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Expression of Bradykinin B_2 Receptor in the Mouse Ovary

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ABSTRACT—The amounts of [1-5]-bradykinin in ovary extracts were determined using gonadotropin-treated immature female mice. The bradykinin levels in the ovary were high at 2, 6, and 48 hr after injection of human chorionic gonadotropin (hCG) into pregnant mare's serum gonadotropin (PMSG)-treated mice. Northern blot analysis of total RNAs isolated from the PMSG/hCG-treated mouse ovaries indicated that the B_2 receptor mRNA was constitutively expressed. Bradykinin B_2 receptor protein was detected by Western blot analysis of the ovary extracts. *In situ* hybridization analysis revealed that the B_2 receptor mRNA is expressed in the granulosa cells of all growing follicles of ovaries from both gonadotropin-treated immature and mature female mice. The effect of bradykinin on the expression of the B_2 receptor gene was examined by RT-PCR analysis with the ovary previously cultured in the presence of bradykinin. Bradykinin treatment of immature female, gonadotropin-treated immature female, and mature female mouse ovaries brought about no apparent changes in the B_2 receptor mRNA level. The present data indicate that the level of B_2 receptor expression in the ovary is fairly constant, and that the biological effect elicited by bradykinin in this organ may be dependent upon concentrations of the ligand produced by operation of the kinin-kallikrein system.

Key words: mouse, ovary, bradykinin B_2 receptor, expression

INTRODUCTION

Bradykinin is a nonapeptide produced by the operation of the kinin-kallikrein system, which consists of kallikreins, kinins, kininogens, kallikrein inhibitors, kininases, and kinin receptors (Bhoola *et al*., 1992). The peptide is a potent mediator of a wide variety of responses in mammalian tissues, including smooth muscle contraction, vasodilation, increased vascular permeability, inflammation, and pain (Bhoola *et al*., 1992). Bradykinin is also known to play some roles in the reproductive processes, such as embryo implantation and follicular contraction at ovulation (Espey, 1980; Clements *et al*., 1997; Allen *et al*., 2002; MaRae, 1998), and uterine contraction at parturition (Liebmann *et al*., 1991; Senior and Whalley, 1976). These effects are mediated by specific receptors, particularly the B_2 receptor subtype (Bhoola *et al*., 1992; Marceau and Bachvarov, 1998).

Most previous studies regarding the effect of bradykinin on the ovary have focused on the ovulatory process. It has been established that bradykinin induces ovulation in perfused rabbits (Lambertsen *et al*., 1976; Yoshimura *et al*., 1988) and rat ovaries (Hellberg *et al*., 1991; Brannstrom and Hellberg, 1989) and that bradykinin potentiates the action of LH (Brannstrom and Hellberg, 1989). Levels of expression of the tissue kallikrein gene family have been determined in the ovaries of untreated control and gonadotropin-treated immature female rats (Clements *et al*., 1995; Holland *et al*., 2001). An increase in ovarian kinin-producing activity during ovulation has also been reported (Espey *et al*., 1986; Espey *et al*., 1989; Gao *et al*., 1992; Tanaka *et al*., 1992; Brann *et al*., 1995; Brann *et al*., 2002). In support of these findings, we recently demonstrated that the follicular fluid of porcine ovaries contains three protein components required for the production of bradykinin: factor XIIa, plasma kallikrein, and high-molecular weight kininogen (Kihara *et al*., 2000; Kimura *et al*., 2000). We also showed that bradykinin and its degraded peptide [1-5]-bradykinin are indeed detectable in the follicular fluid (Kihara *et al*., 2000). More recently, we reported the localization of the B_2 receptor in the porcine ovary by *in situ* hybridization and immunohistochemical staining (Kimura *et al*., 2001). Interestingly, bradykinin treatment of the isolated granulosa cells was found to induce gene expression of some matrix metalloproteinases (MMPs), such as MMP-3 and MMP-20. This finding may provide a clue for elucidating the mechanism of follicle rupture during ovulation induced by bradykinin. However, it has

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proven difficult to clarify the detailed molecular mechanism using this domestic animal, since the available ovaries are almost exclusively in the follicular phase. Therefore, the present study was initiated to establish a basis for approaching the above problem using mice. The specific aim of this study was to localize the B_2 receptor in the mouse ovary, which has not yet been studied in this context.

