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Suppression of Protein Kinase C Signaling by the Novel Isoform for Bovine $PGF_{2\alpha}$ Receptor[1]

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A cDNA clone for a novel isoform of prostaglandin (PG) $F_{2\alpha}$ receptor (FP) was isolated from the cDNA pool of the bovine corpus luteum. The sequence analysis revealed that the new FP isoform (FP_a) encodes a 295-amino acid protein carrying a specific 28-amino acid sequence from the middle of transmembrane segment VI to the carboxyl terminus. Because only one copy gene has been identified for FP, FP_a was generated by alternative mRNA splicing at the middle of the VI transmembrane region, resulting in the lack of a VII transmembrane segment and an intracellular carboxyl tail. The RT-PCR analysis for FP and FP_a indicated that both mRNAs are expressed similarly during the estrous cycle and pregnancy. The $PGF_{2\alpha}$ stimulation drastically enhanced protein kinase C (PKC) activity in the COS-7 cell transfected with FP, whereas no PKC activation was detected in FP_a-transfected cells. Cotransfection of an excess amount of FP_a markedly reduced FP-mediated PKC activity, suggesting that the novel FP isoform might play a role as a negative regulator to attenuate normal FP function.

Key Words: **prostaglandin $F_{2\alpha}$; receptor; isoform; protein kinase C.**

Prostaglandin (PG) $F_{2\alpha}$ is known as one of the bioactive agents produced from arachidonic acid by the cyclooxygenase system (1) to cause a wide range of physiological functions. In mammalian reproductive tissues, $PGF_{2\alpha}$ is predominantly secreted from the uterus or ovary and is responsible for luteal regression during the estrous cycle (2, 3). Pharmacological experiments have revealed that luteal regression is partially caused by luteal cell apoptosis induced by $PGF_{2\alpha}$ (4–6). Thus, as an initiator of luteolysis, $PGF_{2\alpha}$ is known to inhibit steroidogenesis by increasing intracellular Ca^{2+} concentrations, followed by activation of protein kinase C through its binding to a specific receptor (FP) on the transmembrane (7–9). Recent reports have revealed that FP-deficient female mice exhibit a failure of parturition, indicating that $PGF_{2\alpha}$ play a critical role in the female delivery system via its receptors in the uterus (10).

Currently, a number of subtypes and splicing variants involved in multiple physiological functions have been reported for various PG receptors. It is generally understood that PGE_2 receptors can be pharmacologically classified into four subtypes, EP1, EP2, EP3, and EP4, which are linked to different physiological roles by coupling with different G-proteins and signaling pathways (11–14). In addition to these subtypes, several isoforms have been identified in the bovine, mouse, and rabbit as well as human EP3 subtypes (15–18). Because only one copy gene has been identified for EP3, these isoforms appear to be produced by alternative splicing resulting in variations in C-terminal sequences and the coupling of different G-proteins (19–21). Recently, similar splicing variant has been identified for the ovine $PGF_{2\alpha}$ receptor. In addition to an original $PGF_{2\alpha}$ receptor (FP_A), one isoform, namely FP_B, was isolated from the ovine ovarian cDNA library (22). Kristen *et al.* have reported that this new FP isoform, which is generated by mRNA splicing at the C-terminal region, lacks 46-amino acids intracellular tail and demonstrates normal biochemical characteristics of ligand-binding activity, IP_3 accumulation, and intracellular Ca^{2+} mobilization (22). Furthermore, recent reports have revealed that the lack of a C-terminus containing Thr319, Ser337, Ser341, and Thr353, potential phosphorylation sites for protein kinase C, results in different sensitivities to desensitization between these two receptors (23–25). Following the PKC signaling pathway that is induced by $PGF_{2\alpha}$, further tyrosine phosphorylation and mitogen-activated protein kinase (MAPK) activity have been de-

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence database with Accession No. AB061544.

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tected in bovine luteal cells (26) or in osteoblastic MC3T3-E1 cells (27).

To further understand the molecular mechanisms leading to the multiple physiological roles of $PGF_{2\alpha}$, it is critical to determine the diversity of signaling pathways mediated by FP or the FP-related receptor. In our initial approach, we isolated a novel FP isoform from the cDNA pool of the bovine corpus luteum by the 3′-RACE method. This isoform, namely FP_a, was found to be identical to the original FP throughout the six transmembrane domains (7), but diverges 28 amino acids into the carboxyl terminus, which may result in an attenuation of the FP-mediated signaling pathway.

