPOTHALAMUS, PITUITARY, CATTLE.

Jpn J Anim Reprod 36 54–59, 1990

Immunoreactive cholecystokinin (CCK) and its COOH-terminal octapeptide (CCK-8) have been found in the brains of many animal species (Dockeray, 1976; Weiner *et al.*, 1988) in amounts comparable to those found in the gastrointestinal tract, the traditional site of production for this peptide. Intraventricular injections of CCK induced a suppression of luteinizing hormone (LH) release in ovariectomized rats, whereas CCK failed to induce any LH changes *in vitro* from incubated male rat pituitaries (Vijayan *et al.*, 1979). These results indicate that CCK inhibits release of LH by acting through the hypothalamus on the adenohypophysis. In contrary to these results, implantation of powdered CCK-8 into the medial preoptic area (MPO) enhanced the circadian rise in serum LH in the ovariectomized estrogen-primed rat (Kimura *et al.*, 1983; Hashimoto and Kimura, 1986).

It is of interest to investigate those intricate results as

to whether the CCK stimulates or inhibits release of LH, or whether the CCK stimulates or inhibits indirectly through the hypothalamus or directly on the adenohypophysis using superfused castrated bull hypothalamo-pituitaries. Further, effects of CCK on LH secretion have never been studied in domestic animals. It is also of interest to see the effects of CCK on LH release in ruminant animals.

Materials and Methods

Superfusion methods

The medial basal hypothalamus (MBH) and adenohypophysis were removed from castrated bulls at a local slaughter house within 1 hr of death, and brought to the laboratory in ice cold Krebs-Ringer bicarbonate buffer within 1 hr.

The adenohypophysis was cut sagittally into halves before immersion into the buffer. For the perfusion

medium 10 mM glucose and 100 mg/l streptomycin sulfate (Meiji Seika Inc., Tokyo) were added to Krebs-Ringer bicarbonate buffer (118.5 mM NaCl, 4.74 mM KCl, 2.54 mM CaCl_2 , 1.18 mM MgSO_4 , 1.18 mM KH_2PO_4 , 24.9 mM NaHCO_3). Bacitracin (Nacarai Tesque Inc., Kyoto, Japan) was added to the medium (modified KRB solution) to prevent peptide degradation (0.11 mM). At the end of the perfusion the tissues were tested for viability by perfusing a medium enriched in K^+ (123.2 mM KCl, 2.54 mM CaCl_2 , 1.18 mM MgSO_4 , 1.18 mM KH_2PO_4 and 24.9 mM NaHCO_3). The perfusion system consisted of a serial double chamber superfusion system (modified from Miyake and Yen, 1981; Zolman and Convey, 1972): the first chamber was made from a 3 ml polyethylene disposable syringe; the second chamber was made from a 1 ml syringe. In this system, the effluent from the first chamber perfused the second chamber by means of a peristaltic pump (Minipuls-2, GILSON) at a flow rate of 18 ml/hr. Both the medium delivered and that contained in the two chambers were saturated with a mixture of O_2/CO_2 gas (95/5 %). The chambers were maintained at 38 °C in a water bath. The medial basal hypothalamic tissue prepared for perfusion was limited dorsally 3 mm deep, frontally by the caudal border of the optic chiasm, laterally by 1 mm lateral to the tuber cinereum, and caudally by the rostral border of the

mamillary body. The adenohipophysial tissues used for perfusion were five pieces of 1.5 mm cubes from a central part (mainly from the zona tuberalis) of the adenohipophysis. The mean wet weights and standard errors of the MBH and pituitary tissues after perfusion in 10 experiments were 712.9 ± 44.3 mg and 44.9 ± 4.6 mg, respectively. The MBH was placed in the first chamber, and the pituitary tissues were in the second chamber after repeated rinse of the tissues. The effluent from the second chamber was collected in 3 ml fractions at 10 min intervals. The flow rate used was 18 ml/hr. The MBH was placed in the first 1.7 ml chamber, and the five pieces of pituitary tissues in the second 0.5 ml chamber. In other studies, the first chamber was kept empty, and the pituitary tissues were placed alone in the corresponding chamber of the series. After tissues were placed in the appropriate chambers, the system was equilibrated for 3 hr before initiating to collect samples. Six samples of 3 ml each were collected over a period of 1 hr as controls, and then 10^{-8}M , 10^{-7}M or 10^{-6}M CCK-8 (Peptide Institute Inc. Osaka, Japan) in the modified KRB solution were perfused for 100 min. After the perfusion of CCK-8, the tissues were perfused for 100 min with the modified KRB solution, which was followed by a K^+ solution for 30 min. Collected fractions were stored at -20 °C until assayed.

