Gonadotropin-dependent regulation of bovine pituitary adenylate cyclase-activating polypeptide in ovarian follicles prior to ovulation

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Abstract

To study the regulation of bovine pituitary adenylate cyclase-activating polypeptide (PACAP) in preovulatory follicles prior to ovulation, PACAP cDNA was isolated by RT-PCR. Its open reading frame (ORF) is composed of 531 bp, and encodes for a 176-amino acid protein that bears 76–90% identity with other PACAP homologs. Using bovine preovulatory follicles obtained between 0 and 24 h after human chorionic gonadotropin (hCG) and semiquantitative RT-PCR/Southern blot, we demonstrate that levels of PACAP mRNA were low at 0 h, markedly increased at 6 and 12 h ($P < 0.05$), and declined 18 and 24 h after hCG. Levels of PACAP mRNA were high in the bovine pituitary, testis, intestine and uterus, but moderate to low in other tissues. Analyses performed on isolated preparations of granulosa and theca cells showed a significant increase of PACAP transcripts in both cell types after hCG, whereas primary granulosa cell cultures revealed high levels of PACAP as well as its receptors PAC-1 and VPAC-2 mRNA after forskolin treatment. Overexpression of the catalytic subunit of protein kinase A (PKA) in granulosa cells stimulated, but treatment with H89 or PKA inhibitor protein inhibited PACAP mRNA expression, whereas PACAP overexpression stimulated an increase in abundance of transcripts for PGHS-2, PGES, EP2 receptor, progesterone receptor, and ADAMTS-1, but not for P450-side chain cleavage and P450 aromatase. Thus, this study demonstrates the gonadotropin-dependent regulation of PACAP mRNA in bovine preovulatory follicles, the importance of PKA activation in the expression of PACAP in granulosa cells, and stimulating effect of PACAP on gene expression during the ovulatory process.

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide that was first isolated from the ovine hypothalamus (Miyata *et al.* 1989). It belongs to the family of the secretin/glucagon/vasoactive intestinal peptide (VIP; Vaudry *et al.* 2000), and exists in two secreted isoforms, referred to as PACAP-27 and PACAP-38. Both isoforms are generated from tissue-specific proteolytic processing and alternative α -amidation of the same 176-amino acid precursor (Vaudry *et al.* 2000); and share identical N-terminal 27 amino acids of which expression of PACAP-38 predominates in most mammalian tissues (Miyata *et al.* 1989). To mediate its action, PACAP binds with marked affinity to three distinct G protein-coupled seven transmembrane receptors, referred to as PACAP receptor-1 (PAC-1), VIP/PACAP-1 (VPAC-1) and VPAC-2 (Vaudry *et al.* 2000). PAC-1 is highly selective for PACAP, and its binding is followed by activation of both adenylate

cyclase and phospholipase C (Morrow *et al.* 1993, Pisegna & Wank 1993, Spengler *et al.* 1993). VPAC-1 and VPAC-2 bind equally PACAP and VIP, and are almost exclusively coupled to adenylate cyclase (Spengler *et al.* 1993, Rawling & Hezareh 1996, Jaworski & Proctor 2000). The expression of PACAP and its receptors can be found in many tissues, including hypothalamus, pituitary, testis and ovary (Vaudry *et al.* 2000), and the role of PACAP in reproduction has been suggested by studies showing its involvement in the synthesis and release of gonadotropins in pituitary cells *in vivo* and *in vitro* (Hart *et al.* 1992, Osuga *et al.* 1992, Perrin *et al.* 1993).

Ovulation is a complex process triggered by luteinizing hormone (LH)/gonadotropin stimulation that ultimately leads to the rupture of follicles and the release of the germ cell. In rodent ovaries, this stimulation may induce the expression and secretion of PACAP, but its role during the ovulatory process is not clearly understood (Gras *et al.* 1999, Ko *et al.* 1999). It is suggested

that PACAP may be involved in modulation of ovarian functions, including steroidogenesis, accumulation of cAMP, production of plasminogen activator, maturation of the oocyte, and survival of granulosa cells (Vaudry *et al.* 2000). Likewise, mice deficient in PACAP display a reduction of fertility (Shintani *et al.* 2002). Little is known about the regulation of PACAP in preovulatory follicles of monoovulatory species, and there is no evidence of PACAP-regulated genes involved in the ovulatory process. Thus, the objectives of the study were to characterize the PACAP cDNA and its gonadotropin-dependent regulation in bovine preovulatory follicles prior to ovulation, and to determine the effect of PACAP on the expression of genes known to be involved in ovulation.