MATERIALS AND METHODS

Animals. Corpora lutea containing ovaries were collected at local abattoir from Holstein cows or Japanese Blacks within 1 h of slaughter. The estrous stage of the corpus luteum was determined by morphological observation of the ovary as previously reported (28), and was characterized as being at an early (3–5 days after ovulation), middle (8–12 days), late (15–18 days), or regressed (20–21 days) stage. After classification of the luteal stage, total RNA was extracted using the guanidinium thiocyanate method as previously described (29). The pregnant stages were classified into three groups (characterized as early; PE 2–3 months, middle; PM 5–6 months, late; PL 8 months) by morphological observation, and total RNAs were isolated.

Cloning of the bovine novel FP isoform by the 3′-RACE method. The total RNAs prepared from each estrous stage were mixed, and were used for cDNA synthesis by M-MLV reverse transcriptase (Sawady) and the d(T) 18 primer. After RNaseH (Takara) treatment, the second-strand cDNA was synthesized by the use of DNA polymerase (Takara) at 16°C for 2 h. The cDNA was purified by NucleotrapCR (Macherey-Nagel), and PCR amplification was then performed with the Super Taq Premix Kit (Sawady) and a pair of primers: HBFCS1, 5′-ATCAAAGACTGGGAAGATAG-3′ and 3PT18T2, 5′-ACTAGCGGCCGCGTCGACTAG(T)18-3′. The PCR products were purified by NucleotrapCR, and second PCR amplification was then performed using the next pair of primers: HBFCS2T, 5′-TAACTAGTCGACGCGGCCGCGTC-AGCAGCACAGACAAGGC-3′ and 3PT18T2. These FP-specific primers were originally designed from the bovine FP genomic sequence (30). The PCR amplification was achieved as follows: first PCR, 95°C for 1 min, 48°C for 1 min, and 74°C for 90 s for 40 cycles; second PCR, 95°C for 1 min, 55°C for 1 min, 74°C for 90 s for 40 cycles. The PCR product was subcloned into pBluescript SK(+) at the *Not*I site. The nucleotide sequence was determined using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) following the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Two micrograms of total RNA from the bovine corpus luteum at each stage of the estrous cycle or pregnancy was reverse-transcribed by M-MLV reverse transcriptase and the d(T) 18 primer. PCR was performed using the following primers: common sense primer, BPGFP31 (5′-AACCAAGCATGTTAAAATG-3′); isoform-specific antisense primers, GSPBFCTA1 (5′-ACTAGCGGCCGCGAATTCGGTGCTTGTTTGCTGAGTTA-3′), and BFPRV-zA1 (5′-ACCATAGATCCTTACCAG-3′). The PCR amplification was carried out for 30 cycles at 95°C for 45 s, 55°C for 45 s, and 74°C for 1 min. The PCR products were electrophoresed on 1% agarose gel and stained by ethidium bromide.

Semi-competitive RT-PCR. The cDNA pools derived from each stage of the estrous cycle or pregnancy were used as templates for the RT-PCR. For the semi-competitive PCR, 50 fg of competitor DNA, the pUC19 plasmid containing rat β-actin cDNA was added to the above cDNA templates, and PCR amplification was carried out using the following primer sets: for FPs, BPGFP31 and GSPBFCTA1 or BFPRV-zA1; for β-actin, M13-M4 (5′-GTTTTCCCAGTCACGAC-3′) and M13-RV (5′-CA-GGAAACAGCTATGAC-3′). The PCR product was then electrophoresed on 1% agarose gel and transferred onto a NC filter (S&S) for Southern hybridization. A total of three separate experiments were performed using independent corpus luteum for each stage of the estrous cycle or pregnancy.

Southern blot analysis. The cDNA fragment encoding FP or β-actin was labeled by ^{32}P and used as the probe DNA. Hybridization was carried out in a medium containing 50% (w/v) formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% (w/v) SDS, and 100 μg/ml salmon sperm DNA at 42°C for 15 h. The filter was then washed with solution containing 0.1 × SSPE and 0.5% (w/v) SDS at 60°C for 30 min. Radioactivity was detected by a BAS-2000 Bioimage analyzer (Fujifilm).

