Direct effects of bromocriptine on the steroidogenic capability of porcine granulosa cells

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Bromocriptine (12-Br-α-ergocryptine mesylate), a dopamine agonist, is a specific inhibitor of prolactin release from the anterior pituitary. While bromocriptine has proven effective in treating hyperprolactinemic anovulation, some normoprolactinemic patients have also responded to bromocriptine. Therefore, bromocriptine has been widely used clinically not only in hyperprolactinemic cases, but also in cases of amenorrhea (1) and luteal insufficiency (2) in normoprolactinemic states. Its mechanism of action is considered to involve the restoration of ovarian function by a decrease in serum prolactin levels (3, 4). It has also been reported that the effectiveness of bromocriptine in normoprolactinemic anovulatory patients may be due to the stimulation of secretion of gonadotropin-releasing hormone (GnRH) or gonadotropins (1). However, the direct action of bromocriptine on the ovary has not generally been studied.

Recent research suggests that ovarian granulosa cells, localized in vivo in an avascular area defined by the basement membrane, may be under the control of autocrine, paracrine and endocrine systems, and that some unknown substances may be involved in the regulation of ovarian function. To elucidate whether bromocriptine acts directly on the ovary, the present study was designed to investigate the effect of bromocriptine on steroidogenesis in cultured porcine granulosa cells.

Materials and methods

Drugs and reagents

Bromocriptine was supplied by Sandoz Pharmaceutical Co. (Basel, Switzerland). Dulbecco’s modified Eagle’s medium (DMEM) was obtained from the Nissui Pharmaceutical Co. (Tokyo, Japan). Dulbecco’s phosphate-buffered saline (D-PBS), Hanks’ balanced salt solution and fetal calf serum (FCS) from the Grand Island Biological Co. (Grand Island, NY), and tissue culture multiwells and flasks from Falcon Plastics Oxnard, CA. Fura-2 acetoxymethylester was purchased from Dojin Chemicals (Kumamoto, Japan) and PBS-containing crystallized trypsin from the Mochida Pharmaceutical Co. (Tokyo, Japan). All other reagents used in the present study were obtained from Sigma (St Louis, MO).

Culture of porcine granulosa cells

Ovaries were obtained from four to six-month-old pigs from a local slaughterhouse. Granulosa cells were isolated from small (<3 mm), medium (3–5 mm) and large (>5 mm) follicles by the needle aspiration method. Granulosa cells harvested from small-medium and large follicles will be abbreviated as S–M and L cells, respectively. The cells were rinsed twice in D-PBS, and then suspended in DMEM supplemented with 10% FCS and penicillin-G 100 mg/l. Unless otherwise stated, DMEM supplemented with these components will hereafter be referred to as “medium”. The number of viable granulosa cells was counted by the dye exclusion method using 0.4% trypan blue. To examine the secretion of progesterone and estradiol the cells were plated at a relatively high density (4–5 × 10^5 viable cells/well) in each well of 24-well plates. The cultures were incubated in a humidified 95% air–5% CO_2 incubator at 37°C, followed by culture for three days. The medium was
changed and bromocriptine was added on the third day after inoculation.

For the measurement of cell growth, the cells were inoculated at a low density (1 x 10^5 viable cells/well). The cultures were incubated as described above. After incubation for 48 h with bromocriptine, the medium was discarded and the cells were washed twice in D-PBS, followed by incubation for 20 min at 37°C with PBS-containing crystallized trypsin 200 NF unit/ml. The number of cell nuclei present in the dishes was then determined by the critical acid-crystal violet method (5).

**Determination of progesterone and estradiol**
The spent culture media were stored at -20°C until assayed. Their progesterone content was determined by RIA using a commercial kit (Daichii Radioisotope Laboratories, Tokyo, Japan). The within- and between-assay coefficients of variation were 5.8% and 6.6%, respectively. Estradiol content was also determined by RIA using a commercial kit (Diagnostic Products Corporation, L.A.). The within- and between-assay coefficients of variation did not exceed 10%. All samples in the same experiment were assayed at the same time.