Materials and Methods

Cloning of the bovine PACAP cDNA (cDNA)

To isolate the bovine PACAP cDNA, RT-PCR was performed using total RNA extracts (100 ng) obtained from preovulatory follicles (Sirois 1994), sense (5'-ATG ACC ATG TGT AGC GGA GCG-3') and antisense (5'-CTA CAA ATA CGG TAT TCG CCG-3') primers, and a OneStep RT-PCR kit (Qiagen) as directed by the manufacturer. The sense primer was designed from a highly conserved 5'-end region identified after sequence alignments of human (GenBank Accession number: NM_001117), mouse (GenBank Accession number: NM_009625) and rat (GenBank Accession number: NM_016989) homologs, whereas the antisense primer was designed from a 3'-end region of a partial sequence of the bovine PACAP (GenBank accession number: U7889). RT-PCR reactions consisted of 1 cycle of 50 °C for 30 min and 95 °C for 15 min, and 35 cycles of 94 °C for 30 s, 56 °C for 1 min, and 72 °C for 1 min. PCR products were subcloned into pGEM-T Easy vector and sequenced (Centre de service de séquençage, Université Laval, Québec, Canada). By this means, the full-length of bovine PACAP cDNA was obtained.

Cloning of VPAC-2 cDNA fragment

A fragment of bovine VPAC-2 receptor cDNA was isolated by RT-PCR using total RNA extracts (100 ng) obtained from primary granulosa cells 24 h after forskolin (FSK) treatment, a OneStep RT-PCR kit as directed by the manufacturer, and sense (5'-GCC TCT TCA GGA AGC TGC ACT GC-3') and antisense (5'-GCA ACA CCA TGT AGT GGA CGC C-3') primers designed from highly conserved regions identified after sequence alignments of human (GenBank Accession numbers: NM_003383) and rat (GenBank Accession numbers: NM_009511) VPAC-2 receptor. Reaction products were subcloned into pGEM-T Easy vector and sequenced.

In vivo model of ovulation

Holstein heifers of 2–3 years of age exhibiting normal estrous cycles were used as a model of hCG-induced ovulation to study the regulation of PACAP during the ovulatory process *in vivo*, as previously described (Sirois 1994). Briefly, bovine preovulatory follicles were obtained after induction of luteolysis on day 7 of the estrous cycle, and i.v. administration of an ovulatory dose of hCG (3000 IU) 36 h after induction of luteolysis. The ovary bearing the preovulatory follicle was isolated by ovariectomy (via colpotomy) from individual heifers at 0 to 26 h after hCG (Sirois 1994). The interval of time from hCG administration to ovulation is 26–28 h in this animal model. The preovulatory follicles were dissected from the ovary with a scalpel, and pieces of follicle wall (i.e. theca interna with attached granulosa cells) were prepared and further dissected into isolated preparations of granulosa and theca cells (Sirois 1994). All tissue samples were stored at 70 °C. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Université de Montréal and were consistent with the guidelines of the Canadian Council of Animal Care.

PACAP construct, granulosa cell cultures and DNA transfection

To produce the PACAP expression construct, full-length PACAP was amplified by RT-PCR using total RNA extracts (100 ng) obtained from preovulatory follicles, sense (5'-GTG AAT TCG CCG CCA CCA TGG CCA TGT GTA GCG GAG CG-3') and anti-sense (5'-ACC TCT AGA CTA CAA ATA CGG TAT TCG CCG-3') primers, and a OneStep RT-PCR kit. Reaction products were subcloned into the expression vector pcDNA 3.1(+) (Invitrogen Life Technologies), and sequenced.