Fig. 1. Dose-response curves for purified pituitary preparations and serum with use of ovine LH antiserum.

Radioimmunoassays and antiserum to LH

LH in these fractions were measured by a double antibody radioimmunoassay (Scaramuzzi *et al.*, 1972). Polyethylene glycol 6,000 (mol. wt., 7,500) was added to the anti-guinea pig gamma globulin serum to insure a complete precipitation with the dilution rate of 1 % polyethylene glycol at the final incubation.

For a generation of an antiserum to LH, four guinea pigs were immunized with sc injections of 0.91 mg of NIH-LH-S18 emulsified in 2 ml of a 1/1 mixture of Freund's complete adjuvant and saline. This procedure was repeated several times in addition to a booster iv injection. On the day 135 the serum obtained from a female contained the best antibody as determined by testing of titers, specificity and affinity of serum samples.

Dose-response curves demonstrating the decrease in amount of antibody-bound ovine LH-¹²⁵I which occurred with increasing levels of pituitary hormones and bovine and rabbit plasma are shown in Fig. 1. The region where the quantity of ovine LH-¹²⁵I bound was equal to 20-85 % of that bound in PBS-BSA control tubes was used for estimating relative potencies. In this area the curves for all preparations tested were parallel each other in slope. Percentages of cross reaction from bovine LH (NIH-LH-B8) with the rabbit LH (Papkoff, Ex-130-GB), porcine TSH (LER-H-p TSH), crude rabbit pituitary fraction (Parlow, WP-360-A), porcine FSH (LER-441-2) and ovine PRL (NIH-PS 11) were 33.8, 2.9, 1.2, 0.5 and 0.2 % respectively. Percentages of cross reaction from the rabbit LH (Papkoff, EX-130-GB) with the porcine TSH (LER-H-p TSH), porcine FSH (LER-441-2) and ovine PRL (NIH-PS 11) were 8.7, 1.5, and 0.4 % respectively. NIH-LH-S18 contained 0.033 USP units TSH/mg and less than 0.050 NIH-FSH-S1 units/mg.

The reference standard used was NIH-LH-B8, which contained less than 0.050 NIH-FSH-S1 units/mg and 0.19 USP units TSH/mg. The mean potency of NIH-LH-B8 was 1.03 NIH-LH-S1 units/mg. The minimum detectable LH concentration in 17 RIAs was 0.200 ± 0.33 ng/tube. The within-and between-assay coefficient of variation in the present assays were 4.1 % and 12.5 % respectively.

Mean and standard error of LH peak values during LH surge in 36 Japanese Black cows was 51.8 ± 2.9 ng/ml as blood samples were collected every 2 hr during estrus, while LH values during early proestrus were 2.65 ± 0.16 ng/ml in 36 cows. The mean LH concentra-

Fig. 2. LH changes in the effluents from the pituitary in series with the hypothalamus (solid line) or pituitary alone (broken line) with the medium containing 10^{-8} M CCK for 100 min. The mean LH concentration of 6 fractions collected during 1 hr before CCK perfusion was taken as percentage changes from the control value in each group. Note that the 10^{-8} M CCK perfusion decreased LH concentrations. Each points represent mean and SEM. Asterisks represent a significant change from the control level (*, $P<0.05$; **, $P<0.01$). Solid stars: hypothalamus-pituitary perfusion group; open stars: pituitary perfusion alone.

tions of the resting level in the perfusion efflux of all experiments was 25.0 ± 2.6 ng/ml, while the treatments with a high K^+ increased the LH concentrations to 180.0 ± 2.5 ng/ml.

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical analyses were performed with the Student's t-test. The parallelism of slopes between dose-response curves of the standard and other hormones were calculated based on formulas of Bliss(1952). Cross-reactions were expressed in terms of the mass of unlabelled compounds which were required to displace 50 % of the labelled antigen.

Percent of cross reaction = $x/y \times 100$, where x=mass of antigen at 50 % displacement, y=mass of cross-reacting compound at 50 % displacement.

Results

As shown in the Fig. 2, LH concentrations in the perfusion efflux from the pituitary in series with the hypothalamus declined progressively and the decreased levels become significant ($P<0.05$) at 90 and 100 min after the initiation of perfusion of 10^{-8} M CCK as

Fig. 3. LH changes in the effluents from the pituitary in series with the hypothalamus (solid line) or pituitary alone (broken line) with the medium containing 10^{-7} M CCK for 100 min. See also Fig. 2 for explanations of the figure.

compared with control levels. This significant reduction in LH concentrations induced by CCK perfusions were continuously effective even after cessation of CCK perfusions for 50 min (after 10 and 20 min: $P < 0.05$; after 30, 40, and 50 min: $P < 0.01$). In the similar perfusion LH concentrations in the efflux from the pituitary alone have shown a progressive decline and the decrease become significant after 70 and 90 min ($P < 0.01$) and after 100 min ($P < 0.05$). This reduction in LH concentration induced by CCK was significant ($P < 0.05$) 50 min after the cessation of CCK perfusion. After high K^+ treatments, the LH levels in the efflux increased sevenfold from the resting levels.