PKC assay. The receptor-mediated signaling pathway was analyzed by measuring the PCK activity in COS-7 cells. Approximately 1×10^5 cells were transfected with 1.5 μg of pEFBOS-BC2211XSTP1 (FP expressing vector), pEFBOS-FP_a (FP_a expressing vector), or pIRES-neo (carrior DNA) by using FuGene6 (Rhose diagnostics). After a 24-h incubation, the cells were treated with 0, 10^{-8}, or 10^{-6} M of $PGF_{2\alpha}$ (Ono Pharmaceutical Co.) for 1 h at 37°C. The cells were then harvested and incubated on ice for 30 min in lysis buffer (50 mM Tris–HCl (pH 7.5), 0.3% β-mercaptoethanol, 5 mM EDTA, 10 mM EGTA, 50 μg/ml PMSF, 10 mM benzamidine, and 1 μg/ml leupeptin). After centrifugation, the cell lysates were collected and used for the PKC assay. PKC activity was measured using the protein kinase C enzyme assay system (Amersham Pharmacia) following the manufacturer's instructions.

RESULTS AND DISCUSSION

Isolation of a cDNA clone for the novel isoform of bovine FP. Screening of the new FP isoform was carried out by the 3′-RACE method using the cDNA pools derived from the bovine corpora lutea in the estrous cycles. The first PCR was performed using a pair of primers, the FP-specific sense primer (HBFCS1) and the d(T)18 primer. After removal of extra primer by NucleotrapCR, another FP-specific primer, HBFCS2T, which is located downstream from HBFCS1, was used for the second PCR with the d(T)18 primer. The PCR products were then subcloned into pUC19 at the *Not*I site and the resultant clones were size-selected by PCR followed by Southern hybridization using the FP-specific probe. After the screening of a total of 150 clones, four FP-positive clones containing identical inserts were isolated. The sequence analysis revealed that this clone encodes a 295-amino acid protein carrying a specific 28-amino acid sequence from the middle of transmembrane segment VI to the carboxyl terminus. Because only one copy gene has been identified for bovine FP (30), this clone, namely FP_a, was characterized as a novel FP isoform that is generated by alternative mRNA splicing. As shown in Fig. 1, the potential splicing site was localized between ntds 800 and 801, the middle region of transmembrane domain VI. Compared to FP (7), this new FP isoform lacks the

