Localization of Bradykinin B₂ Receptor in the Follicles of Porcine Ovary and Increased Expression of Matrix Metalloproteinase-3 and -20 in Cultured Granulosa Cells by Bradykinin Treatment

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ABSTRACT

We have recently shown that not only bradykinin, but also all components for the production of bradykinin, can be detected within the follicle of porcine ovaries. To elucidate the relevance of the intrafollicular bradykinin-producing system to its physiological role, we investigated the distribution of bradykinin receptor (B₂R) mRNA and the protein in porcine ovaries. A cDNA encoding porcine B₂R was first cloned from a porcine uterus cDNA library. The receptor mRNA was scarcely detected in the ovary by Northern blot analysis. Polymerase chain reaction analysis with total RNAs isolated from the ovary and from granulosa cells of small and large follicles demonstrated the ovarian expression of B₂R mRNA. The B₂R protein was detected by Western blot analysis in extracts of isolated granulosa cells. In situ hybridization of B₂R mRNA and immunohistochemical analysis of the protein revealed that the receptor is expressed in the theca and granulosa cells of all growing follicles. The effect of bradykinin on the expression of some matrix metalloproteinase (MMP) genes was examined using isolated granulosa cells. Bradykinin treatment induced MMP-3 and MMP-20 gene expression to an extreme degree. The expression of MT1-MMP was also affected by bradykinin treatment. These results suggest that MMPs play a role in follicle rupture during ovulation. The present study provides new information regarding the mechanisms of bradykinin-induced ovulation in porcine ovaries.

MATERIALS AND METHODS

Materials

Porcine ovaries and other tissues were obtained from a local slaughterhouse within 30 min of death of the animals, and were transported to the laboratory on ice.

Complementary DNA Cloning of Porcine B₂R

We obtained a cDNA fragment of porcine B₂R by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from porcine uterus using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol. The first-strand cDNA was synthesized from the RNA using a Superscript II Preamplification System (Life Technologies Inc., Rockville, MD). The PCR reaction was performed with a primer pair of 5'-CAGAGATCTACCTGGGGAAC-3' and 5'-GCCCAGTCCTGCAGTTTGT-3' designed from the sequence of human B₂R. The conditions were 35 cycles at 94°C for 30 sec, 62°C for 30 sec, and 72°C for 2 min.

The sequence of the 5' end was obtained from porcine uterus total RNA by the 5' rapid amplification of cDNA ends method [24] using the 5'RACE (rapid amplification of cDNA ends) System (Life Technologies). The primers used were as follows: 5'-AAGCAGATGCTGCT-3' (pB2RT; nucleotides 595 to 582) for RT reaction, 5'-TCATGGTGTTCATGGTGT-3' (pB2RA1; nucleotides 564 to 545) for the first PCR, and 5'-CGAAATGTGTTGCGATGTTG-3' (pB2RA2; nucleotides 519 to 500) for the second PCR (Fig. 1). The PCR was performed under the condition of 35 cycles at 94°C for 30 sec, 62°C for 30 sec, and 72°C for 2 min.

The sequence of the 3' region was obtained by PCR using porcine uterus total RNA with sense primers of porcine B₂R and antisense primers designed from 3' untranslated sequences of rat, mouse, and human B₂R. The primer pairs were 5'-TCAAGGAGAATCCAGACGG-3' (pB2SS1; nucleotides 931 to 950) and 5'-CTGAGTTCATGGTGGTCTG-3' (pB2degAS1; a degenerate primer designed from human,
FIG. 1. Nucleotide and deduced amino acid sequences of porcine B₂R. Arrows indicate the positions of the primers. An open box is the sequence obtained first by PCR using human B₂R primers as described in Materials and Methods. The 5' portion was amplified by the 5'-RACE method, and the 3' portion was generated by PCR with degenerate primers. The seven transmembrane domains, designated TM1-7, are shown in white letters in black boxes. Amino acid residues in black and white squares represent the putative phosphorylation sites for protein kinases A and C, respectively. White triangles indicate the putative N-glycosylation sites and black ones the putative palmitoylation site. The nucleotide sequence is available from the DDBJ/EMBL/GenBank database with the accession number AB051422.

