Biochemical and Biophysical Research Communications **277,** 270–279 (2000)
doi:10.1006/bbrc.2000.3667, available online at http://www.idealibrary.com on IDEAL®

Progesterone Receptor Activation Mediates LH-Induced Type-I Pituitary Adenylate Cyclase Activating Polypeptide Receptor (PAC_1) Gene Expression in Rat Granulosa Cells

Chemyong Ko and Ok-Kyong Park-Sarge
Department of Physiology, University of Kentucky, Lexington, Kentucky 40536

Received September 12, 2000

We have previously reported that the pituitary adenylate cyclase activating polypeptide (PACAP) gene is regulated in ovarian granulosa cells by the autocrine and/or paracrine interaction between progesterone and its nuclear receptor progesterone receptor (PR). To initiate studies on the functional significance of the progesterone-induced PACAP production in luteinizing granulosa cells, we sought to determine the expression and hormonal regulation of PACAP receptors in the rat ovary. The relative mRNA levels of three known PACAP receptor subtypes (PAC_1, $VPAC_1$, and $VPAC_2$) were determined in ovaries of immature rats treated with gonadotropins, by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assays. Results show that all PAC_1, $VPAC_1$, and $VPAC_2$ transcripts are expressed at a detectable level in immature rat ovaries. Importantly, the ovarian level of PAC_1, but not $VPAC_1$ or $VPAC_2$, mRNA notably changes during gonadotropin challenges. Ovarian PAC_1 mRNA expression decreases during the pregnant mare's serum gonadotropin (PMSG)-induced follicular phase but substantially increases during the human chorionic gonadotropin (hCG)-induced periovulatory period. Because the hCG-induced increase in ovarian PAC_1 mRNA expression is attributable to the hormone-induced PAC_1 mRNA expression in granulosa cells of the preovulatory follicles, we next examined whether hCG regulates PAC_1 mRNA expression by directly acting on granulosa cells. When granulosa cells isolated from PMSG (40 h)-primed immature rats were challenged with hCG (or forskolin), PAC_1, but not $VPAC_1$ or $VPAC_2$, mRNA expression significantly increased within 6 h. Because the LH-induced PAC_1 mRNA expression (6 h) proceeds PR activation (3 h) in granulosa cells as the LH-induced PACAP mRNA expression (6 h) does, we further determined the cause–effect relationship among LH, PR activation and PAC_1 receptor gene expression, by examining the effect of PR antagonist, ZK98299, on the ability of LH to increase PAC_1 mRNA levels in luteinizing granulosa cells. Results show that ZK98299 inhibited the stimulatory effect of hCG (or forskolin) on PAC_1 mRNA expression, at the level of all known splice variants of PAC_1 mRNA in granulosa cells. In summary, our results demonstrating that PR activation is critical for the LH-induced PAC_1 gene expression in luteinizing granulosa cells suggest that PR activation regulates the finely tuned expression of the PACAP/PACAP receptor genes in luteinizing granulosa cells and thus dictates the timing of the autocrine and/or paracrine function of PACAP in preovulatory follicles. © 2000 Academic Press

Key Words: **PR; PACAP; PACAP receptor; granulosa cells.**

The LH-induced PRs in the granulosa cells of preovulatory follicles (1, 2) are known to mediate the autocrine/paracrine effect of progesterone in luteinizing granulosa cells (3, 4). Although the infertile phenotype of PR null mutant mice (5, 6) underscores the importance of PR activation in the ovary, the scarce knowledge of events downstream from PR activation has hampered our understanding of the functional role(s) that PRs play in luteinizing granulosa cells. We have recently reported PACAP gene expression as one such PR downstream event (7). The preovulatory LH surge induces PACAP mRNA (7–9) and protein (10) in the granulosa cells of Graafian follicles. PACAP mRNA expression follows PR expression in the granulosa cells of preovulatory follicles *in vivo* during proestrous evening and during hCG-induced periovulatory period, as well as in cultured granulosa cells undergoing hCG-induced luteinization (7). Importantly, a progesterone receptor antagonist, ZK98299 inhibits the LH-induced PACAP mRNA expression without affecting PR expression in cultured granulosa cells (7). In addition, a progesterone synthesis inhibitor 3β-hydroxysteroid dehydrogenase (epostane) also blocks the LH-induced PACAP mRNA expression in preovulatory follicles (11). Thus, it is tempting to suggest that PACAP may

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play an intermediate role(s) in progesterone-stimulated preovulatory processes.

In granulosa cells cultured *in vitro*, LH stimulates not only PACAP gene expression (7) but also PACAP secretion into media (10, Ko and Park-Sarge, unpublished). Thus, it is likely that the preovulatory LH surge will increase intrafollicular concentration of PACAP which can be taken up by cells expressing its receptors and, in turn, exert autocrine and/or paracrine intraovarian actions. PACAP binding activities in granulosa/luteal cells, cumulus cells, and oocytes (12) may be responsible for the PACAP-regulated granulosa cell functions (10, 12–14) as well as oocyte meiosis (15). The question of which PACAP receptor subtypes(s) represents PACAP binding activities in these cells is not clear. Among the three different subtypes of PACAP receptors, type I (PAC_1), type II ($VPAC_1$), and type III ($VPAC_2$) (16–19), PAC_1 has been demonstrated to be expressed in granulosa cells of preovulatory follicles (20, 21), oocytes (12, 15, 22), and even in primordial germ cells (23). We have extended these studies by determining the expression and hormonal regulation of PACAP receptors in the rat ovary. Our results demonstrate that PAC_1, but not $VPAC_1$ or $VPAC_2$, mRNA expression is regulated in luteinizing granulosa cells by the preovulatory LH surge and is also tightly regulated by PR activation. This progesterone-regulated, finely tuned expression of PACAP/PACAP receptor in luteinizing granulosa cells should provide important insight into molecular cascades of progesterone-induced periovulatory processes.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle Media (DMEM), Hams-F12 and antibiotics for tissue culture were from Gibco-BRL (Gaithersburg, MD). Restriction enzymes and reverse transcriptase were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Intergrated DNA Technology Inc. (Coralville, IA). The nucleotide, [α-^{32}P]dCTP and [α-^{35}S]UTP, were from New England Nuclear (Boston, MA) or Amersham Pharmacia Biotech (Piscataway, NJ). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Animals and hormone treatments. All animals were handled according to the NIH guidelines for care and use of animals, and all protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee. Twenty-one-day-old Sprague-Dawley female pups with nursing mothers were purchased from Harlan Breeding Company (Indianapolis, IN) and housed in a photoperiod of 14 h light/10 h darkness with light on at 0500 h. For gonadotropin challenges, 22–23 day old rats were injected s.c. with 15 IU PMSG in 0.1ml PBS and subsequent hCG (10 IU) injections were performed i.p. for those rats primed with PMSG (15 IU) for 48 h. Ovaries of these rats were isolated for ovarian RNA isolation, granulosa cell isolation, or *in situ* hybridization histochemistry.