Primary cultures of bovine granulosa cells were prepared as previously described (Liu *et al.* 1999, Sayasith *et al.* 2004). Briefly, pairs of bovine ovaries bearing a newly formed corpus luteum and a follicle of 8–12 mm in diameter (dominant follicle of the first wave of the estrous cycle) were obtained from a slaughterhouse. Granulosa cells were isolated from the largest follicle as previously described (Liu *et al.* 1997), seeded at a density of 2×10^6 in the 100 mm Petri-dish containing 10 ml of minimal essential medium (MEM; Invitrogen Life Technologies) supplemented with L-glutamine, nonessential amino acids, 2% fetal bovine serum, insulin (1 µg/ml), transferrin (5 µg/ml), and penicillin (100 units/ml)-streptomycin (100 µg/ml) and incubated at 37 °C in a humidified atmosphere of 5% CO₂. To study the regulation of PACAP and its receptors, cells were serum-starved overnight in MEM and then incubated in serum-free MEM containing FSK (10 µM). After various times of incubation, cells were harvested, and total RNA extracts were isolated. In some

experiments, granulosa cells were transfected with the vector expressing bovine PACAP, catalytic subunit of PKA (cPKA) or PKA inhibitor (PKI) polypeptide (4 µg/Petri-dish) using LipofectAMINE PLUS (Invitrogen Life Technologies) in accordance with the manufacturer's protocol. cPKA and PKI polypeptide expression vectors, pRSVcPKA α and pRSVPKI respectively, were generously provided by Dr Richard Maurer (Oregon Health Science University, Portland, Oregon, USA). After 3 h transfection, cells were incubated in serum-free MEM in the absence or presence of FSK (10 µM) or PKA inhibitor H89 (Calbiochem, La Jolla, CA, USA) for 24 h, from which total RNA were extracted.

RNA extraction and semiquantitative RT-PCR/Southern blot

All tissues were obtained from a slaughterhouse, and total RNA was isolated from bovine preovulatory follicles, granulosa cell cultures and tissues using the TRIzol reagent, and a Kinematica PT 1200C Polytron Homogenizer (Fisher Scientific, Montréal, Canada), according to manufacturer's instructions. Levels of PACAP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were analyzed by semiquantitative RT-PCR/Southern blot using RNA extracts (100 ng), a OneStep RT-PCR kit and sense (5'-ATG ACC ATG TGT AGC GGA GCG-3') and antisense (5'-GCG TCC TTT GTT TTTAAC CC-3') primers specific for bovine PACAP, generating a 513-bp DNA fragment, and sense (5'-GTT TCCAGTAGA TTC CAC CC-3') and antisense (5'-TCCACC ACC CTG TTG CTG TA-3') primers specific for bovine GAPDH, producing an 850-bp DNA fragment. The reaction conditions were one cycle of 50 °C for 30 min and 95 °C for 15 min followed by 26 cycles for PACAP of 94 °C for 30 s, 53 °C for 1 min, and 72 °C for 2 min, or 13 cycles for GAPDH of 94 °C for 30 s, 59 °C for 1 min, and 72 °C for 2 min. The number of cycles used was optimized for each gene to fall within the linear range of PCR amplification. PCR products were electrophoresed on 2% agarose gels, transferred to nylon membrane and hybridized with corresponding ³²P-labeled PACAP and GAPDH cDNA fragments using QuikHyb hybridization solution (Stratagene, La Jolla, CA, USA). Membranes were exposed to X-OMAT Kodak films with the intensification screen, and signals were quantified by densitometer using the ImageQuant software.

RT-PCR analysis

The expression of transcripts for prostaglandin (PG) G/H endoperoxide-2 (PGHS-2), PG synthase (PGES), PG E2 receptor (EP2), progesterone receptor (PR), disintegrin and metalloprotease with thrombospondin-like motifs-1 (ADAMTS-1), cytochrome P450-side chain cleavage (P450sc), cytochrome P450 aromatase (P450arom),

PAC-1, and VPAC-2 was analyzed by RT-PCR using RNA extracts (100 ng), a OneStep RT-PCR kit (Qiagen) and appropriate primers (see Table 1). RNA extracts were isolated from untransfected and PACAP-transfected granulosa cells cultured in the absence or presence of FSK (10 µM) for 24 h. Each reaction was performed at one cycle of 50 °C for 30 min and 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 59 °C for 1 min, and 72 °C for 2 min. PCR products were electrophoresed on 2% Tris-acetate/EDTA-agarose gels, and the intensity of the band was quantified by densitometer using the ImageQuant software.