Northern Blot Analysis

Fifty micrograms of total RNAs isolated from porcine uterus, ovary, liver, testis, adrenal gland, and pituitary gland were electrophoresed on a formaldehyde/agarose gel and transferred to a Nytran membrane (Schleicher & Schuell, Dassel, Germany). The blot was hybridized for 18 h with a32P-labeled probe at 42°C in 50% formamide, 53Denhardt solution, 53NaCl/Na-PO₄/EDTA, 1% SDS, and 100mg/ml herring sperm DNA. The probe was prepared by PCR amplification using a sense primer of 5'-CAGAGATCTACCTGGGGAAC-3' (pB2SS4; nucleotides 430 to 449) and an antisense primer of 5'-GGTACTTGATGATGCAGGCG-3' (pB2AS2; nucleotides 801 to 782). The membrane was washed at 50°C in 0.1% SDS/0.1% SSC and exposed to Kodak Biomax film. A probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control, which was prepared by RT-PCR as described in the section RT-PCR Analysis.

RT-PCR Analysis

Total RNAs were extracted from granulosa cells of small-sized (1-2 mm) or large-sized (>5 mm) follicles that had been isolated from about 30 porcine ovaries as described previously [25]. Five micrograms of the RNAs were then reverse-transcribed using a Superscript II Preamplification System (Life Technologies). One-fortieth of the RT reaction was employed for PCR using a sense primer pB2SS2, 5'-ATCACCATGCCAACCACACTTC-3', corresponding to nucleotides 498 to 517, and an antisense primer pB2AS1, 5'-AGGAAGGTGCTGATCTGGAA-3', complementary to nucleotides 1030 to 1011 (Fig. 1), under the following conditions: 40 cycles at 96°C for 20 sec, 57°C for 40 sec, and 72°C for 60 sec. As an internal control, a transcript of porcine GAPDH gene was amplified. The primers used were 5'-ATGCTGGTGCTGAGTATGTC-3' and 5'-AGCTCATTTCCTCGTACGAC-3'. The PCR products were fractionated on a 1.5% agarose gel and stained with ethidium bromide.

Western Blot Analysis

The granulosa cells were prepared from the small-sized and large-sized follicles of 30 porcine ovaries as described above. The granulosa cells were homogenized in 60 mM Tris-HCl (pH 6.8), 2.5% SDS, 0.7 M 2-mercaptoethanol, and 10% glycerol. The samples were separated by SDS-polyacrylamide gel electrophoresis [26] using a 10% gel under reducing conditions, and separated proteins were transferred to polyvinylidene difluoride membrane [27]. After immersion in Block Ace solution (Dainippon Seiyaku, Tokyo, Japan), the blot was incubated with a murine antibody directed against human bradykinin type 2 receptor (Transduction Laboratories, Lexington, KY) at a 1:1000 dilution and subsequently with biotinylated anti-mouse immunoglobulin (Ig) G antibody. The membrane was further incubated with avidin-conjugated horseradish peroxidase. Immunoreactive signals were detected using an ECL Western blot detection kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) according to the manufacturer's protocol.