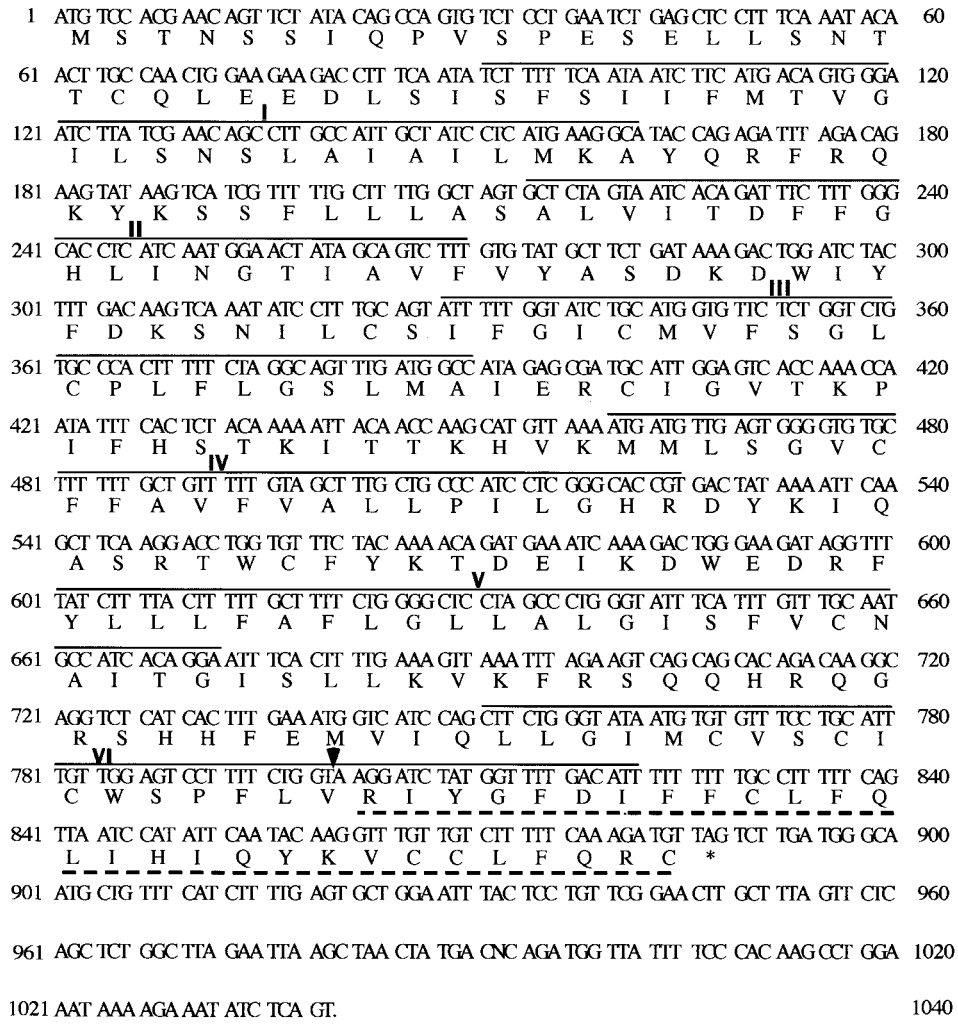

FIG. 1. Nucleotide and deduced amino acid sequences of FP_a. The novel FP_a isoform was cloned by the 3′-RACE methods (see Materials and Methods). The asterisk indicates the termination signal of translation. The arrowhead indicates the potential splicing site. The FP_a receptor has a unique sequence of 28 amino acids at the carboxyl terminus as indicated by the dotted line. The lines above the nucleotide sequence and roman numbers indicate the transmembrane domains.

VII transmembrane segment and the intracellular carboxyl tail.

The ovine FP isoform (FP_B) possesses sequences identical to those of the original FP (FP_A) throughout the seven transmembrane domains, diverging by only nine amino acids (22). In contrast to FP_A, whose carboxyl terminus continues for another 46 amino acids beyond the nine shared residues, the FP_B terminates after only one amino acid. Thus, the ovine FP_A isoform appears to arise by a failure to utilize a potential splice site, while bovine FP_a is generated by alternative mRNA splicing.

According to Kye–Doolittle methods (31), the hydrophobicity of this new isoform was analyzed and compared with that of known FP (Figs. 2a and b, lower panels). As indicated in the graph, FP_a has a truncated C-terminus lacking a deduced VII transmembrane domain and an intracellular carboxyl tail. Because hydrophobic amino acids are colocalized around the truncated region, the secondary structure of FP_a can be predicted as if the C-terminus buried inside the plasma membrane (Fig. 2b, upper panel) or protruding into the extracellular region. In general, the G-coupled proteins are known to associate with cytosolic domains of the receptor, including the carboxyl tail (19, 20), therefore it's hard to believe that the receptor-defecting intracellular C-terminus can effectively couple to the G-protein to allow for a normal signaling pathway.

The rEP_1-variant, an isoform of rat EP_1, is also produced by a failure of mRNA splicing at the predicted splicing site (32). This clone encodes EPI-identical sequences, except for 49 amino acids stretching from the middle of transmembrane segment VI to the carboxyl terminus. Because the hydrophobic amino acids colocalize around the carboxyl terminus, this isoform possesses a transmembrane segment VII-like structure