Statistical analyses

One-way ANOVA was used to test the effect of time after hCG on levels of PACAP mRNA in samples. PACAP mRNA levels were normalized with the control gene GAPDH prior to analysis. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC, USA). When ANOVA indicated a significant *F* value (*P* < 0.05), Dunnett's test was used for multiple comparisons of individual means.

Results

Cloning of bovine PACAP cDNA

To clone bovine PACAP cDNA, RT-PCR was performed using RNA extracts isolated from preovulatory follicles obtained 24 h after hCG treatment (Sirois 1994), and primers designed from the partial bovine PACAP cDNA and a highly conserved 5'-end region identified after sequence alignments of human, mouse and rat homologs.

Table 1 Details of primer sets used in RT-PCR analyses.

Genes	Primer sequences	PCR products (bp)
PGSH-2	5'-CACAGTGCCTACTACTTACC-3' (sense) 5'-GTCTGGAACAACCTGCTCATCGC-3' (antisense)	735
mPGES	5'-TGATGAACGCCAGGTGCTC-3' (sense) 5'-ATGCCACGGTGTGTACCATACGG-3' (antisense)	330
EP2	5'-CTGCTGGATCATTGGAAGTATGC-3' (sense) 5'-TCCATCTCGCTGTCCACGTG-3' (antisense)	439
PR	5'-TAGAAAGTGCTGTACGGCTGG-3' (sense) 5'-ATAGAAACGCTGTGAGCTAGG-3' (antisense)	742
ADAMTS-1	5'-GGGACACCGAACAGGAAGTGG-3' (sense) 5'-GCACTACTACAGGGTGACAGGGC-3' (antisense)	628
P450sc	5'-AGAGAATGGACTTTCGCCACATC-3' (sense) 5'-AGGGACACTGGTGTGGAACATC-3' (antisense)	525
P450arom	5'-CACATCCTCAATACAGGTCCC-3' (sense) 5'-GGT-GTCCAGCATGATGCG-3' (antisense)	469
PAC-1	5'-GTGAGATGGTCTGGTGGTTC-3' (sense) 5'-ACCATCCAGCCTTGATTCC-3' (antisense)	497
VPAC-2	5'-GCCTCTTCAGGAAGCTGCACTGC-3' (sense) 5'-GCAACACCATGTAGTGGACGCC-3' (antisense)	589

PGHS-2, prostaglandin G/H synthase-2; mPGES, microsomal prostaglandin E synthase; EP2, prostaglandin E2 receptor; PR, progesterone receptor; ADAMTS-1, a disintegrin and metalloprotease with thrombospondin-like motifs-1; P450sc, cytochrome P450-side chain cleavage; P450arom, cytochrome P450 aromatase; PAC-1, PACAP receptor-1; VPAC-2, vasoactive intestinal peptide/PACAP receptor-2.

Results showed that the full-length bovine PACAP cDNA consisted of 513 bp (Fig. 1A). Its predicted amino acid sequence encodes a 176-amino acid protein that is the same length as human and sheep, and one amino acid residue longer than mouse, rat, and chicken homologs (Fig. 1B). The nucleotide sequence of bovine PACAP was submitted to GenBank with Accession number AY924308. In comparison to other species, bovine PACAP deduced amino acid sequence is relatively similar to human (77%), mouse (79%), rat (76%), sheep (90%; GenBank Accession number NM_001009776) and chicken (76%; GenBank Accession number NM_001001291) homologs, and conserves putative elements for its functions, including the signal peptide, a PACAP-related peptide region, dibasic residues at positions 129–130, 159–160 and 170–171 involved in post-translational peptide cleavage to release PACAP-27 and PACAP-38 isoforms, and an amino acid residue at position 131 required for PACAP activity (Fig. 1B).

Tissue expression of bovine PACAP transcripts and its gonadotropin-dependent regulation in preovulatory follicles

To investigate the relative expression of PACAP mRNA in various bovine tissues, an RT-PCR/Southern blot was performed, and results showed that levels of the PACAP transcript were high to very high in pituitary, testis, intestine, uterus and preovulatory follicle isolated 12 h after hCG, and moderate to low or undetectable in other tissues tested (Fig. 2A). In contrast, expression of GAPDH mRNA (control gene) was relatively constant in these tissues (Fig. 2A).