In Situ Hybridization

Porcine ovaries frozen in isopentane-dry ice were cut into 14-μm sections and thaw-mounted onto silane-coated slides. The sections were fixed...
TABLE 1. Primers used to detect genes induced by bradykinin.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>GenBank accession number</th>
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<tr>
<td>MMP-1</td>
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<td>X54724</td>
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<td>Antisense 5'-TCACGAAATGGTCTGATGATG-3'</td>
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<td>MMP-7</td>
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<td>AB031323</td>
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<tr>
<td></td>
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with 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan) in PBS for 15 min, and treated with 1 μg/ml proteinase K (Roche Molecular Biochemicals, Mannheim, Germany) in PBS for 3 min at room temperature. After being fixed in the same fixative, the sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine/HCl (Sigma, St. Louis, MO). After 2 h prehybridization at room temperature in 50% formamide, 6× SSPE, 5× Denhardt solution, and 500 μg/ml yeast transfer RNA, the sections were incubated for 18 h at 55°C in the same buffer containing 100–200 ng/ml digoxigenin (DIG)-labeled probes. The probes were prepared by in vitro transcription of the same fragment as used in the Northern blot analysis with T3 or T7 RNA polymerase. The hybridized sections were washed three times in 0.2× SSC at 55°C for 20 min each. Signals were detected with a DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals). A total of three animals were used.

**Immunohistochemistry**

The cryosections were prepared as described above. The sections were fixed in 4% paraformaldehyde in PBS, treated with 3% hydrogen peroxide in methanol for 15 min at room temperature, and incubated in Block Ace solution for 30 min at room temperature. Mouse anti-human bradykinin type 2 receptor antibody (Transduction Laboratories) was applied at a 1:200 dilution on the slides and remained for 24 h at room temperature. After being washed in PBS, the specimens were treated with biotinylated anti-mouse IgG antibody and then with ABC reagents (Vector Laboratories) as described in the manufacturer’s protocol. A total of three animals were used.

**Granulosa Cell Culture**

Porcine granulosa cells were collected by aspiration from the follicles of approximately 30 ovaries using 21-gauge hypodermic needles and syringes. The cells were suspended in Dulbecco modified Eagle medium (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 0.3 mg/ml l-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Life Technologies) and centrifuged at 300 × g for 5 min. After being washed three times in fresh medium, the cells were plated on surface-modified 24-well dishes (Becton Dickinson Labware, Bedford, MA) at a density of 1 × 10^6 cells per well and cultured for 24 h. The bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (Peptide Institute, Osaka, Japan) was then added at various concentrations to the medium without serum. After being cultured for another 24 or 48 h, the cells were collected and subjected to RNA extraction and RT-PCR analysis. The PCR reaction was conducted under three different conditions: 1) 25 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min; 2) 30 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min; and 3) 35 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min. The primers used are listed in Table 1. It was found that by using 0.5 μg of total RNAs extracted from granulosa cells, the PCR amplification was linear under conditions of 30 cycles for all of the genes with the exception of MMP-1. The products were electrophoresed with 1.5% agarose gel, except for MMP-13. The product of MMP-13 was only ~200 base pairs (bp), and was fractionated with 5% polyacrylamide gel for better separation. All the PCR products were stained with ethidium bromide.

**Statistical Analysis**

Data were shown as the mean ± SEM. All data were analyzed by one-way ANOVA, followed by the Dunnett test. Data were considered significantly different from the control at P < 0.05.

**RESULTS**

**Cloning of Porcine B2R cDNA**

RT-PCR and RACE-PCR were used to clone cDNA fragments encoding the porcine homologue of B2R, as described in Materials and Methods. The deduced amino acid sequence of the porcine open reading frame derived from the cDNAs shares high sequence homology with human (85.5%) [28], rat (84.9%) [29], mouse (87.8%) [30], rabbit (86.3%) [31], and guinea pig (85.2%) [32] B2R (Fig. 1). The DNA sequence of the complete cDNA has been deposited in the DNA Data Bank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank (accession number AB051422).

**Expression of B2R in Granulosa Cells Isolated from Porcine Ovarian Follicles**

Figure 2 shows the results of Northern blot analysis of total RNAs isolated from various types of porcine tissue. Signals were detected at a position of 4.2 kilobase (kb) with the uterus, testis, adrenal gland, and pituitary RNAs. However, the ovary and liver RNAs gave no detectable signal. These results were similar to those in previous studies with rat [29], human [28], rabbit [31], and mouse B2R [30], except that ovary RNA had not been examined previously by Northern blot analysis.