10^{-7} M CCK induced neither inhibitory nor stimulatory effects on LH concentrations in the perfusion efflux from the pituitary in series with the hypothalamus except one fraction at 50 min after the initiation of perfusion with 10^{-7} M CCK (Fig. 3). Initial LH rise after CCK perfusion, however, was observed in 3 out of 5 experiments in the pituitary perfusion in series with the hypothalamus. 10^{-7} M CCK induced LH rise only 2 out of 5 experiments, when the pituitary was perfused alone.

As shown in Fig. 4, LH concentrations in the perfusion efflux from the pituitary in series with the hypothalamus increased significantly 50 and 60 min after the initiation of 10^{-6} M CCK as compared with the control level ($P < 0.05$). LH concentrations in the efflux from the pituitary alone increased significantly ($P < 0.05$) 40, 50, 60 and 80 min after the initiation of

Fig. 4. LH changes in the effluents from the pituitary in series with the hypothalamus (solid line) or pituitary alone (broken line) with the medium containing 10^{-6} M CCK for 100 min. Note that the 10^{-6} M CCK elevated LH concentration significantly in the effluents of the two groups. See also Fig. 2 for explanations of the figure.

perfusion with 10^{-6} M CCK.

Discussion

To our knowledge, the present study in the first demonstration of the effects of CCK on LH release in cattle. In the present study CCK inhibited release of LH from bovine pituitaries at a dose level of 10^{-8} M, regardless of whether the pituitary tissues were perfused with or without the hypothalamus. The inhibitory effect of CCK on LH release has been shown using an intraventricular injection method in the rat (Vijayan *et al.*, 1979). They failed to show a significant effect of CCK on LH levels in cultured pituitary cells. From these results they concluded that the CCK inhibited pituitary LH release acting via the hypothalamus. Frequent samplings in the superfusion system of the present study were able to demonstrate an inhibitory effect of CCK on pituitary LH release acting directly on the adenohypophysis. This direct inhibition by 10^{-8} M CCK of the pituitary from releasing LH could be interpreted as a physiologic effect.

On the contrary to an inhibitory effect of 10^{-8} M CCK on pituitary LH release, 10^{-6} M CCK stimulated LH release. Kimura *et al.* (1983) reported that implantation of powdered CCK into the medial preoptic area induced the elevation of LH values in ovariectomized estrogen-primed rats. This increase in LH was blocked by a dopamine receptor blocker, pimozide (Hashimoto

and Kimura 1986). These results were at variance with those reported by Vijayan *et al.* (1979) who found the inhibitory effect of CCK on the pituitary LH release in an indirect effect through the hypothalamus. The present results in 10^{-6} M CCK perfusion of the pituitary with the MBH tissues were in agreement with the earlier observations reported by Kimura *et al.* (1983). They implanted a powdered CCK directly in the brain tissue. Therefore, an extremely high concentration of CCK might have affected tissues around the site of implantation on the MPO. It is not known so far the implanted CCK had diffused or picked up to the pituitary portal system. Yet water soluble CCK might have reached to a considerable distance, although they could claim that the implanted CCK in the MPO enhanced LH release *in vivo* on the brain tissue. In the present study the increased release of LH induced by 10^{-6} M CCK could be interpreted as a pharmacologic effect; and this stimulative effect might not be unlike that of Kimura *et al.* (1983). The stimulating effects of 10^{-6} M CCK on LH release, however, were effective regardless of whether the adenohypophysial tissues were perfused with or without the MBH tissues. While CCK was found to have a biphasic effect on LH release depending on its concentrations, perfusions of the pituitary-hypothalamus or pituitary alone with an intermediate dose level of CCK (10^{-7} M) induced neither inhibitory nor stimulatory effects on LH release except one fraction. The reason remains undetermined why 10^{-7} M CCK failed to show a significant effect on pituitary LH release. It has been reported that the major hypothalamic source of CCK is from neurons of the paraventricular nucleus (Beinfeld *et al.*, 1981). Brain CCK receptors show high densities in the ventromedial hypothalamic nucleus, supraoptic and paraventricular nuclei, and also in the pituitary (Day *et al.*, 1986). The inhibition of LH release after intraventricular injection of CCK (Vijayan *et al.*, 1979) and the stimulatory effect of hypothalamic implanted CCK on LH release (Kimura *et al.*, 1983) in rats were consistent with the present results, which showed an inhibitory effect of 10^{-8} M CCK on pituitary LH release and a stimulatory effect of 10^{-6} M CCK on LH release in perfused adenohypophysial tissues *in vitro* in cattle.