**Determination of extracellular cAMP**
To measure the extracellular accumulation cAMP, porcine granulosa cells were cultured for 4 h in a medium containing methyl isobutyl xanthine 0.5 mmol/l, a phosphodiesterase inhibitor. The spent culture media were stored at -20°C until assayed by RIA. The samples were then reconstituted with distilled water and succinylated, after which they were assayed for cAMP using a commercial RIA kit (Yamasa, Chiba, Japan). The within- and between-assay coefficients of variation were 6.0% and 10.0%, respectively.

**Measurement of cytosolic free Ca^{2+} concentration ([Ca^{2+}],)***
To examine [Ca^2+], 3 x 10^5 viable cells were inoculated in flasks. After incubation in medium for five days, porcine granulosa cells were trypsinized and incubated with 3 μmol/l fura-2 acetomethyl ester at 37°C for 45 min in Hanks’ balanced salt solution. The cells were then washed and suspended in a sodium solution (130 mmol/l NaCl 1.5 mmol/l CaCl_2, 5 mmol/l KCl, 1 mmol/l MgCl_2, 20 mmol/l HEPES) supplemented with 10 mmol/l glucose and 0.5 g/l BSA (1.6-2.0 x 10^5 cells/ml). Ca^{2+}-fura-2 fluorescence was measured at 37°C in Hitachi F-2000 spectrofluorimeter (excitation: 340 nm and 380 nm, emission 505 nm) equipped with a thermostated cuvette holder and stirring apparatus. [Ca^{2+}] values were calculated according to the method of Grynkiewicz et al. (6).

**Statistics**
Data are expressed as the mean ± standard error (mean±SEM). Student’s t-test or Dunnett’s multiple comparison test was performed to determine the significance of the difference, with a level of p<0.05 accepted as statistically significant.

**Results**
Fig. 1 shows the effect of bromocriptine on the basal secretion of progesterone by porcine granulosa cells during 48 h of incubation. Porcine granulosa cells were cultured for 48 h in the presence of increasing concentrations of bromocriptine (10^{-4} to 10^{-5} mol/l). Bromocriptine significantly increased the basal secretion of progesterone by S-M granulosa cells at concentrations exceeding 10^{-7} mol/l. The stimulatory effect of bromocriptine was dose-dependent. A significant stimulatory effect of bromocriptine on the basal secretion of progesterone by L granulosa cells was also observed at concentrations exceeding 10^{-6} mol/l. As shown in Fig. 2, bromocriptine dose-dependently augmented the FSH-stimulated secretion of progesterone by S-M granulosa cells for 48 h of incubation at concentrations exceeding...

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*Fig. 1. Concentration-related stimulatory effect of bromocriptine on basal secretion of progesterone by porcine granulosa cells for 48 h of incubation. The amount of progesterone secreted by S-M (•—•) and L (O—O) granulosa cells was assayed by RIA. Values are the mean ± SEM of four experiments, each performed in quadruplicate. The secretion of progesterone in control cultures (without bromocriptine) by the S-M granulosa cells was 144.9 ± 7.0 nmol/l and by L granulosa cells 53.3 ± 1.6 nmol/l. ***p<0.001; **p<0.01; *p<0.05 vs controls.*
The secreted amount of progesterone by S-M granulosa cells was time-dependent during the basal secretion of progesterone by porcine granulosa cells (S-M and L) during 144 h of incubation. The basal secretion of progesterone by S-M granulosa cells was significantly increased by the addition of bromocriptine in both a time-dependent and concentration-dependent manner.

Fig. 4 shows the effect of bromocriptine on the basal secretion of estradiol by porcine granulosa cells for 48 h. Porcine granulosa cells were cultured for 48 h in the presence of increasing concentrations of bromocriptine (10^{-9} to 10^{-5} mol/l) and Δ^4-androstenedione 10^{-6} mol/l. The basal secretion of estradiol by S-M granulosa cells was significantly decreased at concentrations exceeding 10^{-7} mol/l (Fig. 4). These inhibitory effects of bromocriptine were dose-dependent. Bromocriptine also decreased the basal secretion of estradiol by L granulosa cells at concentrations exceeding 10^{-6} mol/l. As shown in Fig. 5, a similar inhibitory effect of bromocriptine onestradiol secretion by porcine granulosa cells for 48 h.