MATERIALS AND METHODS

Animals

Immature female mice (C57BL/6N) were obtained from Charles River Inc., Yokohama, Japan. The animals were kept under controlled conditions of 24°C and a 14-hr light/10-hr dark cycle and allowed free access to food and water. Twenty-eight-day-old mice were injected with 5 IU of pregnant mare's serum gonadotropin (PMSG) (Sigma, St. Louis, MO) to stimulate follicle growth and 48 hr later with 5 IU of human chorionic gonadotropin (hCG) (Sigma) to induce ovulation. The mice were sacrificed at 0 and 48 hr after PMSG injection, and 2, 6, 12, 24, and 48 hr after hCG injection. The ovulation cycles of mature 8-week-old mice were examined by daily testing of vaginal smears. Experimental procedures used in the current study were approved by the committee of the Center for Experimental Plants and Animals, Hokkaido University.

[1-5]-Bradykinin assay

A total of 21 immature female mice were used. They were divided into 7 groups (n=3 each) for PMSG/hCG treatment. A pair of ovaries obtained from each treated animal were crushed in ice-cold ethanol and centrifuged at 500×g for 30 min at 4°C to recover the supernatants. After another extraction of the precipitates with 80% ethanol, the two resulting supernatants were combined and evaporated to dryness. The pellets were dissolved in 200 µl of distilled water and the solutions were adjusted to pH 2.0 by adding 15 µl of 0.1 N HCl. The solutions were washed twice with diethyl ether and the aqueous phases were dried again by evaporation. The resulting precipitates were dissolved in 100 µl of the buffer C included in a Markit-M [1-5]-BK kit (Dainippon Seiyaku, Tokyo, Japan). The quantities of [1-5]-bradykinin, Arg-Pro-Pro-Gly-Phe, in the samples were estimated by enzyme-linked immunosorbent assay (ELISA) using the kit described above. Three independent experiments were carried out.

Northern blot analysis

Total RNAs were isolated from ovaries of gonadotropin-treated immature female mice using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The RNAs (100 µg/lane) were electrophoresed on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a Nytran membrane (Schleicher & Schuell, Dassel, Germany). A total of eighteen mice were used to prepare one membrane. The blot was hybridized with the [α-^{32}P]-labeled probes for 18 hr at 42°C in 50% formamide, 5 × Denhardt's solution, 5×0.15 M NaCl/8.65 mM NaH_2PO_4/1.25 mM EDTA (SSPE), 1% SDS, and 100 µg/ml herring sperm DNA. The membrane was washed twice at 50°C in 2 ×standard saline-sodium citrate (SSC) buffer/0.05% SDS and twice at 50°C in 0.1 × SSC /0.1% SDS. The signals were visualized by autoradiography using a BAS 2000 Image Analyzer (Fuji, Tokyo, Japan). This experiment was conducted twice using two different membranes.

The cDNA probes for the B_2 receptor (518-bp), a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS-1) (463-bp), cathepsin L (400-bp), MMP-19 (364-bp), tissue-type plasminogen activator (tPA) (1002-bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (306-bp) were prepared by reverse transcriptase-polymerase chain reaction (RT-PCR) with mouse ovary total RNA, which was isolated from ovaries of PMSG/hCG-treated immature mice. The primer pairs used are indicated in Table 1. The amplified products were subcloned into pBluescript II plasmid (Stratagene, La Jolla, CA) and sequenced using an ABI automatic sequencer, model 377 (Perkin-Elmer/Applied Biosystems, Foster City, CA). The probes were excised out by restriction enzymes and purified. The labeling reactions were conducted with [α-^{32}P]-dCTP using a Random Primer DNA Labeling kit (Takara, Tokyo, Japan).

In situ hybridization

The probes for the B_2 receptor were prepared by RT-PCR using mouse ovary total RNA isolated at 6 hr after hCG injection of PMSG-treated immature mice. We used the primer pairs indicated in Table 1 for amplification of the B_2 receptor. Antisense and sense probes were synthesized by *in vitro* transcription of the plasmid clones using digoxigenin (DIG)-UTP and T3 or T7 RNA polymerase.

Mouse ovary sections (10 µm) were cut on a cryostat and thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides. All sections were fixed with 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan) in phosphate-buffered saline (PBS) for 20 min and washed with PBS for 5 min three times. The sections were