Granulosa cell isolation and culture. Granulosa cells of PMSG (15 IU, 40 h)-primed immature rats were isolated by the method of follicular puncture (24), with minor modifications (7). Ovaries were collected in cold serum-free 4F medium consisting of 15 mM HEPES (pH 7.4), 50% DMEM, and 50% Ham's F12 with bovine transferin (5 μg/ml), human insulin (2 μg/ml), hydrocortisone (40 ng/ml), and antibiotics. Cells were collected and plated in 4F medium supplemented with 5% fetal bovine serum (FBS, Gibco) at a density of approximately 1–1.5 $\times$ 10^6 cells per 60 mm dish, and incubated in the humidified atmosphere of 5% CO_2 at 37°C until hormone treatment.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from whole ovaries and cultured granulosa cells was isolated by homogenization in a guanidium thiocyanate solution and subsequent centrifugation through a cesium-chloride gradient ultracentrifuge. RT-PCR was performed essentially as previously described (7). Total RNA (2–3 μg) was reverse-transcribed at 37°C in a 20 μl reaction volume using random hexamer (500 ng) and MMLV reverse transcriptase (10 units) (New England BioLabs, Boston, MA). Complementary DNA (cDNA) samples (2 μl) were added to a total 25 μl reaction mix containing the primers (20 nM each), [α-^{32}P]dCTP (1 μCi at 3000 Ci/mmol), and Taq DNA polymerase (2.5 U) in 1$\times$ PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin). All PCR amplifications were carried out for 20, 25, 30, and 35 cycles on a Perkin Elmer Cetus thermocycler (Perkin Elmer Cetus, Norwalk, CT) using annealing temperatures provided for each primer set (see below). The samples were then electrophoresed on 8% nondenaturing polyacrylamide gels. The intensity of the expected PCR band was analyzed using a Molecular Dynamics Phosphoimager and ImageQuant version 3 software (Molecular Dynamics, Sunnyvale, CA). Quantitation of each gene product was obtained from the cycles in a linear range of amplification. Typically, PACAP and S16 PCR products were saturated at 30 cycles whereas PACAP receptor PCR products remained in a linear range of amplification even during 25–30 cycles. PACAP and PACAP receptor RT-PCR signals were normalized to those of the ribosomal protein S16 internal control. The sequences and annealing temperatures for individual gene products are as following: the rat PACAP (25) (5′-ctt tgc ccg ccg tcc tat tta-3′ and 5′-gtg aag atg ccg tcc gag tgg-3′, 452 bp, 62C), rat PAC_1 (16) (R1F, 5′-ttt cat cgg cat cat cat cat cct t-3′ and R1R, 5′-cct tcc agc tcc tcc att tcc tct t-3′, 289 bp for short and 373 bp for Hip or Hop splice variant forms, 62C), rat $VPAC_1$ (26) (5′-cat agg gac aca ctc ctt tgc cg-3′ and 5′-agg tag aag aac tca ccc acc cag aag-3′, 350 bp, 62C), rat $VPAC_2$ (27) (5′-ccc agt gtg tgt ata ggt gca-3′ and 5′-caa aca cca tgt agt gga cgc-3′, 308 bp, 62C), and rat S16 (28) (5′-tcc aag ggt ccg ctg cag tc-3′ and 5′-cgt tca cct tga tga gcc cat t-3′, 100 bp, 62C).

Southern blotting. RT-PCR products using combinations of primers {R1F (5′-ttt cat cgg cat cat cat cat cct t-3′), R1R (5′-cct tcc agc tcc tcc att tcc tct t-3′), Hop1 (5′-tcc agc atc tac ttc agc tg-3′), Hop2 (5′-tcc agc atc tac ttc tgc gt-3′), Hip (5′-tgc cct cag acc agc att ca-3′), EC1F (5′-acg acc tga tgg gac taa acg-3′), and EC1R (5′-act gtg tag aga gcc ttc acc-3′, 60C)} were separated on a 1.8% agarose gel, transferred onto a nytran membrane (S & S), and baked at 80C for 2 h. The blots were hybridized with [α-^{32}P]dCTP-labeled probes generated from the PAC_1 clones corresponding to the probe A (353pb corresponding to the nucleotides 152–505), the probe B (289 bp corresponding to the nucleotides 1080–1369), or the probe C (1134 bp; nucleotide no.152–1369, L16680, respectively; for probe location, see Fig. 5) at 42°C for 8 h in a solution consisting of 50% formamide, 5$\times$ SSC, 2$\times$ Denhardts reagent, 10% Dextran sulfate, 0.1% SDS, and 100 μg/ml salmon sperm DNA. Blots were washed to the stringency of 0.1$\times$ SSC at 68°C and then exposed to a phosphoimager screen for 4 h.

In situ hybridization. Frozen ovaries were cut in 20 μm sections using a MICROM HM 505 E cryostat (Microm Labogerate GmbH, Germany) and mounted onto Superfrost/Plus Microscope slides (Fisher, PA). Sections were fixed in 5% paraformaldehyde, pretreated with triethanolamine followed by acetic anhydride, and hybridized with antisense and sense RNA probes as previously described (Park *et al.*, 1991). [^{35}S]UTP-labeled PACAP RNA probe was synthesized from the clone pSP72-PACAP encoding the rat PACAP (7). For PACAP receptor probes, PAC_1 (nucleotides 152–1369, L16680), $VPAC_1$ (nucleotides 1527–1876, M86835) and $VPAC_2$ (nu-

cleotides 774–1082, D28132) cDNA fragments were cloned into PCR-2.1[TOPO] and the riboprobes were synthesized using T7 or T3 polymerase. Probes ($1–4 \times 10^7$ cpm/ml in hybridization buffer: 50% formamide, 5× SSPE, 2× Denhardt's reagent, 10% dextran sulfate, 0.1% SDS, and 100 μg/ml yeast tRNA) were applied to sections and the sections were incubated in a humidity chamber at 47–55°C for 16–18 h. After hybridization, sections were treated with RNase A (20 μg/ml) at 37°C for 40 min, washed in increasingly lower concentrations of SSC down to 0.1× SSC at 60°C, and dehydrated through an ethanol series. Slides were then exposed to Kodak XAR-5 film (Kodak, Rochester, NY) for 2 days, and the film was directly scanned for inverse image using the Nikon LS-1000 film scanner (Nikon Corporation, Japan).