To determine whether B2R mRNA is expressed in the ovary, we further examined the gene expression by the RT-PCR method. For this purpose, a pair of primers (pB2RSS2 and pB2RAS1) was used to amplify a 533-bp product with the total RNA isolated from small-sized and large-sized follicles of porcine ovariess. As shown in Figure 3A, both RNAs gave products of an expected size. With knowledge of the high degree of amino acid se-

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sequence identity of porcine and human B₂R, we used a well-characterized murine antibody generated against human B₂R to conduct Western blotting on lysates of isolated porcine ovary granulosa cells. This antibody detected a major (42-kDa) and a minor (44-kDa) protein with the cell lysate, both corresponding to the 42-kDa positive control rat pituitary B₂R protein (Fig. 3B).

The results described above indicate that granulosa cells of the porcine ovary indeed express B₂R.

In Situ Hybridization Analysis of B₂R mRNA in the Porcine Ovary

In situ detection of B₂R mRNA in the porcine ovary was carried out with an antisense DIG-labeled B₂R cRNA probe. The probe detected specific signals in virtually all follicles, irrespective of size (Fig. 4). In small-sized follicles without a noticeable thecal layer and antrum, interestingly, follicles, irrespective of size (Fig. 4). In small-sized follicles (SGC, 1–2 mm) or large-sized (LGC, >5 mm) follicles were reverse-transcribed with (+) or without (−) reverse transcriptase. The PCR reaction was as follows: 40 cycles at 96°C for 20 sec, 57°C for 40 sec, and 72°C for 60 sec. The PCR products were separated in 1.5% agarose gel and stained with ethidium bromide. Porcine GAPDH was amplified as an internal control.

Effects of Bradykinin on MMP Gene Expression in the Cultured Granulosa Cells

To obtain insight into the role of bradykinin in the ovary, we examined the effects of bradykinin on the expression of some genes in the primary culture system using porcine ovary granulosa cells. Giving attention to the possible involvement of bradykinin in follicle rupture during ovulation, we chose six porcine MMPs (MMP-1, -3, -7, -13, -19, and MT1-MMP) for which nucleotide sequence data are available from public databases (Table 1). In a preliminary experiment, we observed the products corresponding to all these MMPs, except for MMP-7 (also known as matrixin), at comparable levels when PCR amplification was conducted using 1.5 μg of total RNA extracted from isolated granulosa cells (data not shown). However, changes in the extent of expression of individual genes were detectable in response to reducing the amount of template RNA to 0.5 μg in PCR. Under these conditions, the expression of MMP-1 (also known as interstitial collagenase) and MMP-19 was as follows: 40 cycles at 96°C for 20 sec, 57°C for 40 sec, and 72°C for 60 sec. The PCR products were separated in 1.5% agarose gel and stained with ethidium bromide. Porcine GAPDH was amplified as an internal control.

In the Cultured Granulosa Cells

For 24 h brought about an enhancement of its expression. Bradykinin treatment did not affect the expression of MT1-MMP expression was very low in the untreated cells and incubation alone for 48-h treatment with bradykinin at concentrations of 10 and 100 nM (Fig. 6). The effect of bradykinin on the expression of MT1-MMP was complex. MT1-MMP expression was very low in the untreated cells that had been isolated from the ovary, and incubation alone for 24 h brought about an enhancement of its expression. Bradykinin treatment did not affect the expression of MT1-MMP in these cells. Nor was MT1-MMP expression ob-

FIG. 2. Northern blot analysis of porcine B₂R mRNA. Fifty micrograms of total RNA from the indicated porcine tissues were applied to each lane. The probe was a 372-bp fragment of porcine B₂R cDNA prepared by PCR, and a 4.2-kb B₂R signal is exhibited. Porcine glyceraldehyde 3-phosphate dehydrogenase cDNA was used as an internal control and indicated as GAPDH.