Table 1. Primers used to amplify mouse genes

Genes		Primer sequences	Accession No.	Nucleotide No.
B_2R	Sense	5'-ttcaccaacgtgctgctgaac-3'	X69676	833-1350
	Antisense	5'-actgtttcttccctgcccagtc-3'		
tPA	Sense	5'-tgccttcctcttcctcttctacag-3'	J03520	1181-2182
	Antisense	5'-cattcttgtccccagtgcaaac-3'		
MMP-19	Sense	5'-ctcttgtctgctgggatcatgg-3	AA611442	77-440
	Antisense	5'-gaagatgcggaatgtcaagtt-3'		
Cathepsin L	Sense	5'-atcgggttgcctagaaggacag-3'	M20495	534-933
	Antisense	5'-tggtcgaggttcttgctgctac-3'		
ADAMTS-1	Sense	5'-atcggaatcaaaggggtcc-3'	NM_009621	2216-2678
	Antisense	5'-aaggaaggtctgcacaaggtctg-3'		
GAPDH	Sense	5'-catgaccacagtccatgccatc-3'	M32599	562-867
	Antisense	5'-tagcccaagatgcccttcagtg-3'		

treated with 1 μg/ml proteinase K (Roche Molecular Biochemicals, Mannheim, Germany) for 5–10 min at room temperature and refixed in the same fixative for 10 min. The slides were washed again with PBS and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/HCl, pH 8.0 (Sigma) for 10 min. After prehybridization for 2 hr at room temperature in 50% formamide, 6 × SSPE, 5 × Denhardt's solution, and 500 μg/ml yeast transfer RNA, the sections were incubated at 70°C for 18 hr in the prehybridization buffer containing DIG-labeled RNA probes (sense or antisense, 30–60 ng/50 μl/slide). After hybridization, the sections were washed three times in 0.2 × SSC at 70°C for 20 min. The hybridized probes were detected using a DIG Nucleic Acid Detection kit (Roche Molecular Biochemicals). The slides were incubated in 1% blocking reagent in DIG buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 60 min and in 1:5,000 diluted anti-DIG antibody in DIG buffer 1 containing 1% blocking reagent for 30 min. After washing twice with DIG buffer 1 for 15 min and equilibrating with DIG buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 min, the sections were incubated with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in DIG buffer 3 for 3–18 hr.

Western blot analysis

A total of twenty-two mice were used for the analysis. Ovaries were removed from mice treated with gonadotropins and were homogenized in 60 mM Tris-HCl (pH 6.8), 2.3% SDS, 0.7 M 2-mercaptoethanol, and 10% glycerol. After boiling at 100°C for 10 min, the samples were centrifuged at 13,000 × g at 4°C for 5 min. The resulting supernatants containing 150 μg each were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmili, 1970) using a 10% polyacrylamide gel, and the separated proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) using Towbin's transfer buffer (Towbin *et al.*, 1979). The blotted membranes were then treated with Block Ace (Dainippon Seiyaku) at room temperature for 1 hr. The membranes were incubated with the primary antibody, mouse antibody directed against human bradykinin type 2 receptor (Transduction Laboratories, Lexington, KY), at 1:1,000 dilution for 1 hr and subsequently with biotinylated anti-mouse IgG antibody for 1 hr. They were then incubated with avidin-conjugated horseradish peroxidase for 1 hr, and signals on the membrane were detected using ECL Western Blotting Detection Reagents (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

Organ culture

The organ culture of mouse ovaries was carried out according to the method of Cavanagh (1984) with slight modification. RPMI 1640 tissue culture medium (Life Technologies, Inc., Rockville, MD) containing 0.3 mg/ml L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin (Life Technologies Inc.) was used throughout. Upon being removed from animals, the ovaries were washed with the medium and transferred to 2 ml of the medium in a 35-mm dish. One ovary from each mouse was placed in control medium and the other from the same mouse in the medium containing bradykinin at 100 nM. The cultures were maintained in a humidified atmosphere of 5% carbon dioxide in air at 37°C for 24 hr.

Semi-quantitative RT-PCR analysis

Total RNAs of ovaries cultured with or without bradykinin were isolated using Isogen (Nippon Gene). To determine mRNA levels of the B_2 receptor and GAPDH in ovaries, five micrograms of total RNAs were reverse-transcribed and one five-hundredth of the synthesized cDNA was used for PCR reactions. The reaction conditions were 21–25 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min. The amplified products were electrophoresed with 1.5% agarose gel and detected with ethidium bromide staining.

RESULTS

[1-5]-Bradykinin concentration in the ovary extract of gonadotropin-treated immature female mice

Our previous study demonstrated that bradykinin is present in the follicular fluid of porcine ovaries (Kihara *et al.*, 2000). To examine whether this would be true for mice, we first attempted to detect bradykinin using the ovary extract of immature female mice treated with PMSG/hCG. Because bradykinin is degraded very rapidly *in vivo* soon after its production, we determined the amount of a stable degraded product, [1-5]-bradykinin, by the ELISA method. As shown in Fig. 1, the amount of [1-5]-bradykinin gradually increased after PMSG injection. After hCG treatment, the ovarian [1-5]-bradykinin levels showed a further progressive rise to reach a peak at 6 hr, a time preceding the beginning of ovulation. The [1-5]-bradykinin level then fell at 12–24 hr after hCG stimulation, but rose again at the 48 hr-time point after hCG. The results suggest that bradykinin is produced in the mouse ovary before ovulation.