RESULTS

To initiate studies on the functional significance of progesterone-induced PACAP production in luteinizing granulosa cells, we sought to determine the expression and hormonal regulation of PACAP receptors in the rat ovary. Initially, the relative mRNA levels of three known PACAP receptor subtypes (PAC_1, $VPAC_1$, and $VPAC_2$) were determined, by semiquantitative RT-PCR assays, in ovaries of immature rats treated with PMSG (15 IU for 0, 24, and 48 h) and PMSG (15 IU, 48 h) followed by hCG (10 IU for 1, 3, 6, 9, and 12 h). We amplified PAC_1, $VPAC_2$, and $VPAC_2$ mRNAs from the same cDNA samples for 20–35 cycles using gene-specific primers. The primers R1F and R1R (for location, see Fig. 5) were selected to amplify PAC_1 transcripts because this primer set should amplify all known PAC_1 isoforms with the different third intracellular loop. Upon electrophoresis and quantitation of PCR products, we determined the linear range of amplification of PAC_1, $VPAC_2$, $VPAC_2$, and S16 products. The normalized data (PACAP receptor/S16) from the condition resulting the linear range of amplification (S16 between 20–25 cycles, PAC_1, $VPAC_1$, and $VPAC_2$ between 25–30 cycles) are presented. Results show that immature rat ovaries express all three PAC_1, $VPAC_1$ and $VPAC_2$ mRNA species at a detectable level (Fig. 1, $n = 2$). We identified two clearly amplified PAC_1 bands that correspond to the 289-bp short isoform with neither hip nor hop exon, and the 454-bp hip/hop isoform. Further restriction digestion and DNA sequencing analyses revealed that this 454-bp hip/hop band is a mixture of the hip1/hop1 and hip1/hop2 isoforms. We also identified the expected $VPAC_1$ (350 bp) and $VPAC_2$ (308 bp) RT-PCR products, although they required more cycles to be amplified, suggesting that the $VPAC_1$ and $VPAC_2$ transcripts may be less abundant than the PAC_1 transcript, in the rat ovary. Interestingly, total ovarian PAC_1 mRNA decreases significantly during the PMSG-induced follicular growth while a subsequent injection of hCG induces a transient increase in PAC_1 mRNA expression within 6 h, in agreement with the previous report (20). Importantly, neither $VPAC_1$ nor $VPAC_2$ mRNA levels are significantly altered during hormone treatments. We