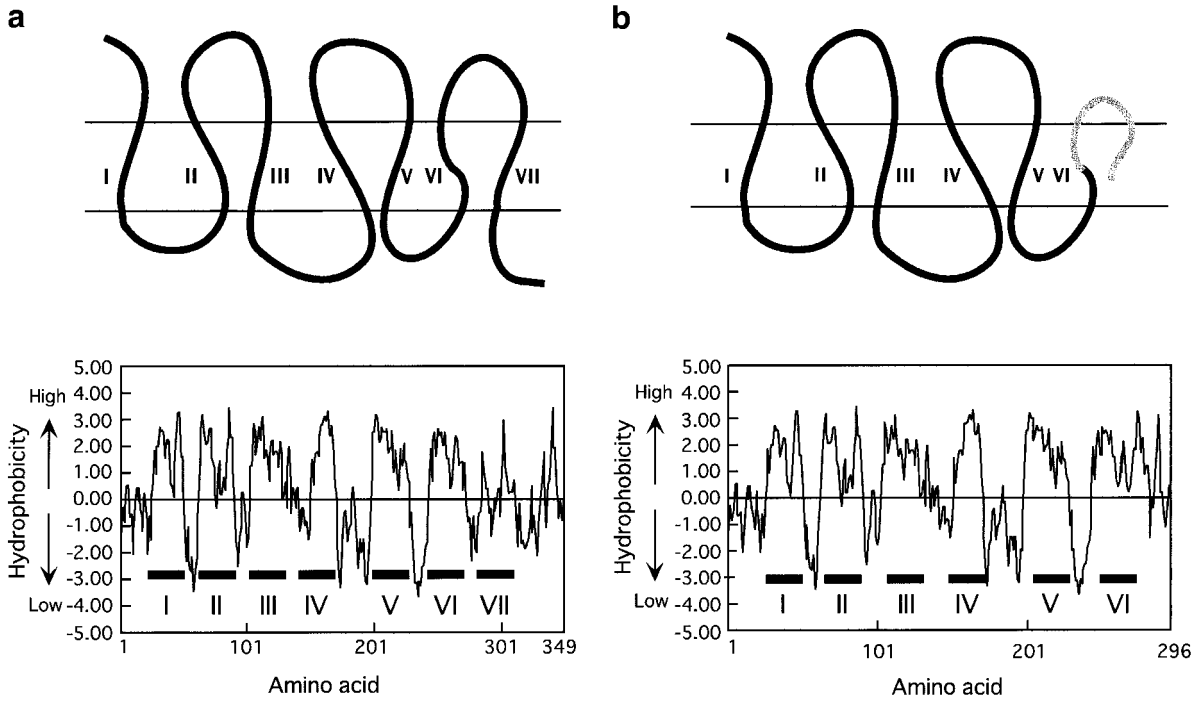

FIG. 2. Deduced two-dimensional structures of the FP_a receptor. The hydrophobicity of FP (a) and the novel FP_a isoform (b) was analyzed by Kyte–Doolittle's method (lower panel), and deduced secondary structures are indicated (upper panel). FP has typical seven-transmembrane motifs, while FP_a contains six potential transmembrane domains with a unique carboxyl-terminus as indicated by the gray line. The roman numbers indicate the transmembrane domains.

and lacks an intracellular carboxyl tail, as similarly observed for bovine FP_a. Pharmacological experiments have indicated that the rEP_1-variant retaines ligand-binding activity with affinity and specificity similar to that of the EPI receptor (32). Although the rEP_1-variant has lost its ability to couple to the signaling pathway by itself, it significantly suppresses the Ca^{2+} mobilization mediated by the original EPI receptor. Thus the EPI isoform receptor might affect the signal coupling of PGE_2 receptors and attenuate the action of PGE_2. Because of the structural resemblance of FP_a to the rEP_1-variant, similar functional characteristics could be predicted for FP_a.

Expression of FP and FP_a in the corpus luteum during the estrous cycle and pregnancy. The mRNA expression of FP_a was then compared to that of FP by the RT-PCR method. The total RNAs prepared from corpora lutea at different stages of estrous cycle or pregnancy were used for cDNA templates for RT-PCR. To avoid the over-amplification of DNA, PCR was performed for a maximum of 30 cycles. In each estrous stage, both FP_a and FP mRNAs were abundantly expressed through the early to late stages (Fig. 3a), while no expression was detected at the regressed phase. Because the RT-PCR for GAPDH showed similar levels of PCR products, these total RNAs were found to be sufficient for use as templates for RT-PCR analysis without any correction (data not shown).

To conveniently analyze the RNA expression during pregnancy, the bovine stages of pregnancy were classified into three groups as described under Materials and Methods, and the total RNAs were prepared from the corpora lutea at each of these stages. As indicated