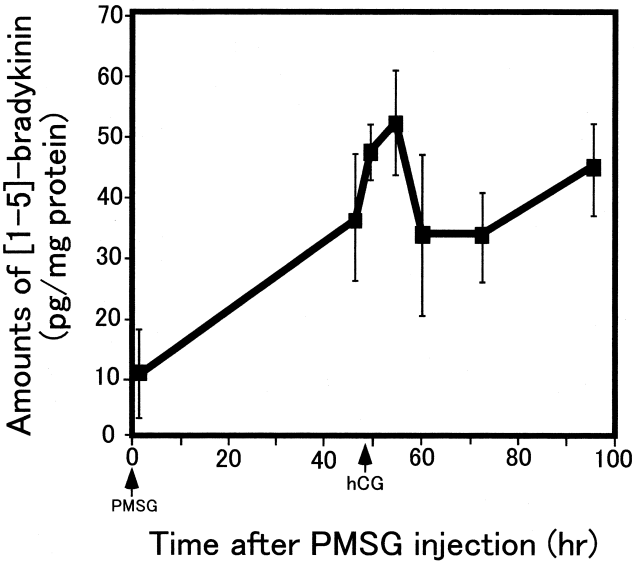

Fig. 1. Quantification of [1-5]-bradykinin in the ovary extracts of gonadotropin-treated immature female mice.
Immature female mice (28 days old) were primed with PMSG and 48 hr later with hCG, and the ovaries were collected at the indicated time points. Extracts prepared from the collected ovaries were used for quantification of [1-5]-bradykinin by the ELISA method. The values are shown as the mean ± SEM of three independent experiments.

mRNA expression of bradykinin receptor in gonadotropin-treated mouse ovaries

That bradykinin may play a role in the mouse ovary suggests that its receptor may also be expressed there. Since two distinct bradykinin receptor subtypes, B_1 and B_2 (Farmer and Burch, 1992; Hall, 1997), are known to exist in mice, we conducted a preliminary PCR experiment to see whether both subtypes of bradykinin receptor are expressed in the ovary. By using specific primer sets for respective

receptor subtypes and total RNAs isolated from ovaries of gonadotropin-treated immature mice, a specific PCR product was amplified only for the B_2 receptor (data not shown), indicating that the B_2 receptor is solely expressed in this organ. Northern blot analysis was conducted for the receptor using total RNAs isolated from the gonadotropin-treated mouse ovaries (Fig. 2). The signals of the B_2 receptor mRNA were detected at all time points examined. However, hCG treatment brought about a slight increase of the receptor expression at 6 hr after treatment.

We also examined the mRNA expression of some genes that are thought to play a role in ovulation. The genes examined were tPA (Beers, 1975; Beers *et al*., 1975), MMP-19 (Hägglund *et al*., 1999), cathepsin L (a lysosomal cysteine protease), and ADAMTS-1 (Robker *et al*., 2000; Espey *et al*., 2000). The expression of MMP-19 and ADAMTS-1 was notably induced at 12 hr after hCG injection (Fig. 2), the time of ovulation, confirming previous results (Hägglund *et al*., 1999; Robker *et al*., 2000; Espey *et al*., 2000). tPA expression was also slightly induced after hCG injection. In contrast, cathepsin L mRNAs were expressed at a rather constant level throughout the periovulatory period.

Localization of the B_2 receptor mRNA in gonadotropin-treated mouse ovaries

We next analyzed the temporal and cell-specific expression of the B_2 receptor mRNA in the mouse ovary during gonadotropin-induced ovulation by the *in situ* hybridization method. The ovaries were collected at different time points after gonadotropin treatment, and the ovary sections were hybridized with antisense or sense probes of mouse B_2 receptor. Positive signals were detected in all ovaries examined when hybridized with an antisense probe, whereas no significant signals were detected with a sense probe (Fig. 3). The receptor mRNAs were specifically localized in the granulosa cells of basically all of the growing follicles of gonadotropin-treated mice. The ovarian follicles of untreated immature mice (PMSG 0h) appeared to show a weaker signal compared with those of treated mice.