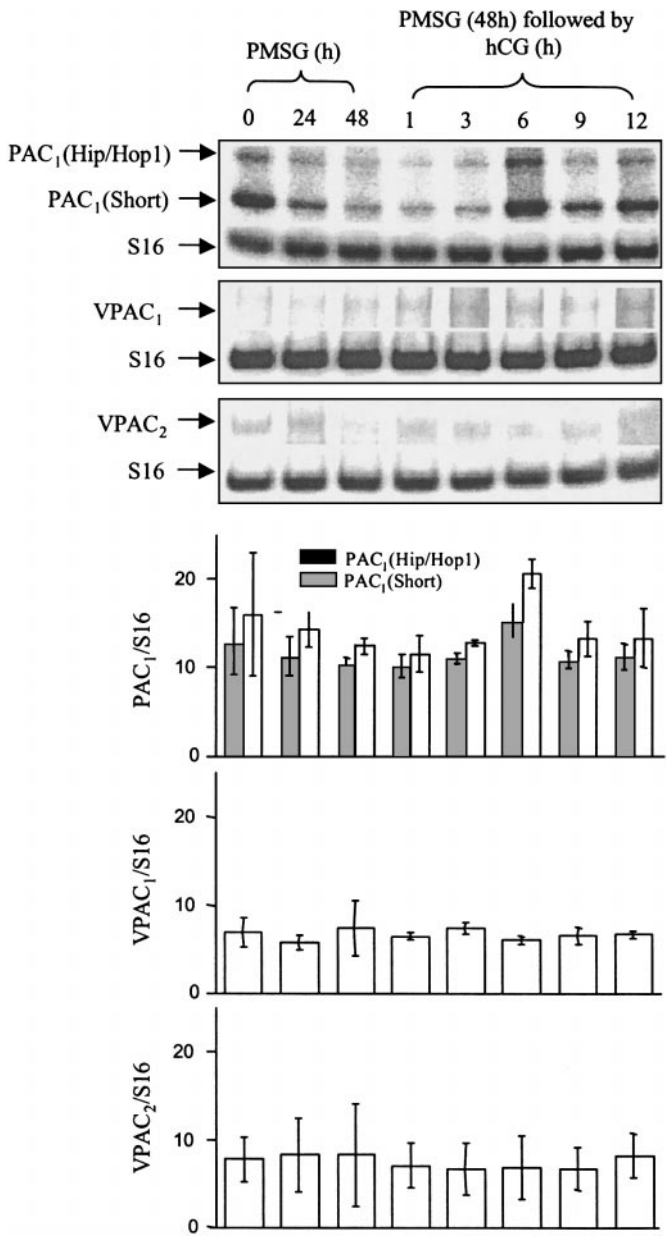


FIG. 1. Gonadotropins regulate PAC_1, but not $VPAC_1$ or $VPAC_2$ mRNA expression in the ovary. Ovaries were collected from immature rats that were untreated (control), treated with PMSG (15 IU) for 24 or 48 h, and PMSG (15 IU, 48 h) followed by hCG (10 IU) treatment for 3, 6, 9, 12, and 24 h. RNA (2 μg) from these ovaries was assayed for PAC_1, $VPAC_1$, or $VPAC_2$ mRNA along with an internal control S16 using semiquantitative RT-PCR reactions. PCR products were taken from each sample after 20, 25, 30, and 35 cycles of amplification to ensure the linear range of amplification of each product. Shown are PAC_1, $VPAC_1$, and $VPAC_2$ RT-PCR products after 25 cycles of amplification along with S16 RT-PCR products after 20 cycles of amplification, both of which are within the linear range of amplification. A representative autoradiogram of polyacryamide gels is shown on the top, along with quantitation data on the bottom. PAC_1, $VPAC_1$, and $VPAC_2$ signals were normalized using S16 signals in each sample, after band intensity was estimated using ImageQuant version 3 software on a phosphorimager. The data shown are the mean ± SD of the two independent experiments. Notice that PAC_1, but $VPAC_1$ or $VPAC_2$ mRNA expression is modulated by gonadotropins.

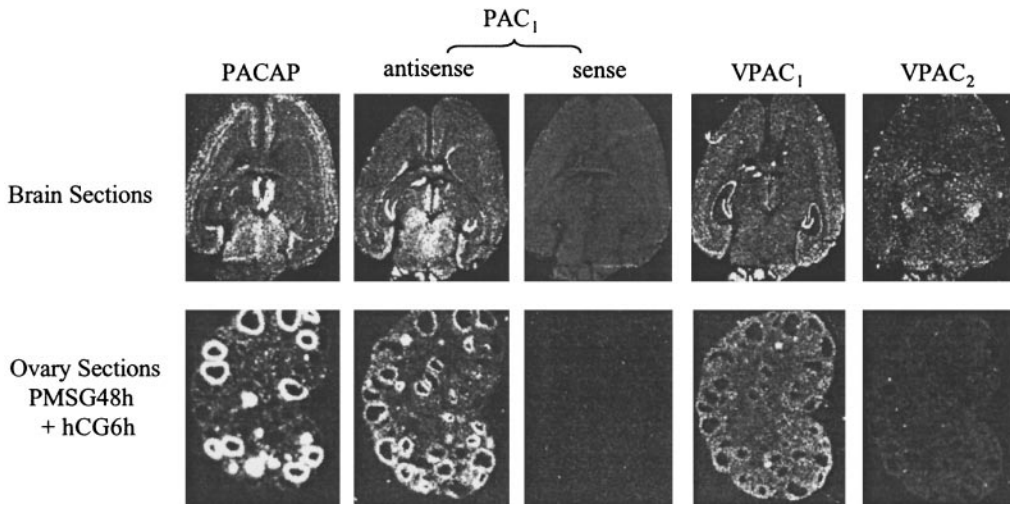


FIG. 2. PAC_1, but not $VPAC_1$ or $VPAC_2$ mRNA expression is cell-specific in the ovary. Ovarian sections of immature rats primed with PMSG (15 IU, 48 h) followed by hCG (10 IU, 6 h) were used to localize PAC_1, $VPAC_1$, and $VPAC_2$ mRNAs at a cellular level using *in situ* hybridization. Brain sections of adult female rats were used to demonstrate the specificity of the probes used. ^{35}S-labeled antisense probes against PAC_1, $VPAC_1$, and $VPAC_2$ mRNAs along with an ^{35}S-labeled sense probe against PAC_1 were hybridized to ovarian and brain sections. ^{35}S-labeled PACAP antisense probe was used to orient preovulatory follicles. After hybridization, tissue sections were exposed to Kodak XAR-5 film for 2 days and the film was directly scanned for inverse images using the Nikon LS-1000 film scanner. Magnifications were ×5 for brain sections and ×100 for ovarian sections. Notice that PAC_1, but not $VPAC_1$ or $VPAC_2$ mRNA expression is cell-specific in the ovary.

further examined ovarian PAC_1, $VPAC_1$, and $VPAC_2$ mRNA expression at a cellular level, by using *in situ* hybridization histochemistry in immature rats primed with PMSG (15 IU, 48 h) followed by hCG (10 IU, 6 h), the time of the peak expression of the PAC_1 gene. In order to demonstrate the sensitivity and specificity of the probes used, we also used brain sections of randomly cycling adult rats. Under the conventional *in situ* condition specifically detecting PAC_1 mRNA in brain sections (17, 29–30), we detected ovarian PAC_1 mRNA expression in a cell-specific manner (Fig. 2). In agreement with the previous report (20, 31), PAC_1 mRNA was easily detected in granulosa cells of virtually all healthy small follicles. Lower PAC_1 mRNA expression was observed in later stages of nonpreovulatory antral follicles. Importantly, PAC_1 mRNA was easily detected in the granulosa cells of preovulatory follicles that are defined to express PACAP mRNA (7), suggesting that hCG induced PAC_1 mRNA expression in the granulosa cells of preovulatory follicles. In contrast, under the conventional *in situ* condition specifically detecting $VPAC_1$ and $VPAC_2$ mRNA species in brain sections as reported previously (17, 30, 32), neither $VPAC_1$ nor $VPAC_2$ mRNA expression was cell-specific in ovarian sections. $VPAC_1$ or $VPAC_2$ mRNA was detected at low level in agreement with our RT-PCR assays (Fig. 1). Taken together, these results suggest that the PAC_1 gene is expressed in the undifferentiated granulosa cells of growing follicles, inhibited by PMSG-induced signals, and stimulated by hCG in luteinizing granulosa cells of preovulatory follicles.