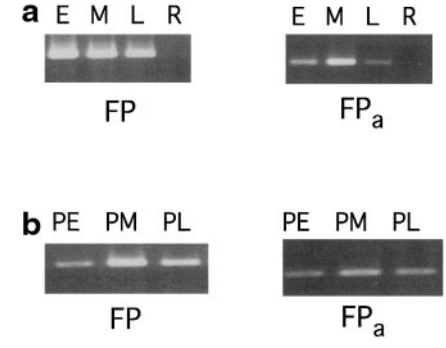

FIG. 3. RT-PCR analysis for FP and FP_a. RT-PCR was performed using the cDNA templates derived from total RNA of the bovine corpus luteum during the estrous cycle and pregnancy. Each cDNA was amplified using common sense primer (BPGFP31) and isoform-specific antisense primers (GSPBFCTA1 or BFPRV-zA1). The PCR product was electrophoresed on 1% agarose gel and stained by ethidium bromide. (a) Estrous cycle: left, FP; right, FP_a, (b) pregnancy: left, FP; right, FP_a. Each estrous stages and pregnant phases were classified as follows: E, early (3–5 days after ovulation); M, middle (8–12 days); L, late (15–18 days); R, regressed (20–21 days); PE, early pregnancy (2–3 months after fertilization); PM, middle pregnancy (5–6 months); PL, late pregnancy (8 months).

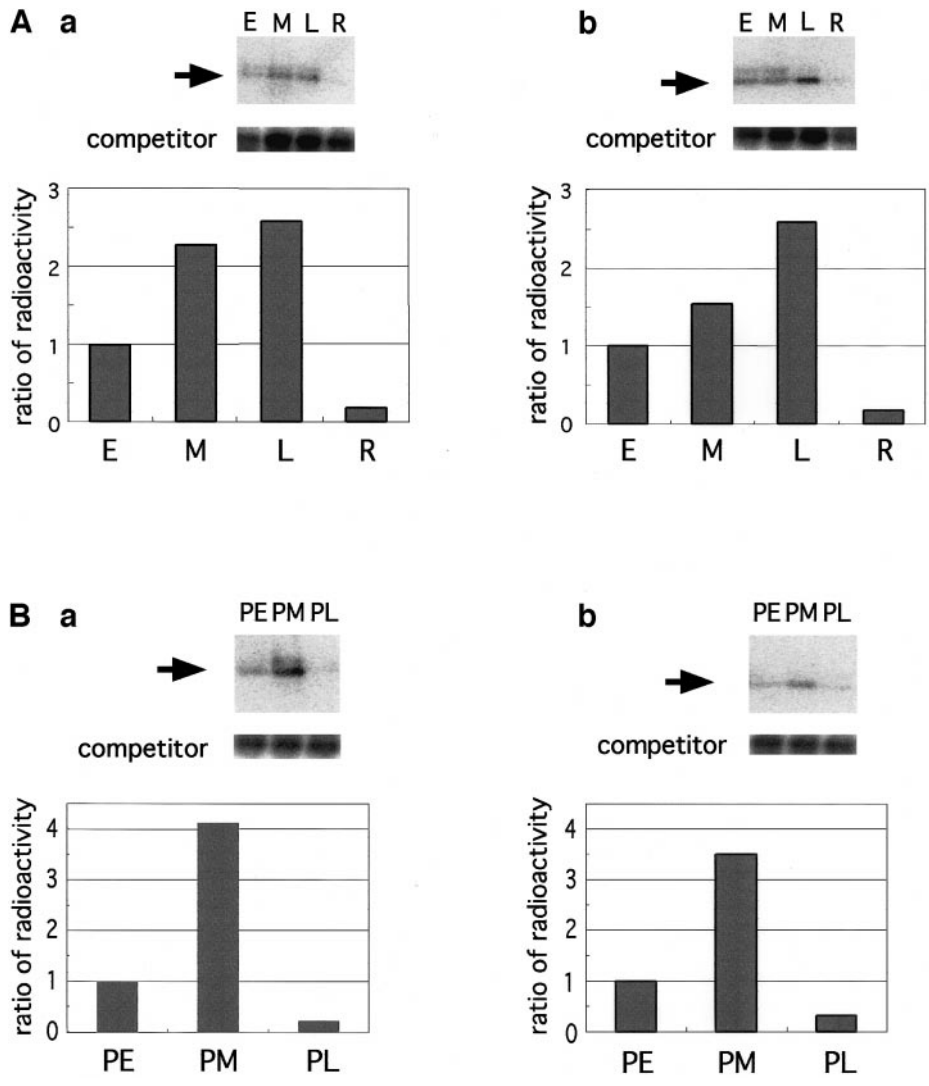

FIG. 4. Semi-competitive RT-PCR analysis for FP and FP_a. To compare the RNA levels of FP (a) and FP_a (b), semi-competitive RT-PCR was performed using total RNAs prepared from bovine CL during the estrous cycle (A) and pregnancy (B). For the semi-competitive PCR, competitor DNA (pUC19 containing rat β-actin cDNA) was added to each PCR mix. The PCR products were detected by Southern blot hybridization (each of the upper panels). Arrows indicate hybridized signals for FP (a) and FP_a (b). Hybridized signals for β-actin are indicated as competitors. Radioactivities were quantitatively measured by BAS-2000 Bioimaging analyzer, and radioactive ratios are plotted on the graph.