In addition, bromocriptine led to a dose-dependent increase in the FSH-stimulated secretion of progesterone by L granulosa cells at concentrations exceeding 10^{-6} mol/l. At a dose 10^{-5} mol/l bromocriptine increased the basal secretion of progesterone by S-M granulosa cells by 1.7-fold, and that of the FSH-stimulated cells by 3.9-fold. A similar concentration-related stimulatory effect of bromocriptine on the time course of the basal secretion of progesterone by porcine granulosa cells (S-M and L) was also shown in Fig. 4. Estradiol secretion by porcine granulosa cells was 2132.0 ± 136.5 pmol/l and by the L granulosa cells 916.1 ± 17.3 pmol/l. These inhibitory effects of bromocriptine were dose-dependent. Bromocriptine also decreased the basal secretion of estradiol by L granulosa cells at concentrations exceeding 10^{-6} mol/l. As shown in Fig. 5, a similar inhibitory effect of bromocriptine onestradiol secretion by porcine granulosa cells for 48 h.
the FSH-stimulated secretion of estradiol was observed in the S-M and L granulosa cells.

We determined whether bromocriptine would stimulate the proliferation of porcine granulosa cells. In contrast to the significant effect of bromocriptine on steroidogenesis in porcine granulosa cells, there was no growth-promoting effect of bromocriptine by S-M and L granulosa cells (Fig. 6). Porcine granulosa cells cultured at 1 × 10^5 viable cells exhibited little increase in cell number throughout the culture period.

To examine the possible stimulation of cAMP by bromocriptine, porcine granulosa cells were cultured for 4 h in the presence of increasing concentrations of bromocriptine (10^{-8} to 10^{-6} mol/l) and methyl isobutyl xanthine. Bromocriptine increased the basal and FSH-stimulated accumulation of cAMP by porcine granulosa cells (S-M and L) in a dose-dependent manner (Fig. 7).

The bromocriptine-induced mobilization of Ca^{2+} in porcine granulosa cells was assessed by measuring the change in [Ca^{2+}], employing fura-2 acetoxyethyl ester as a Ca^{2+} indicator. Bromocriptine 10^{-6} mol/l did not induce any change in [Ca^{2+}], of S-M and L granulosa cells (Fig. 8).

**Discussion**

Various changes in steroidogenesis have been observed in studies involving the administration of bromocriptine. For instance, in the rat ovary, 20α-hydroxysteroid dehydrogenase activity increased after administration of a single dose of bromocriptine by injection on day 5, but not when it was given on day 16 of pregnancy. This effect was counteracted by injecting prolactin (3). These findings were considered due to a reduction of circulating prolactin by bromocriptine rather than to its direct effect. In contrast, another in vitro study demonstrated that dopamine stimulates the formation of cAMP in parathyroid gland cells and stimulates the release of parathyroid hormone, suggesting a direct effect of dopamine on the parathyroid gland (7).

There are numerous reports on the direct inhibitory effects of prolactin on the ovary. Veldhuis et al. (8, 9) and Wang et al. (10) have shown that high levels of prolactin can inhibit the FSH induction of aromatase activity, thus reducing estradiol in porcine and rat granulosa cells. In other studies (11, 12), high levels of prolactin were also shown to inhibit the secretion of progesterone by human granulosa cells in vitro. However, the role of prolactin on the ovarian activity of patients with normoprolactinemic states has received little attention. McGarrigle et al.
Fig. 7. Concentration-related stimulatory effect of bromocriptine on cAMP accumulation in conditioned media for 4 h of incubation in the presence of methyl isobutyl xanthine. The content of cAMP in the media in the absence or presence of FSH 50 mIU were assayed by RIA. Values are the mean ± SEM of three experiments, each performed in quadruplicate. The basal accumulation of cAMP in control cultures (without bromocriptine) was 0.59 ± 0.09 nmol/l and that of FSH-stimulated cAMP 5.20 ± 1.1 nmol/l. **p < 0.01; * p < 0.05 vs controls.