To understand the mechanisms by which the preovulatory LH surge stimulates PAC_1 gene expression in the granulosa cells of preovulatory follicles, we first determined whether LH induces PAC_1 mRNA expression by directly acting on granulosa cells of preovulatory follicles. Granulosa cells were isolated from PMSG (15 IU, 40 h)-primed immature rats and treated with hCG (1 IU/ml, data not shown) or forskolin (10 μM, Fig. 3). PAC_1 mRNA expression, as estimated by RT-PCR assays, was significantly induced by these hormonal agents and peaked in 6 h. In contrast, $VPAC_1$ and $VPAC_2$ mRNA expression was low and unchanged by these hormonal agents. The close temporal correlation between PACAP and the PAC_1 mRNA expression, both of which peak within 6 h after hCG (or forskolin) stimulation, but with a significant temporal gap from PR expression which peaks within 3 h after hCG (7, 33), prompted us to examine whether PR activation is critical for the LH-induced PAC_1 mRNA expression. Granulosa cells were isolated from PMSG (15 IU, 40 h)-primed immature rats, cultured *in vitro,* and received ZK98299 (10 μM) or vehicle 1 h prior to forskolin (10 μM) or hCG (10 IU/ml). Cells were incubated for additional 6 h, the time required for the peak of the hCG-induced PAC_1 mRNA expression. Both hCG and forskolin effectively induced PAC_1 mRNA expression, as estimated by RT-PCR assays, in these cells (Fig. 4). Importantly, ZK98299 effectively blocked the hCG- or forskolin-induced PAC_1 mRNA expression, indicating the critical role that PRs play for the LH-induced PAC_1 gene expression in luteinizing granulosa cells. In con-

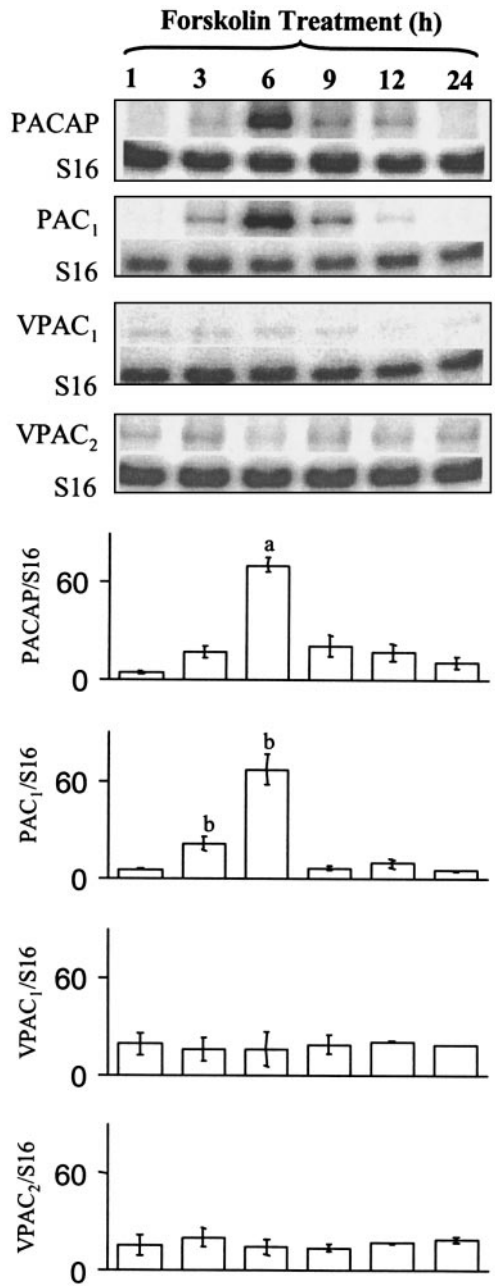


FIG. 3. Forskolin induces PAC_1 mRNA expression in granulosa cells cultured *in vitro*. Granulosa cells from PMSG (15 IU, 40 h)-primed immature rats were incubated with vehicle or forskolin (10 μM) for 1, 3, 6, 9, and 12 h. RNA from these granulosa cells was assayed for PAC_1, $VPAC_1$, or $VPAC_2$ mRNA along with an internal control S16 using semiquantitative RT-PCR reactions. PACAP mRNA was also assayed as comparison. PCR products were taken from each sample after 20, 25, 30, and 35 cycles of amplification to ensure the linear range of amplification of each product. Shown are PACAP, PAC_1, $VPAC_1$, and $VPAC_2$ RT-PCR products after 25 cycles of amplification along with S16 RT-PCR products after 20 cycles of amplification, both of which are within the linear range of amplification. A representative autoradiogram of polyacryamide gels is shown on the top, along with and quantitation data on the bottom. PAC_1, $VPAC_1$, and $VPAC_2$ signals were normalized using S16 signals in each sample, after band intensity was estimated using Image-Quant version 3 software. The data shown are the mean ± SEM of the three independent experiments. Data were analyzed by ANOVA followed by Student's Newman-Keul test. Groups with different superscript letters are significantly different from control (hCG 1 h) (a, for PACAP; b, for PAC_1) ($P < 0.001$).

trast, hCG, forskolin, or ZK98299 affected neither $VPAC_1$ nor $VPAC_2$ mRNA.

We sought to further determine whether the LH-induced and PR-mediated PAC_1 mRNA expression reflects changes in specific PAC_1 variants for the following reasons. First, multiple PAC_1 variants are produced with minor differences in the sizes of alternative exons. Second, some of these PAC_1 variants may use alternative signaling pathways (16–17). Third and importantly, we (Fig. 1) and others (20, 34) detected short and Hip/Hop PAC_1 isoforms in the ovary, both of which are induced by hCG. In order to differentiate PAC_1 mRNA encoding three and six possible variants in the first extracellular domain and the third intracellular loop, respectively, we used six different combinations of primer pairs for RT-PCR assays (their locations shown in Fig. 5A). The primer pair of EC1F + R1R will result in products ranging between 961 bp and 1299 bp, depending on the splice variant forms (9 bands of different sizes can be produced). The primer pair of EC1F + EC1R would detect all three potential splice variant isoforms in the N-terminal extra cellular domain (35–36) and should result in a 184bp PCR product for the isoform PAC_1very short (PAC_1vs), a 289-bp PCR product for the isoform PAC_1short (PAC_1s), and a 354-bp PCR product for the isoform PAC_1normal (PAC_1n). For the detection of splice variants in the third intracellular loop domain, four different combinations of PCR primer pairs were used. The primer pair of R1F + R1R should result in a 289-bp PCR product for the isoform Short, a 370-bp PCR product for the isoform Hop2, and a 373-bp PCR product for the isoform Hip or Hop1. The primer pair of Hip + R1R should result in a 243-bp PCR product for the isoform Hip, a 327-bp PCR product for the isoform Hip-Hop1, and a 324-bp PCR product for the isoform Hip-Hop2. The primer pair of Hop1 + R1R should result in a 310-bp PCR product for the isoform Hop1. The primer pair of Hop2 + R1R should result in a 310-bp PCR product for the isoform Hop2. PCR products were analyzed on a 1.8% agarose gel and transferred onto a nytran membrane, and hybridized with probes. The probe A should detect isoforms PAC_1vs (184 bp), PAC_1s (289 bp), and PAC_1normal (352 bp). The probe B should detect isoforms Short (289 bp), Hip (373 bp), Hop1 (373 bp), Hop2 (373 bp), Hip-Hop1 (454 bp) and Hip-Hop2 (254 bp). The probe C should detect all possible PAC_1 mRNA variants generated by alternative splicing of the PAC_1 hnRNA. The specificity of this RT-PCR/Southern blotting approach was determined by comparing RT-PCR products generated from the hypothal-