FIG. 3. Expression of B₂R in granulosa cells of porcine ovary. A) RT-PCR detection of B₂R mRNA in total RNA isolated from porcine ovary granulosa cells. Five micrograms of the total RNA from granulosa cells of small-sized (SGC, 1–2 mm) or large-sized (LGC, >5 mm) follicles were reverse-transcribed with (+) or without (−) reverse transcriptase. The PCR reaction was as follows: 40 cycles at 96°C for 20 sec, 57°C for 40 sec, and 72°C for 60 sec. The PCR products were separated in 1.5% agarose gel and stained with ethidium bromide. Porcine GAPDH was amplified as an internal control. B) Immunological detection of B₂R protein in porcine ovary granulosa cells. Twenty-four micrograms of porcine granulosa cell extracts or 5 μg of rat pituitary lysate were applied to each lane and subjected to SDS-PAGE under reducing conditions. The proteins were blotted, and specific signals were detected with anti-B₂R antibody.
FIG. 4. In situ detection of B2R mRNA in porcine ovary. Sections of porcine ovary were hybridized with DIG-labeled antisense (A, C, and E) or sense (B, D, and F) probes of porcine B2R. Positive signals were detected with the antisense probe in the follicular granulosa and theca cells regardless of the follicle size. No positive signals were detected with the sense probe. Follicle sizes (in diameter) are 17 μm in A and B, 2 mm in C and D, and 3.5 mm in E and F. Bars = 10 μm in A and B and 30 μm in C–F.
FIG. 5. Immunohistochemical localization of B₂R in porcine ovary. Sections of porcine ovary were stained with anti-B₂R antibody (A, C, and E). Specific signals were detected in the granulosa and theca cells of all follicles. Staining without the antibody was carried out as a negative control (B, D, and F). Follicle sizes (in diameter) are 0.5 mm in A and B, 2.2 mm in C and D, 3.5 mm in E and F. Bars = 30 μm.
FIG. 6. Induction of MMPs by bradykinin in cultured porcine ovary granulosa cells. A) Granulosa cells of porcine ovaries were cultured as described in Materials and Methods, and treated with 0 (lanes 2 and 6), 0.1 (lanes 3 and 7), 1 (lanes 4 and 8), and 10 nM (lanes 5 and 9) bradykinin. After being cultured for another 24 h (lanes 2–5) or 48 h (lanes 6–9), the cells were collected and subjected to RNA extraction followed by RT-PCR analysis. The primers used are listed in Table 1. The RT-PCR was conducted using 0.5 µg of the total RNAs under the conditions of 30 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 2 min. For lane 1, the cells were collected before bradykinin treatment. The PCR products were separated in 1.5% agarose gel except for the product of MMP-13, which was electrophoresed using a 5% polyacrylamide gel. The bands were revealed by ethidium bromide staining. B) Signal intensities of the bands shown in A were quantified by densitometric analysis using NIH Image (National Institute of Health, Bethesda, MD), and data from PCR bands were normalized with the control gene GAPDH. Results are presented as ratio MMP or B2R to GAPDH (mean ± SEM, triplicate samples). Columns marked with an asterisk are significantly different (P < 0.05) from lane 1.

served in the cells incubated for 48 h in the absence of bradykinin. However, an incubation of the cells with bradykinin induced the expression of this MMP. On the other hand, MMP-13 (also known as collagenase-3) expression was not affected by the treatment, and disappeared during the 48-h incubation. The pattern of B2R gene expression resembled those of MMP-3 and MMP-20 gene expression. Compared with the clear bands of B2R PCR products shown in Figure 3A, no visible band was seen in the 0-h control lane of B2R in Figure 6A. This is due to the difference in the PCR reaction conditions employed. Neither MMP-7 and PK gene were expressed in the granulosa cells irrespective of whether bradykinin was included in the culture (data not shown).