Expression of the B_2 receptor protein in gonadotropin-treated mouse ovaries

To identify the expression of the B_2 receptor at the protein level, we carried out a Western blot analysis using mouse anti-human B_2 receptor antibody. The antibody was raised against a 15-residue-peptide of the human receptor, and was confirmed to interact with the mouse B_2 receptor protein. Immature 28-day-old female mice were treated with PMSG and 48 hr later with hCG. At different time points after hormone treatment, ovaries were collected, and their extracts were prepared for the analysis. As shown in Fig. 4, a 42-kDa band was specifically detected in all lanes. Rat

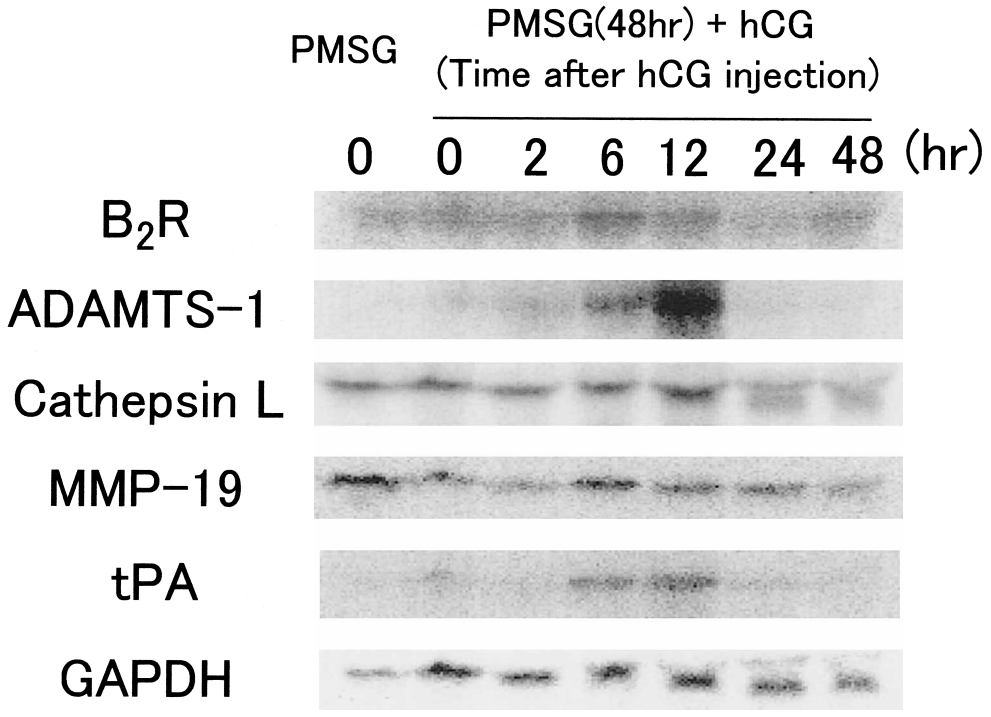

Fig. 2. Northern blot analysis of mouse B_2 receptor, ADAMTS-1, cathepsin L, MMP-19, and tPA mRNAs in the ovaries of gonadotropin-treated immature mice.
One hundred micrograms of total RNAs, which were isolated from gonadotropin-treated mouse ovaries at the indicated time points, were applied to each lane. The blotted membrane was hybridized with the [^{32}P]-labeled probes and the signals were detected by autoradiography. Only one kind of band was visualized with each probe and their sizes are indicated with the names of genes on the left. A probe for mouse GAPDH was used as an internal control. This experiment was conducted twice using two different membranes, and a representative result is shown.