amus, ovary, and granulosa cells of the rats primed with PMSG (48 h) followed by hCG (6 h). Results (Fig. 5B) show similar expression of PAC_1 mRNA variants among the hypothalamus, ovary, and granulosa cells. Interestingly, we detected predominant expression of the PAC_1normal variant (353 bp) among the three known isoforms in the first extracellular domain. We also detected all six expected PCR products (PAC_1-Short, PAC_1-Hip, PAC_1-Hop1, PAC_1-Hop2, and PAC_1-Hip/Hop1 or PAC_1-Hip/Hop2) in the third intracellular loop of the PAC_1 receptor were detected. Additional sequencing and restriction enzyme analyses on the PCR products confirmed the identities of all six PAC_1 mRNA isoforms, indicating that luteinizing granulosa cells express all six PAC_1 mRNA isoforms with differences in the third intracellular loop. We then examined the effect of forskolin on the level of PAC_1 mRNA variants in the presence and absence of ZK98299. Experiments were done in an identical manner to the procedures described for Fig. 4. Upon 25 or 30 cycles of PCR amplification, PCR products were separated on a 1.8% agarose gels, transferred onto a nytran membrane, and hybridized with the probe C. Results (Fig. 5C) show that forskolin induced expression of all tested PAC_1 mRNA variants in granulosa cells and that ZK98299 treatment effectively blocked the stimulatory effect of forskolin on the expression of PAC_1 mRNA variants.

Taken together, our results show that all three PAC_1, $VPAC_1$, and $VPAC_2$ are expressed in rat ovary, that PAC_1 is specifically expressed in the granulosa cells of preovulatory follicles under LH regulation, and that the LH-induced PAC_1 mRNA expression requires PR activation in granulosa cells.

DISCUSSION

The autocrine/paracrine action of the LH-induced production of follicular progesterone (3–4) and its nuclear receptors (1–2) is critical for ovulatory processes, as evident from the infertile phenotype of PR null mutant mice (5–6). The ligand-induced activation of PRs is assumed to initiate a cascade of gene expression in luteinizing granulosa cells leading to follicular rupture. Included among the PR downstream genes in luteinizing granulosa cells are PACAP (7), ADAMTS-1 protease (37), and cathepsin L protease (37). Like the PR gene (1), the PACAP, ADAMTS-1, and cathepsin L protease genes are transiently expressed in the granulosa cells of preovulatory follicles during the hCG-induced preovulatory period. Interestingly, the time of PACAP mRNA peak expression (6 h after hCG) differs from that of ADAMTS-1 and cathepsin L proteases (12 h after hCG). Thus, it is tempting to suggest that PACAP gene expression is an earlier event occurring in response to the ligand-induced PR activation in luteinizing granulosa cells. As PACAP is secreted into media