DISCUSSION

Based on the idea that the ovulatory process resembles an inflammatory reaction, the involvement of the kallikrein-kinin system in ovulation has been of intense interest for many researchers. Previous studies conducted along this line have shown an increase in ovarian kinin-producing activity during ovulation [16–18]. Also, it has been established that bradykinin induces ovulation in perfused rabbit [19, 20] and rat ovaries [21] and potentiates the action of LH [22]. We have very recently demonstrated that when using porcine ovaries, not only all components of the bradykinin-producing system but also its product, bradykinin, are present within the follicles [15]. Despite increasing evidence for the role of bradykinin in mammalian ovaries, little is known about the cellular distribution and regulation of its receptors in this organ. Thus, the primary purpose of the present study was to determine the temporal and spatial distribution of B2R in the ovary. To this end, we used porcine ovaries for the following two reasons: 1) previous studies had been conducted in our laboratory with ovaries from this species, and our knowledge drawn from these studies, including information on the kallikrein-kinin system, should be helpful in developing a better understanding of the biological meaning of intrafollicular production of bradykinin; and 2) a large number of granulosa cells can be isolated from porcine ovaries so that further experiments in the primary culture system using the cells can easily be carried out.

We have cloned and sequenced B2R from porcine uterus mRNA. It is highly homologous to B2R cloned from five other species. Seven transmembrane domains and putative sites for glycosylation, palmitoylation, and phosphorylation are all found in the current sequence (Fig. 1). In addition, two acidic residues (Asp269 and Asp287, the residue numbers are based on the porcine sequence) and four hydrophobic residues (Leu105, Val109, Ile113, and Phe262) that are postulated to interact with the N-terminal and C-terminal parts of bradykinin, respectively, based on the rat model of the bradykinin/B2R complex [33], are also conserved in the porcine receptor.

In the present study, we could not detect a clear signal for B2R by Northern blot analysis. This result does not mean that the B2R gene is not expressed at all in the porcine ovary, however. Indeed, the message was detected by the PCR amplification method with RNA isolated from gran-
ulosa cells of the ovary. Expression of the B$_2$R protein was further confirmed by Western blotting with the specific B$_2$R antibody. Although a ligand-binding assay and pharmacological characterization are also necessary for identification, we tentatively presume that these polypeptides represent porcine B$_2$R proteins expressed on the plasma membranes of granulosa cells.

Two immunoreactive bands detected in the Western blot analysis indicated the presence of two B$_2$R protein isoforms. These proteins may be produced due to different posttranslational modifications (for example, glycosylation or limited proteolysis) of a single B$_2$R gene product. Another possibility for the multiplicity of the B$_2$R protein is that its primary transcript could have undergone different kinds of RNA splicing. It is not known at present which explanation is more plausible. In this context, AbdAlla et al. [34] have demonstrated that for human, rat, and mouse B$_2$R, an in-frame ATG codon, which is upstream of the previously reported translation start site, is actually used for initiation of translation. In contrast, there is no such in-frame ATG at the analogous region in the porcine sequence, indicating that synthesis of porcine B$_2$R is initiated at a single ATG codon at position 228. From these considerations, we can at least exclude the possibility that two porcine B$_2$R proteins are generated due to usage of different translation start sites in the pig.

Consistent with the results of the RT-PCR experiments, the in situ hybridization and immunohistochemical analysis of the ovary demonstrated a localization of B$_2$R in the same granulosa cells. The morphological studies revealed that theca cells also express B$_2$R. It is interesting that all growing follicles uniformly express B$_2$R, suggesting that expression of the receptor gene is controlled by one or more factors governing folliculogenesis. A number of factors are known to be involved in this process. Previous studies have clearly demonstrated that FSH, LH, estrogen, and prolactin play a central role in the mechanisms controlling the temporal pattern of gene activation required for the process of folliculogenesis [35]. A recent report of Murone et al. [36] described the localization of B$_2$R in rat uterus and the effects of estrogen and progesterone on the level of B$_2$R in the organ. The authors suggested that B$_2$R levels in the rat uterus are regulated by estrogen, and possibly progesterone, in both myometrium and endometrium. The same explanation may be applicable to the regulation of B$_2$R gene expression in the porcine ovary. Alternatively, the fact that all growing follicles express B$_2$R may indicate that the receptor expression is constitutive and not at all influenced by factors regulating follicular growth. Further detailed studies are necessary to determine which explanation is true.