To study the regulation of PACAP mRNA in bovine follicles prior to ovulation, total RNA extracts were prepared from the wall of preovulatory follicles (theca interna with attached granulosa cells) isolated at 0, 6, 12, 18 and 24 h after hCG treatment ($n=4$ distinct follicles (each from a different animal) per time point; except $n=2$ at 6 h post-hCG). Representative results from RT-PCR/Southern blot revealed that levels of PACAP mRNA were very low or undetectable before the hCG treatment (0 h), markedly increased at 6 and 12 h, and decreased at 18 and 24 h post-hCG (Fig. 2B). Results from several follicles expressed as ratios of PACAP to GAPDH indicated that a significant increase in levels of PACAP mRNA was observed from 6 to 18 h post-hCG as compared to 0 h, with a maximum induction 6 and 12 h after hCG ($P<0.05$; Fig. 2C).

Cellular localization of PACAP transcripts in preovulatory follicles prior to ovulation

To determine the cellular localization of bovine PACAP mRNA in preovulatory follicles, RT-PCR/Southern blot was performed using isolated preparations of granulosa

and theca cells obtained from preovulatory follicles 0, 6, 12, 18 and 24 h after hCG treatment ($n=2$ distinct follicles (each from a different animal) per time point). In granulosa cells, results showed that the expression of PACAP mRNA was undetectable at 0 h, markedly increased in levels from 6 to 12 h, and decreased between 18 and 24 h post-hCG (Fig. 3A). When results from several follicles were expressed as PACAP and GAPDH ratios, a significant increase of PACAP transcripts was observed at 6 and 12 h as compared to 0 h post-hCG, with a peak at 12 h post-hCG (Fig. 3B). In theca cells, results revealed that levels of PACAP mRNA were undetectable at 0 h, increased at 6 h, and declined between 12 and 24 h post-hCG (Fig. 3C). When results from multiple follicles were expressed as PACAP and GAPDH ratios, a marked increase in levels of PACAP mRNA was observed from 6 to 18 h as compared to 0 h post-hCG, with a peak at 6 h post-hCG (Fig. 3D).

PKA pathway-dependent regulation of PACAP transcripts in granulosa cells

To study the regulation of PACAP mRNA *in vitro*, granulosa cells were isolated from preovulatory follicles and cultured in the absence or presence of FSK for various times. RT-PCR/Southern blot analyses showed that levels of PACAP mRNA were low at 0 h, markedly increased from 6 to 48 h, with a peak at 12 h post-FSK (Fig. 4A). When results from three independent experiments were expressed as ratios of PACAP to GAPDH, a significant increase in levels of PACAP mRNA was observed after FSK treatment ($P<0.05$; Fig. 4B).

To characterize the role of PKA activation in the expression of PACAP mRNA, granulosa cells, either mock transfected or those transfected with the vector expressing cPKA or PKI, were cultured in the absence or presence of FSK and PKA inhibitor H89 for 24 h. RT-PCR analyses showed that levels of PACAP mRNA were low in cells before treatments (control), but greatly increased by FSK or cPKA (Fig. 4C and D, lanes 2 and 5 vs 1), whereas levels of FSK-inducible PACAP mRNA were significantly reduced by H89 and PKI (Fig. 4C and D, lanes 3 and 4 vs 2).

Effect of PACAP overexpression on PGHS-2, PGES, EP2, ADAMTS-1, PR, P450scc and P450arom mRNA in bovine granulosa cells

To investigate if PACAP induction may have an effect on the expression of PGHS-2, PGES, EP2, ADAMTS-1, PR, P450scc and P450arom mRNA, granulosa cells, either mock transfected or transfected with the PACAP expression vector, were cultured in the absence or presence of FSK for 24 h. In untransfected cells, results from RT-PCR analyses showed that basal expression of mRNA for PGHS-2, PGES, EP2, ADAMTS-1, PR and P450arom was low or undetectable, but markedly increased by FSK (Fig. 5, lanes 2 vs 1). In

PACAP-transfected cells, overexpression of PACAP stimulated basal expression of mRNA for each gene, except for P450scc and P450arom, initially observed in untransfected cells (Fig. 5, lane 3 vs lane 1), whereas the

treatment with FSK enhanced the latter PACAP-dependent stimulation (Fig. 5, lane 4 vs lane 3). In contrast, expression of GAPDH mRNA (control gene) was relatively constant for each samples tested (Fig. 5H).