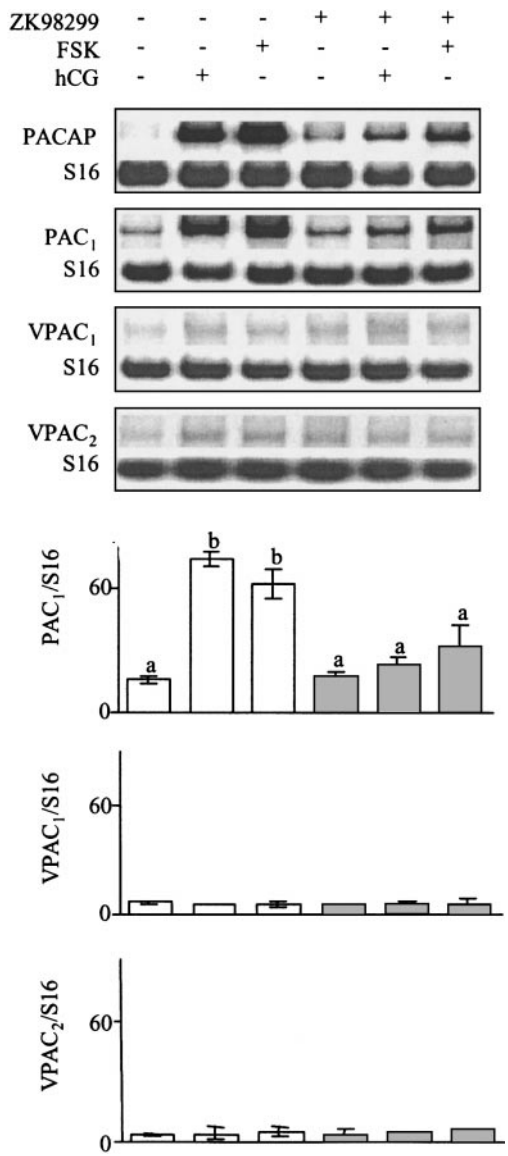
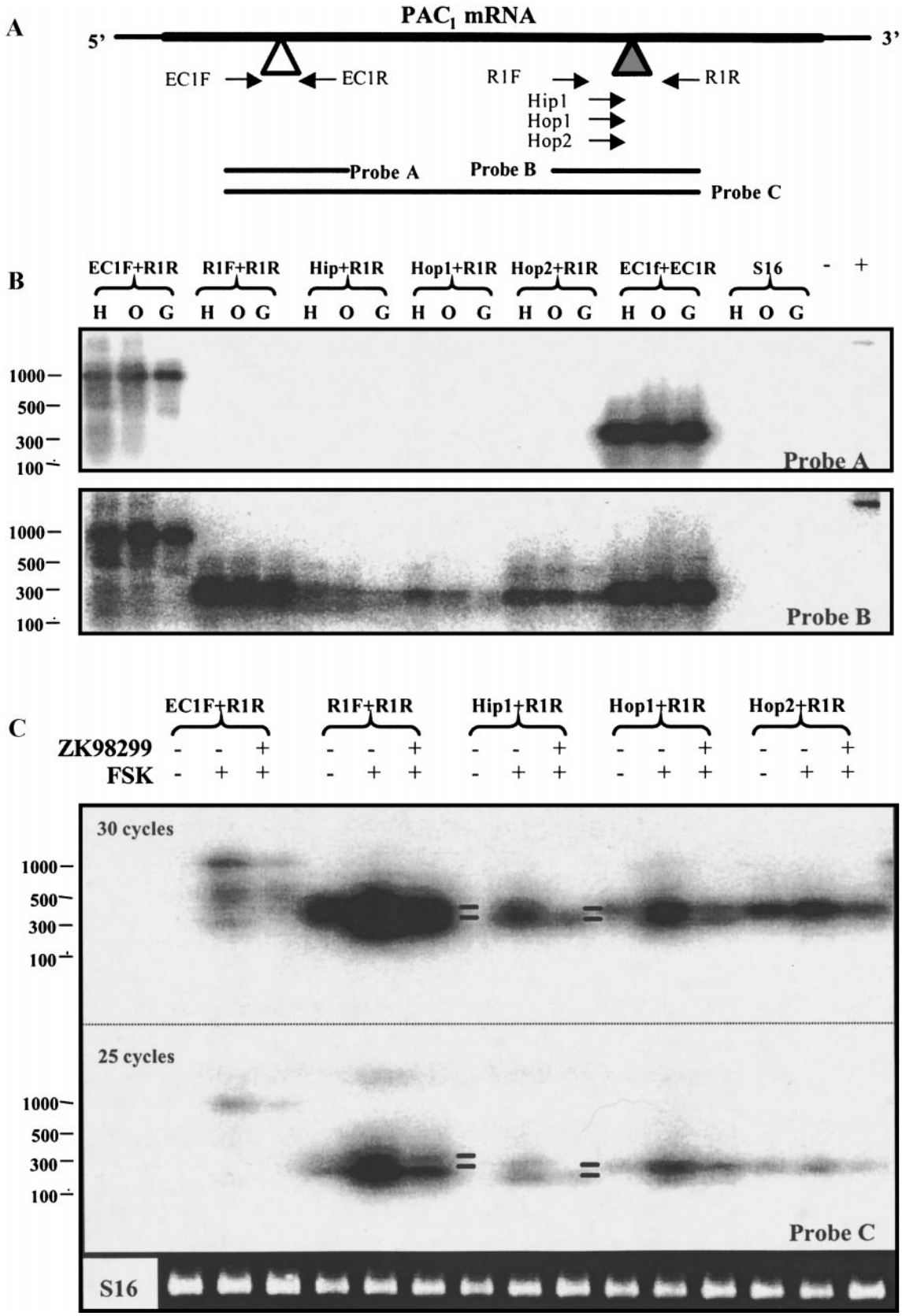


FIG. 4. ZK98299 inhibits the forskolin- or hCG-induced PAC_1 mRNA expression in granulosa cells *in vitro*. Granulosa cells from PMSG (15 IU, 40 h)-primed immature rats received ZK98299 (10 μM) or vehicle for 1 h prior to additional reception of vehicle, forskolin (10 μM, FSK) or hCG (10 IU/ml) for additional 6 h. RNA from these cells was assayed for PAC_1, $VPAC_1$, or $VPAC_2$ mRNA along with an internal control S16 using semiquantitative RT-PCR reactions. PACAP mRNA was also assayed as comparison. PCR products were taken from each sample after 20, 25, 30, and 35 cycles of amplification to ensure the linear range of amplification of each product. Shown are PACAP, PAC_1, $VPAC_1$, and $VPAC_2$ RT-PCR products after 25 cycles of amplification along with S16 RT-PCR products after 20 cycles of amplification, both of which are within the linear range of amplification. A representative autoradiogram of polyacryamide gels is shown on the top, along with and quantitation data on the bottom. PAC_1, $VPAC_1$, and $VPAC_2$ signals were normalized using S16 signals in each sample, after band intensity was estimated using ImageQuant version 3 software. The data shown are the mean ± SEM of the three independent experiments. Data were analyzed by ANOVA followed by Student's Newman-Keul test. Groups with different superscript letters are significantly different from each other ($P < 0.001$).


A
PAC1 mRNA
5'
3'
EC1F
EC1R
R1F
R1R
Hip1
Hop1
Hop2
Probe A
Probe B
Probe C
B
EC1F+R1R
R1F+R1R
Hip+R1R
Hop1+R1R
Hop2+R1R
EC1f+EC1R
S16
H O G
1000
500
300
100
Probe A
Probe B
C
EC1F+R1R
R1F+R1R
Hip1+R1R
Hop1+R1R
Hop2+R1R
ZK98299
FSK
30 cycles
25 cycles
Probe C
S16

from cultured granulosa cells (10, Ko and Park-Sarge, unpublished), PACAP in follicular fluid is likely to increase during the preovulatory period and thus be taken up by luteinizing granulosa cells of preovulatory follicles (8–10, 38, our unpublished data). The question of which membrane-associated, cytoplasmic, and/or nuclear events, including ADAMTS-1 and cathepsin protease gene expression, result from PACAP-PAC_1 interaction in luteinizing granulosa cells remains to be determined. However, our results demonstrating the ability of a specific PR antagonist, ZK98299, to block the stimulatory effect of LH and forskolin on PAC_1 mRNA expression in granulosa cells clearly underscore the importance of the finely tuned progesterone-induced regulation of the PACAP and its receptor PAC_1 genes in luteinizing granulosa cells.