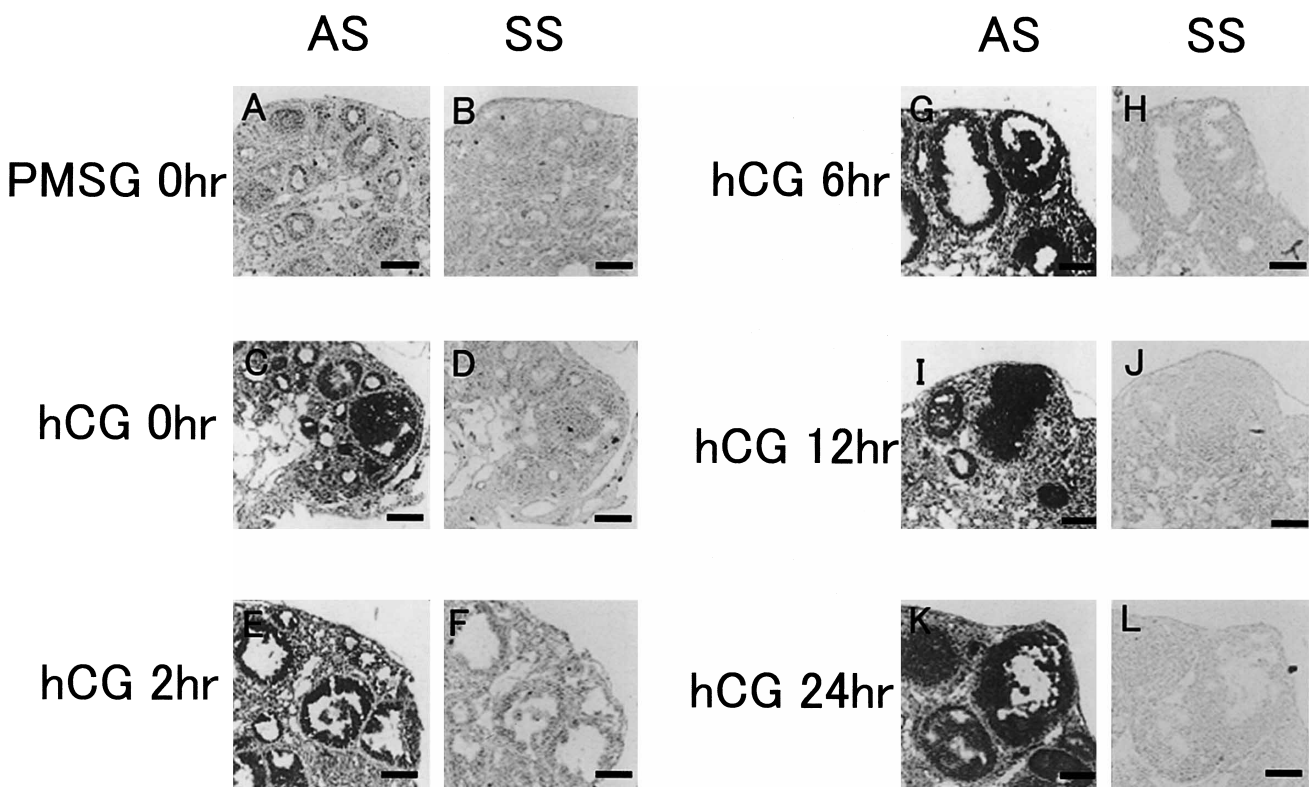

Fig. 3. *In situ* detection of B_2 receptor mRNA in the ovaries of gonadotropin-treated immature mice.
The ovaries were collected from gonadotropin-treated mice at the indicated time points. The sections were hybridized with DIG-labeled antisense (AS; A, C, E, G, I, and K) or sense (SS; B, D, F, H, J, and L) probes of mouse B_2 receptor at 70°C and washed at 70°C. Positive signals were specifically detected with the antisense probe in the follicular granulosa cells and the corpus luteum in all ovaries examined. No significant signals were detectable with the sense probe. The results are from a representative animal. A total of twenty-five animals were used. Bars represent 10 μm.

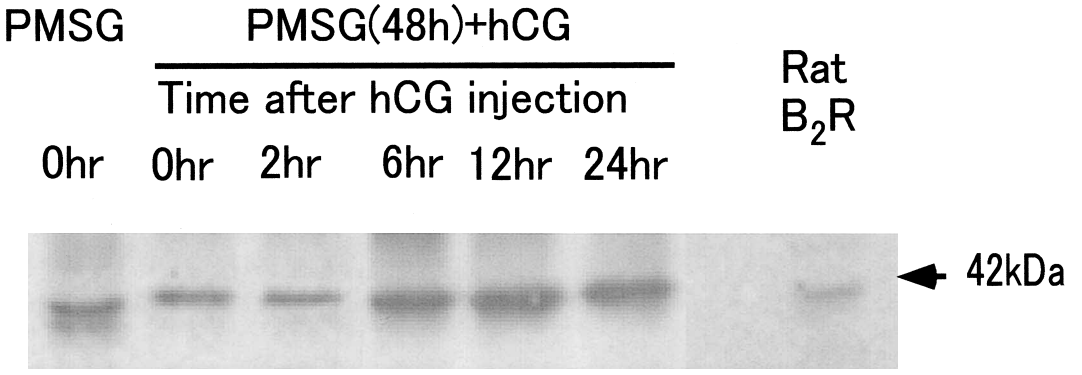

Fig. 4. Immunological detection of the mouse B_2 receptor protein with the ovary extracts of gonadotropin-treated immature mice.
Ovaries were isolated from the gonadotropin-treated immature mice at the indicated time points and their extracts were prepared. Samples containing 150 μg protein of the extracts and 5 μg of rat pituitary lysate (used as a control) were fractionated by SDS-PAGE. The blotted membrane was incubated with mouse anti-human B_2 receptor antibody. A band was detected at 42 kDa, which is indicated by an arrow, in each lane.

pituitary lysate was used as a control. The strength of the signals was relatively higher at 6–24 hr after hCG injection. An immunoreactive material with the same molecular mass was detected when the extract of adult mouse ovary was used (data not shown).