Acknowledgments

We are indebted to Dr. Leo Reichert Jr., Emory University (now in the Albany Medical College) and

NIAMD for their kind supply of purified pituitary hormone preparations and to Dr. H. Papkoff, University of California, San Francisco, Dr. A. V. Parlow, Harbor General Hospital, for rabbit pituitary preparations. We wish to thank Dr. K. Wakabayashi, Institute of Endocrinology, Gunma University for his kind supply of anti-guinea pig gamma globulin serum. The present work was supported by a Grant-in-Aid for Scientific Research No. 62480076 from the Ministry of Education, Science and Culture.

References

- Beinfeld MC, Meyer DK, Eskay RL, Jensen RT, Brownstein MJ (1981) The distribution of cholecystokinin immunoreactivity in the central nervous system of the rat as determined by radioimmunoassay. *Brain Res* 213: 51-57.
- Bliss CI (1952) *The Statistics of Bioassay*. Academic Press, New York.
- Day NC, Hall MD, Clark CR, Hughes J (1986) High concentrations of cholecystokinin receptor binding sites in the ventromedial hypothalamus. *Neuropeptides* 8: 1-18.
- Dockeray GJ (1976) Immunochemical evidence of cholecystokinin-like peptides in brain. *Nature* 264: 568-570.
- Greenwood FC, Hunter WM, Glover JS (1963) The preparation of 131 I labelled human growth hormone of high specific radioactivity. *Biochem J* 89: 114-123.
- Hashimoto R, Kimura F (1986) Inhibition of gonadotropin secretion induced by cholecystokinin implants in the medial preoptic area by the dopamine receptor blocker, pimozone, in the rat. *Neuroendocrinology* 42: 32-37.
- Kimura F, Hashimoto R, Kawakami M (1983) The stimulatory effect of cholecystokinin implanted in the medial preoptic area on luteinizing hormone secretion in the ovariectomized estrogen primed rat. *Endocrinol Japon* 30: 305-309.
- Miyake A, Yen SSC (1981) Direct *in vitro* stimulation of pituitary LH release by alpha melanocyte stimulating hormone. *Life Sci* 29: 2637-2640.
- Scaramuzzi RJ, Blake CA, Papkoff H, Hilliard J, Sawyer CH (1972) Radioimmunoassay of rabbit luteinizing hormone: serum levels during various reproductive states. *Endocrinology* 90: 1285-1291.
- Vijayan E, Samson WK, McCann SM (1979) *In vivo* and *in vitro* effects of cholecystokinin on gonadotropin, prolactin, growth hormone and thyrotropin release in the rat. *Brain Res* 172: 295-302.
- Weiner RI, Findell PR, Kordon C (1988) Role of classic and peptide neuromediators in the neuroendocrine regulation of LH and prolactin. In: *The Physiology of Reproduction Vol. 1* (Knobil E, Neill JD eds.), Raven Press, New York.

Zolman J, Convey EM (1972) Bovine pituitary LH and prolactin release during superfusion *in vitro*. *Pro Soc Exp Biol Med* 140: 194-198.

去勢牛の視床下部一下垂体の *in vitro* 灌流系における 黄体形成ホルモン放出に及ぼすコレシストキニンの効果

兼松 重任・佐藤 尚親・橋爪 力
岩手大学農学部畜産学科家畜繁殖学講座

コレシストキニン (CCK) が家畜の黄体形成ホルモン (LH) の放出に与える影響を検討した。屠場でえた去勢牛の視床下部一下垂体組織を *in vitro* の灌流系に置き、CCK を灌流し、灌流液中の LH をラジオイムノアッセイで測定した。灌流装置には二室があり、第一室には視床下部の内側底部組織を入れ、そこを流れた液が下垂体組織をいれた第二室に流れるようにした (連続)。そのほか下垂体のみの灌流も同時に行った (単独)。灌

流液は修正クレブス液を用い38℃に保った。 10^{-8}M の CCK は連続・単独灌流とも有意に灌流液中の LH 濃度を低下させた ($P < 0.05$ ・ $P < 0.01$)。これに対して、 10^{-6}M の CCK は連続・単独灌流とも逆に有意に LH 濃度を増加させた ($P < 0.05$)。 10^{-7}M の CCK では一定した変化は得られなかった。以上のことから、CCK は牛の腺下垂体に直接作用して LH の放出を調整することが示唆された。