in Fig. 3b, FP_a expression was found to be equivalent to that of FP throughout the stages of pregnancy. The mRNA levels of FP and FP_a in the middle stage of pregnancy were found to be comparatively higher than those of other stages. A total of three separate experiments were carried out using independent corpora lutea from the estrous cycle or pregnancy.

Semi-competitive RT-PCR analysis of FP and FP_a mRNA levels throughout the estrous cycle and pregnancy. To further compare the RNA levels of FP and FP_a quantitatively, semi-competitive RT-PCR was carried out using RNAs prepared from the corpus luteum obtained during the estrous cycle or pregnancy. As the internal competitor of PCR, the pUC19 plasmid carrying rat β-actin DNA was mixed with the luteal cDNA templates with pUC19-specific primers and FP- or FP_a-specific primers, resulting in the generation of DNA fragments specific to β-actin and FP or FP_a. Each PCR product was then detected by Southern blot analysis (Fig. 4A, upper panels). The PCR product for FP_a was found to gradually increase throughout the estrous cycle from early to late, and then to markedly dropped at the regressed phase (Fig. 4A(a)), the same as that of FP (Fig. 4A(b)), suggesting that mRNA expression for both products is commonly regulated during the estrous cycle. Because the levels of competitor DNAs were found to remain stable throughout the estrous cycle, the tendency of mRNA expression seems to be

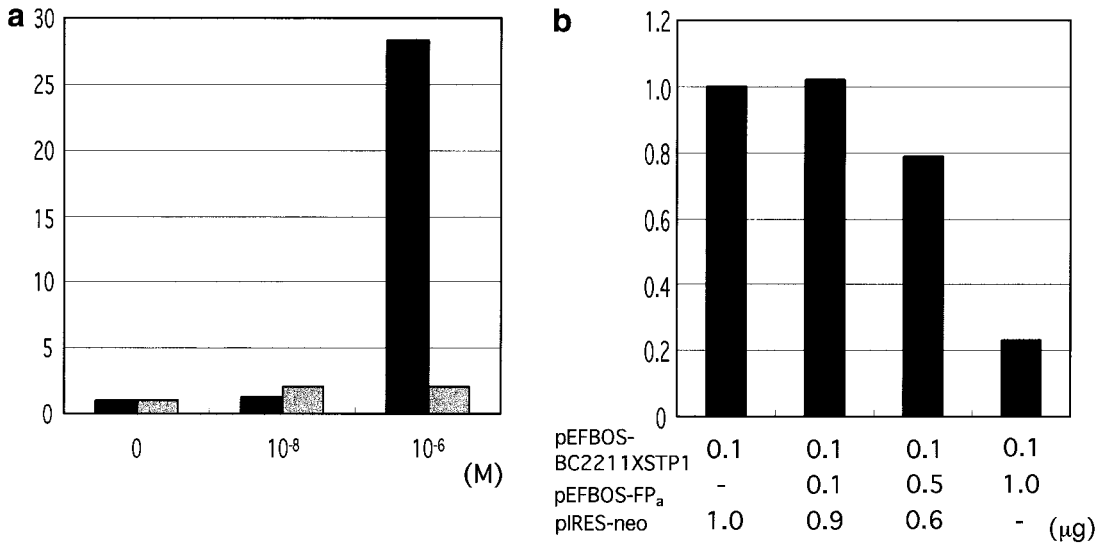

FIG. 5. PKC assay for FP and the FP_a receptor. The COS-7 cells were transiently transfected with pEFBOS-BC2211XSTP1 (FP, FP expressing vector), pEFBOS-FP_a (FP_a, FP_a expressing vector), and/or pIRES-neo by FuGene6. After a 24-h incubation, the cells were treated with 0, 10^{-8}, 10^{-6} M (a) or 10^{-6} M (b) of $PGF_{2\alpha}$ for 1 h, and then cell lysates were prepared for the following PKC assay: (a) PKC activity stimulated by $PGF_{2\alpha}$ in COS-7 cells expressing FP (black bar) or FP_a (grey bar); (b) inhibition of FP-mediated PKC activity by the cotransfection of pEFBOS-FP_a. An excess amount (0, 0.1, 0.5, 1.0 μg) of pEFBOS-FP_a was transfected with 0.1 μg of pEFBOS-BC2211XSTP1, and the resultant PKC activity was plotted on the graph. The pIRES-neo was used as a carrier DNA to normalize the total amount of DNA for transfection.