Detection of B$_2$R expression in all growing follicles indicates that biological responses elicited by bradykinin may be solely dependent on changes in the ligand concentration. We have recently identified all components necessary for bradykinin production in the follicles of porcine ovaries [13, 15]. In addition, the concentrations of bradykinin in the fluid have been shown to fluctuate over a wide range, depending on the size of follicles [15]. Because a number of previous studies have pointed out the role of bradykinin in ovulation [16, 19–23], we were interested in examining its effect on the expression of MMP genes. Using a primary culture system of granulosa cells isolated from porcine ovaries, MMP-3 and MMP-20 expression was found to be notably enhanced in the cells treated with bradykinin (10$^{-8}$ to 10$^{-7}$ M, 48 h). We have also observed that bradykinin treatment causes a moderate increase in MT1-MMP gene expression. It is important to determine whether these MMPs are capable of hydrolyzing collagens, as mammalian ovulation is a process that requires degradation of the collagenous connective tissue in the thecal layers of a mature follicle [37]. MMP-3 [38] and MT1-MMP [39] have been well established as degrading a variety of extracellular matrix (ECM) proteins, including different types of collagens. In contrast, MMP-20 has been shown to cleave not only the major component of teeth ECM, amelogenin [40, 41], but also gelatin, casein, aggrecan, and cartilage oligomeric matrix protein [42, 43]. However, it is not known at present whether MMP-20 can degrade collagens. Nevertheless, our current data tempt us to speculate that the above three bradykinin-inducible MMPs may play a role in the degradation of follicle walls during ovulation. In this regard, we must note some recent reports published by other investigators regarding the involvement of MMPs in ovulation. Haglund and colleagues [44] have examined the ovarian expression of 11 MMPs and three TIMPs during gonadotropin-induced ovulation in the mouse. Based on the observation that, among the MMPs examined, the stromelysin-like enzyme MMP-19 was the only MMP whose expression was induced five to 10 times at the time of ovulation, the authors suggested that MMP-19 may be involved in the tissue degradation occurring during follicular rupture. More recently, the importance of two other proteases, a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS-1) and cathepsin L (a lysosomal cysteine protease), both of which exhibit gene expression in a progesterone-dependent manner, has been documented using mice lacking a progesterone receptor [45]. A significant role of ADAMTS-1 in the inflammatory events of the ovulatory process has also been suggested in the rat [46]. Obviously, further studies are necessary to examine the effects of bradykinin treatment on the expression of MMP-19, ADAMTS-1, and cathepsin L when the nucleotide sequences for the porcine proteases become available.

In this study, we found that MMP-20 is expressed, though at a very low level, in untreated ovary granulosa cells, and that its expression is induced dramatically by bradykinin treatment. These findings were somewhat surprising in view of the general belief that MMP-20 is a tooth-specific protease [40–43]. Because MMP-20 has been proposed to be the predominant protease that processes enamel proteins during the secretory phase of amelogenesis, it would be interesting to examine whether bradykinin could be involved in the enamel protein-processing in teeth through the regulation of MMP-20 gene expression. At any rate, our present data indicate that MMP-20 may play a role not only in the teeth but also in the ovary.

In summary, we have determined the distribution of bradykinin receptor mRNA and its protein using the porcine ovary. Both theca cells and granulosa cells of all growing follicles were found to express the receptor mRNA and the protein. Treatment of isolated granulosa cells with bradykinin induced the expression of the MMP-3 and MMP-20 genes drastically, and MT1-MMP moderately. To our knowledge, this is the first report describing not only the bradykinin receptor localization for a mammalian ovary but also the induction of some MMP genes in the ovary follicle cells in response to bradykinin treatment. These results provide new information for future studies on the mechanisms of bradykinin-induced ovulation in mammalian ovaries.
REFERENCES