(13) demonstrated that any prolactin-related block of gonadotropin stimulation of follicular growth could be overcome if sufficient gonadotropin were given. However, not just a decrease in prolactin levels is essential to ovarian function.

Our findings demonstrate that bromocriptine exerts a direct action on the steroidogenesis by porcine granulosa cells. A significant effect of bromocriptine was observed in both S-M and L granulosa cells. In addition, this stimulatory effect of bromocriptine was demonstrated in basal steroidogenesis by the porcine granulosa cells as well as the FSH-stimulated cells. These direct effects of bromocriptine in vitro are consistent with clinical observations of its effectiveness in the normoprolactinemic state. It has been reported that the well-known ovulation-inducing drugs, such as clomiphene citrate (14) and GnRH analog (15), directly affect the steroidogenesis by the granulosa cells. The bromocriptine-related changes in the present study seem to occur in two different ways: by stimulating the secretion of progesterone, and by inhibiting the secretion of estradiol. It may be speculated that bromocriptine inhibits aromatase activity. In our experiments, significant effects of bromocriptine on steroidogenesis without the increase of cell growth were demonstrated at concentrations of 10^{-9} mol/l through 10^{-5} mol/l. In patients who were administered a single oral dose of bromocriptine 2.5 mg, the serum concentration of bromocriptine reached 10^{-8} mol/l (16). Therefore, the doses of BRC used in this in vitro study are not necessarily pharmacological.

Fig. 8. Ca^{2+}-Fura-2 fluorescence by bromocriptine in S-M granulosa cells. [Ca^{2+}] was measured with a fluorescent indicator, Fura-2, as described in Materials and Methods. Bromocriptine 10^{-5} mol/l was applied at the open arrow, followed by the application of Triton X 0.05% and EGTA 3 mmol/l. [Ca^{2+}] was observed for 5 min.
The ovarian cAMP of the signal-transducing pathway has been extensively studied with regard to granulosa cell and follicular maturation (17, 18). In contrast, the role of the recently described and ubiquitous calcium-sensitive, phospholipid-dependent protein kinase (protein kinase C) effector pathway in ovarian physiology is less well understood. However, a recent report by Wheeler & Veldhuis (19) indicates that the protein kinase C effector pathway is effectively coupled in an inhibitory fashion to both the receptor-mediated and non-receptor-mediated activation of adenylate cyclase. In the present study, we investigated the effects of bromocriptine on cAMP accumulation in media and the cytosolic free Ca$^{2+}$ concentrations in porcine granulosa cells. Our results demonstrate that bromocriptine increased the basal and FSH-stimulated accumulation of cAMP by porcine granulosa cells in a concentration-dependent manner. This evidence suggests that cAMP may be a second messenger for the effect of bromocriptine on steroidogenesis. On the other hand, the lack of stimulation of [Ca$^{2+}$] by bromocriptine in porcine granulosa cells indicates that this agent does not act via the calcium-sensitive and phospholipid-dependent protein kinase C pathway.

One of the most compelling arguments for an important physiological role of dopamine in the periphery is the identification of specific dopamine receptors in a variety of tissues outside the central nervous system. Dopamine receptors have been identified in the renal, mesenteric, coronary and cerebral vascular trees, in the kidney and the exocrine pancreas as well as in the brain and pituitary (20. 21). However, dopamine receptors in the ovary have yet to be elucidated. Nevertheless, the present study demonstrates the significant effects of bromocriptine on steroidogenesis by porcine granulosa cells, suggesting that dopamine may participate in the intracellular regulation of various critical features of granulosa cell differentiation. Although an earlier study (22) supported a possible regulatory role of adrenergic agents in the production of progestrone by the granulosa and luteal cells, the physiological relevance of this finding remained to be clarified. More recent reports suggest that epidermal growth factor (23), insulin-like growth factor-I (24) and interleukin-1 (25) may be involved in the autocrine and/or paracrine regulation of progesterone production in granulosa cells. Thus, it is possible that other novel substances may play a role in the regulation of ovarian function. Our findings indicate that bromocriptine has a direct effect on ovarian granulosa cells and dopamine may be one of the autocrine and/or paracrine regulators.

References


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