We tried to determine the localization of the B_2 receptor in the ovaries of gonadotropin-treated immature and untreated adult mice by immunohistochemical analysis using this antibody. However, reliable staining was not gained because of nonspecific signals.

Localization of B_2 mRNA in adult mouse ovaries

We determined the localization of the B_2 receptor mRNA in the ovaries of adult mice. The stages of the ovu-

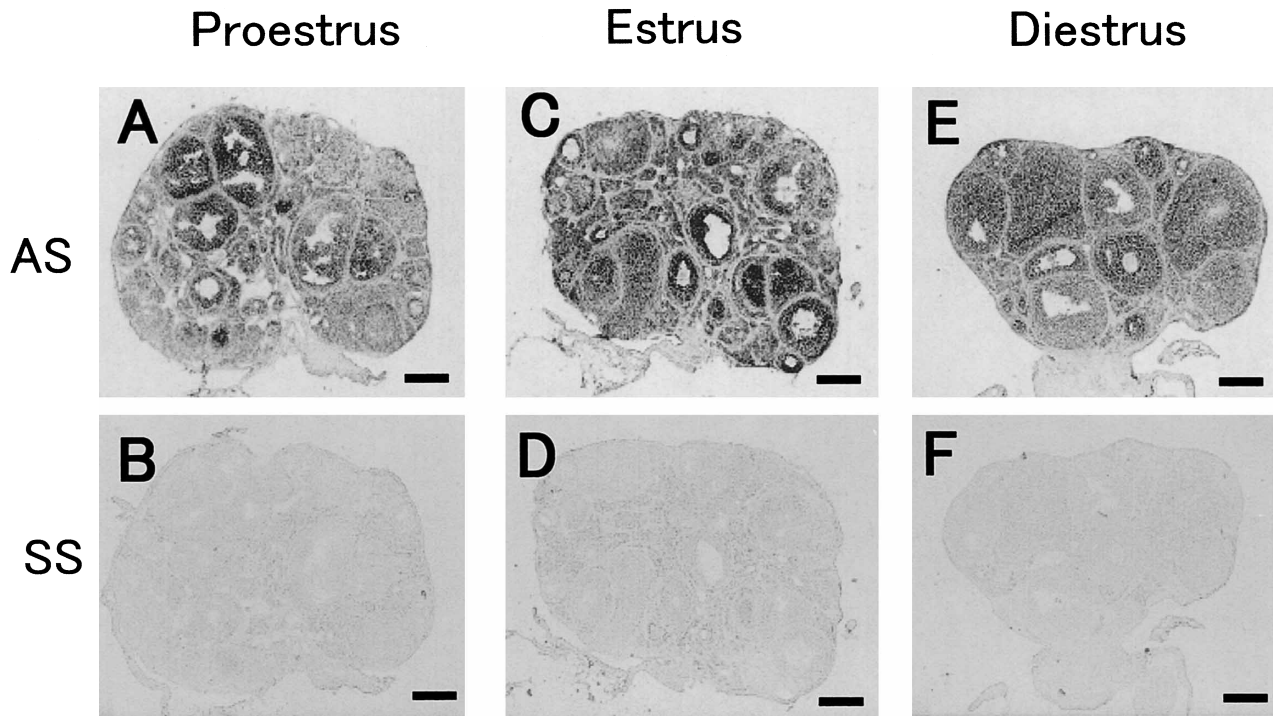

Fig. 5. *In situ* detection of B_2 receptor mRNA in the ovaries of adult mice.
Mature female mice (8 weeks old) were kept and the ovulation cycle was checked every day by smear tests. The ovaries were collected at the stages of proestrus (A and B), estrus (C and D), and diestrus (E and F), and cut into 10-μm sections. The neighboring sections were hybridized with DIG-labeled antisense (AS; A, C, and E) or sense (SS; B, D, and F) probes of mouse B_2 receptor at 55°C and washed at the same temperature. Positive signals were specifically visualized with the antisense probe in the follicular granulosa cells and corpus luteum in each ovary. No significant signals were detected with the sense probe. A total of six animals were used. Bars represent 20 μm.

lation cycle were assessed by means of daily smear tests. Ovaries at the proestrous, estrous, and diestrous stage were collected for *in situ* hybridization analysis. The B_2 receptor mRNA was specifically detected in the granulosa cells and corpus luteum at all stages, although the signal intensities were different from one follicle to another at proestrus (Fig. 5). The results were compatible with those from the gonadotropin-treated immature female mouse ovaries.