equivalent between FP_a and FP during the estrous cycle. We have previously described that FP levels are strictly up-regulated as the estrous cycle progresses, particularly in the late estrous stage, and have discussed that changes in FP expression can act as a trigger for luteal cell apoptosis (33). In addition to the FP expression, FP_a mRNA levels were found to reach a maximum in the late estrous phase, suggesting that this novel isoform participates in cellular function in cooperation with normal FP.

The mRNA expressed in the corpus luteum throughout pregnancy was also examined quantitatively by a semi-competitive RT-PCR method. As indicated in Fig. 4B, the mRNA levels of both FP_a and FP in middle pregnancy were found to be significantly higher than those of the early stage, and these levels then markedly dropped at the late stage. Because the levels of both FP_a and FP mRNAs are dramatically increased in middle pregnancy, some physiological function related to pregnancy can be hypothesized. A total of three independent experiments were performed using separately prepared corpora lutea for the estrous cycle or pregnancy. Comparatively, the overall mRNA levels of FP seemed to be more abundant than those of FP_a.

Taken together, these observations suggest that both bovine FP_a and FP mRNAs are commonly expressed under the same regulatory mechanisms in the corpus luteum during the estrous cycle and pregnancy.

PKC assay for FP and FP_a. Currently, it is well understood that $PGF_{2\alpha}$ activates phospholypase C (PLC) via FP and induces the following signaling pathway. Because PKC is generally involved in receptor phosphorylation, desensitization, and the signaling cascade underlying cellular apoptosis, it is critical to clarify how protein kinase activity is up-regulated by the action of $PGF_{2\alpha}$ and its receptor. To further understand whether the novel FP isoform could be involved in the signaling pathway of $PGF_{2\alpha}$, PKC activity was analyzed in FP_a-transfected COS-7 cell. The FP_a-expressing vector, pEF-BOSFP_a, was transiently transfected in the COS-7 cell by FuGene6, and the PKC activity was measured after stimulation with different amounts of $PGF_{2\alpha}$ (Fig. 5a). As clearly observed in the figure, FP_a-expressing cells did not exhibit PKC activation, while FP-expressing cells showed a strong response to 10^{-6} M $PGF_{2\alpha}$ (Fig. 5a). Because FP_a showed no induction of kinase activity in response to $PGF_{2\alpha}$, PKC activities were analyzed by the co-transfection of FP with an excess of FP_a cDNA in COS-7 cells. As indicated in Fig. 5b, FP-mediated PKC activity was strictly blocked by adding the excess of FP_a DNA, especially by adding a 100-fold excess of FP_a DNA. Those data strongly support the hypothesis that FP_a can suppress or attenuate the signaling pathway induced by $PGF_{2\alpha}$ through its coupling to conventional FP.

As previously described for the ovine FP isoform, FP_B exhibits radioligand-binding properties that are indistinguishable from those of the original FP (FP_A) (22). Furthermore, the FP_B isoform effectively stimulated inositol phosphate accumulation to the same absolute maximum as does FP_A. Moreover, the rEP_1-variant, the truncated receptor isoform generated by

mRNA splicing of the EP1 subtype, also retains specific binding activity with affinity to PGE_2 (32). Although the rEP_1-variant didn't exert the signaling pathway by itself, it interfered with the signal transduction induced by the action of the original FP. Because the bovine FP_a has structural characteristics similar to those of the rEP_1-variant (32), similar functional properties as a modulator of signal transduction can be predicted.

The results of the present study represented the novel function of the isoform of bovine $PGF_{2\alpha}$ receptor in the luteolysis during estrous cycle or in the maintenance of pregnancy. The physiological significance of FP_a expression synchronized to those of FP in the corpus luteum during the estrous cycle and pregnancy is remained to be resolved. Moreover, to clarify the signal transduction of FP_a more precisely, ligand-binding properties such as binding activity with affinity and ligand specificity will be examined for the bovine FP isoform.

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