PAC_1 exists in multiple isoforms arising from alternative-splicing of PAC_1 hnRNA. The presence of two different hip/hop exons in the third intracellular loop of the receptor results in six possible isoforms whereas the presence of two different in the extracellular domain results in 3 possible isoforms (16–17, 35–36, 39). It is of great interest which isoforms are expressed in a particular tissue and/or cell type, because some of these variants bind PACAP 38 and 27 with different affinity and activate both or exclusively one of adenylate cyclase (AC) and phospholipase C (PLC) (16–17, 36, 40–41). Although short and hip/hop PAC_1 isoforms have previously been reported in the rat ovary (12, 20, Fig. 1 of this study), our approach employing semi-quantitative RT-PCR coupled with Southern blotting provides a more sensitive and precise determination of the presence of all six PAC_1 isoforms with different exon combinations in the third intracellular loop (Hip, Hip-Hop1, Hip-Hop2, Hop1 and Hop2) in luteinizing granulosa cells. Thus, it is likely that the PACAP-PAC_1 interaction may evoke an array of intracellular signaling events including cAMP production and phospholipid metabolism. Interestingly, only one splice variant (PAC_1n) of the three potential splice variants in the first extracellular domain was detected in luteinizing granulosa cells. This isoform binds PACAP38 and PACAP27 with similar affinity (35–36), suggesting that all ovarian PAC_1 isoforms should interact with PACAP38 and PACAP27 with equal potency. Thus, ovarian responsiveness to PACAP is likely to be affected by the quantity of PACAP production but not by the nonequal production of PACAP38/PACAP27 (PACAP38 $\gg$ PACAP27) by luteinizing granulosa cells (Ko and Park-Sarge, unpublished observation) and ovaries (42–43). Importantly, our results demonstrating the consistent hCG (or forskolin)-induced PAC_1 mRNA variants inarguably suggest that alternative-splicing events resulting in the production of these PAC_1 mRNA variants are not differentially regulated by hCG. The ability of ZK98299 to block the stimulatory effect of hCG or forskolin to induce these PAC_1 mRNA variants further suggests the possibility that the LH-induced PAC_1 gene expression is primarily attributable to the action of ligand-occupied PRs in luteinizing granulosa cells. It remains to be determined whether PR action occurs at the level of transcription of the PAC_1 promoter or the stability of PAC_1 mRNA.

As we (this study) and others (20–21, 34) have reported, PAC_1 mRNA expression is dynamic during gonadotropin challenges. Prior to the preovulatory LH surge, antral and preovulatory follicles express little PAC_1 mRNA. However, small follicles (preantral) prior to antral formation highly express PAC_1 mRNA in their granulosa cells. It will be important to determine what factor(s) are involved in initiation of PAC_1 gene expression in small follicles. PRs are not expressed in small follicles (1, 7) and thus, non-PR-dependent signals must impinge on the PAC_1 promoter. The functional significance of PAC_1 in small follicles must be overcome in antral and preovulatory follicles since PAC_1 mRNA expression is inhibited during gonadotropin-dependent growth of follicles, mimicked by PMSG in superovulation protocols, regardless of whether the inhibitory signals are generated in differentiating granulosa cells by direct action of PMSG or they are secondary factors generated in antral and preovulatory follicles formed as a result of PMSG action on small follicles.

Unequivocally, PAC_1 has been shown to be the primary PACAP receptor expressed in luteinizing granulosa cells (12, 20, 31, 34, this study). In addition, our results also show $VPAC_1$ and/or $VPAC_2$ gene expression in these cells that is not in total agreement with a

FIG. 5. ZK98299 inhibits the stimulatory effect of forskolin on PAC_1 mRNA variants. (A) A schematic diagram of the PAC_1 mRNA along with primer locations and probe locations. (B) RNA from the hypothalamus, ovary, and granulosa cells from immature rats primed with PMSG (15 IU, 48 h) followed by hCG (10 IU, 6 h) was assayed for PAC1 mRNA variants (primer pairs are indicated on the top) along with an internal control S16 using semi-quantitative RT-PCR reactions with 30 cycles of amplification. RT-PCR products were separated on a 1.8% agarose gel, transferred onto a nytran membrane for Southern blotting. The probes A and B are indicated. V, hypothalamus; O, ovary; G, granulosa cells; −, no reverse transcription; +, plasmid control. (C) Granulosa cells from PMSG (15 IU, 40 h)-primed immature rats received ZK98299 (10 μM) or vehicle for 1 h prior to additional reception of vehicle, forskolin (10 μM, FSK) or hCG (10 IU/ml) for additional 6 h. RNA from these cells was assayed for PAC1 mRNA variants along with an internal control S16 using semiquantitative RT-PCR reactions. PCR products were taken from each sample after 25 and 30 cycles of amplification. S16 RT-PCR after 25 cycles of amplification are shown with ethidium bromide staining. RT-PCR products of the PAC_1 mRNA variants were separated on a 1.8% agarose gel, transferred onto a nytran membrane, and hybridized with the probe C. Black bars indicate two separate bands.

recent report (12) showing $VPAC_2$, but not $VPAC_1$, mRNA expression. The basis of this discrepancy between these studies is unknown, however, it is possible that the radioactivity-incorporated assay employed to detect $VPAC_1$ and $VPAC_2$ transcripts in our analysis may be more sensitive. Indeed, the expression of these genes is fairly low, because it required more cycles of amplification to reach a linear range of amplification of both $VPAC_1$ and $VPAC_2$ transcripts. In addition, *in situ* hybridization results show low signals barely above background. Moreover, neither $VPAC_1$ nor $VPAC_2$ mRNA expression is substantially regulated in luteinizing granulosa cells by the preovulatory LH surge or progesterone. Thus, it is unlikely, although not impossible, that $VPAC_1$ and/or $VPAC_2$ plays a significant role in autocrine/paracrine PACAP action in luteinizing granulosa cells.

Taken together, our results demonstrating that both PACAP and its receptors PAC_1 are expressed during the preovulatory period with a cell-type specific and PR-dependent manner in granulosa cells suggest that PR activation regulates a finely tuned expression of PACAP/PACAP receptor in luteinizing granulosa cells and thus dictates the timing of the autocrine and/or paracrine function of PACAP in preovulatory follicles.

ACKNOWLEDGMENTS

We wish to thank Lisa Savage for help with *in situ* hybridization. The cDNA encoding the PACAP type III receptor ($VPAC_2$) was kindly provided by Dr. Susumu Seino at Chiba University, Japan. ZK98299 was kindly provided by Dr. David Henderson at Schering AG, Germany. This work was supported by NIH Grants HD-30719 and HD-36879 (to O.K.P.S.). O.K.P.S. is a recipient of a NIH Research Career Development Award HD01135.

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