Effect of bradykinin treatment on B_2 receptor expression in the mouse ovary

Ovaries were collected from untreated immature, gonadotropin-treated immature, and untreated adult female mice, and were cultured in the presence of 100 nM of bradykinin for 48 hr. Total RNAs from the cultured ovaries were subjected to semi-quantitative RT-PCR analysis, as described in “Materials and Methods”. No apparent changes in the mRNA levels of the B_2 receptor were observed in bradykinin-treated and untreated mouse ovaries (data not shown). These results indicate that B_2 receptor expression in the mouse ovary is not affected by the ligand.

DISCUSSION

Bradykinin released by proteolytic cleavage of kininogen by kallikrein is thought to be converted rapidly to a stable degraded form of bradykinin, [1-5]-bradykinin. We therefore determined the [1-5]-bradykinin levels of the ovary extract of gonadotropin-treated immature female mice at various time points. Our results showed that the peptide reached its maximum level at 6 hr after hCG treatment. Previous studies (Espey, 1980; Smith and Perks, 1983; Espey *et al*., 1986; Gao *et al*., 1992) raised the possibility that the kinin-kallikrein system may be an important component in the cascade of events that leads to ovulation in the rat. In particular, kallikrein activity in the ovary extract of gonadotropin-primed immature rats was reported to increase just before or at the time of ovulation (Espey *et al*., 1986; Espey *et al*., 1989; Brann *et al*., 2002). Interestingly, the periovulatory pattern of ovarian kallikrein activity in the rat is very similar to that of the [1-5]-bradykinin level in the mouse ovary. These findings strongly suggest that the bradykinin-producing system plays a role in the ovulatory process of the rodent. We have recently identified all components necessary for the generation of bradykinin within the follicle of porcine ovaries (Kihara *et al*., 2000; Kimura *et al*., 2000). We assume that, like porcine ovaries, the individual follicles of the mouse ovary also contain the bardykinin-producing system.

The mouse ovary expresses the bradykinin B_2 receptor but not the B_1 receptor. However, the content of the receptor

mRNA seems quite low in the mouse ovary; application of ovary total RNA of as much as 100 μg was needed for detecting a signal corresponding to the B_2 receptor. Such a low content of the receptor mRNA was found for porcine ovaries. In the pig, no clear signal was detectable by Northern blot analysis using a blot membrane containing 50 μg of ovarian total RNA (Kimura *et al*., 2001). Expression of B_2 receptor in the ovary was slightly induced by the hCG treatment of PMSG-primed mice, the highest level of the receptor mRNA being observed at 6 hr after hCG treatment. In this context, it is interesting to note that the [1-5]-bradykinin level in the ovary extracts of the same gonadotropin-treated mice also reached a maximum at 6 hr after hCG. This increased bradykinin level does not seem to cause a slight induction of B_2 receptor mRNA expression.

Northern and Western blot analyses of the receptor both indicate that the expression of B_2 receptor in the ovaries of gonadotropin-treated immature female mice is enhanced at 6 hr after hCG treatment. These results do not seem to be compatible with that of *in situ* detection of the receptor mRNA. However, it should be noted that the *in situ* hybridization analysis gave no quantitative data of B_2 receptor expression. Indeed, we conducted the present *in situ* analysis so as to produce clear differences in the sense and antisense staining of sections for each time point. We presume that the results of Northern and Western blot analyses represent the *in vivo* expression level of B_2 receptor.

Our present study clearly showed that B_2 receptor mRNA is localized in association with granulosa cells of all growing ovarian follicles of the gonadotropin-treated immature mouse ovary. In the adult mouse, granulosa cells of follicles and cells constituting the corpus luteum express B_2 receptor mRNA. We failed to localize the receptor protein by immunohistochemistry, but the Western blot analysis of the ovary extract revealed its occurrence in the ovary. Based on these results, we conjecture that the receptor protein is present on the plasma membrane of the granulosa cells and luteal cells of the ovary.

In contrast to our previous finding that B_2 receptor mRNA expression in the cultured granulosa cells of porcine ovaries is induced by bradykinin treatment (Kimura *et al*., 2001), the receptor expression in the mouse ovary was not affected by the treatment. This is perhaps due to species difference. The above finding suggests that the *in vivo* elevation of bradykinin concentration in the mouse ovary has no significant effect on the expression of B_2 receptor mRNA. Such a constitutive expression of the receptor leads us to speculate that biological responses elicited by bradykinin may be dependent on changes in the ligand concentration in the ovary.

The detailed mechanism of ovulation in mammals still remains to be elucidated. Since bradykinin induces ovulation, detailed investigation of the bradykinin-induced ovulation could provide useful information of this biologically important process. The present study on the B_2 receptor expression and localization in the mouse ovary definitely helps clarify our knowledge about the mechanisms of ovulation.

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