A

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ATGACCATGTGTAGCGGAGCGAGGC TGGCCCTGCTCGTTTACGGGATACT 50
GATGCACAGCAGCGTCTACGGCTCA CCTGCCGCTCCGGACTCCGGTTCC 100
CGGGGATCAGGCCGAGAACGAGGT GTACGACGAGGACGGAAACCCGCGAG 150
CAGGACTTCTACGATTCGGAGTCTC TGGGCGTGGGAGCCCCGCCTCCGC 200
GCTGCGCGATGCCCTACGCGCTCTAC TACCCCGCGGAGGAAAGAGATGTCTG 250
CCCACGGGATCCTTAATAAGGCCTA CCGCAAAGTGCTGGACGAGCCGTCC 300
GCCAGGAGATCACCTGCAGACGCTC ATGGCCAAGGGCTTGGGTGGGACCC 350
GGGCGGCAGCGCGGACGACGACTCG GAGCCGCTCTCCAAGCGCCACTCGG 400
ACGGCATCTTCACTGACAGCTACAG CCGCTACCGGAAACAAATGGCTGTT 450
AAGAAATACTTGGCTGCTGTTCTAG GGAAAAGGTATAAACAAAGGGTTAA 500
AAACAAAGGACGGCGAATACCGTAT TTGTAG 531

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B

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Bov MTMCSGARLALLVYGILMHSSVYGSPAASGLRFPGIRPENEVYDEDGNFQ 50
Hum .....S.....A.....E.A.G.....L 50
Mou .....SC.....S.....D.A..Q....L 49
Rat .....N..SC.....S.....E.A..Q....L 49
She .....A.....E.....Q 50
Chi .....N..SC.....S.....E.A..Q....L 49

Bov QDFYDSESLGVGSPASALRDAYALYYPAEERDVAHGILNKAYRKVLDQPS 100
Hum P..GG..PP.A.....P.A.A.W.R..GR.....E.....L 100
Mou .....WDPP.....DR.....E...E.....L 99
Rat .....WDPP.A.....DR.....E...E.....L 99
She .....PP.....D.....L 100
Chi .....WDPP.A.....DR.....E...E.....L 99

Bov ARRSPADAHGQGLGWDPPGGSADDDSEPLSKRHSDGIFTDSYSRYRKQMAV 150
Hum .GKHLQSLVAR.V.GSL..G.G..A..... 150
Mou ..KYLQSVVAR.A.ENL...V..PA..T..... 149
Rat ..KYLQSMVAR.M.ENLAAA.V..RA..T..... 149
She ...YLQTLMAK...GT...G.....S..... 150
Chi ..KYLQSMVAR.M.ENLAAA.V..RA..T..... 149

Bov KKYLAAVLGKRYKQRVKNKGRRIPYL 176
Hum ..... 176 (77)
Mou ..... 175 (79)
Rat ..... 175 (76)
She ..... 176 (90)
Chi ..... 175 (76)

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Figure 1 Nucleotide sequence of bovine PACAP and its deduced amino acid sequence in comparison to mammalian homologs. The bovine PACAP cDNA was obtained by the RT-PCR, as described in Materials and Methods. (A) The sequence is composed of an open reading frame of 531 bp. The translation initiation (ATG) and stop (TAG) codons are bolded. Numbers on the right refer to the last nucleotide on that line. (B) The amino acids of bovine PACAP (bov) were deduced from its nucleotide sequence, and aligned with human (hum), mouse (mou), rat, sheep (she) and chicken (chi) homologs. Identical residues are noted by a printed period. Hyphens indicate gaps in protein sequence used to optimize the alignment. The signal peptide is boxed and PACAP-related peptide is overlined. PACAP-38 is bolded and PACAP-27 is underlined. The sites for enzymatic cleavage are indicated by reversed triangles and residue critical for the PACAP activity is marked with an asterisk. Numbers on the right and in parentheses refer to the last amino acid on that line and a degree of identity (percentage) in amino acid residues when comparisons are made with bovine PACAP respectively.

Figure 2 Expression of PACAP mRNA in bovine tissues and its gonadotropin-dependent regulation in preovulatory follicles. (A) Relative expression of bovine PACAP and GAPDH was analyzed by a semiquantitative RT-PCR/Southern blot, as described in Materials and Methods, using total RNA (100 ng) extracted from various bovine tissues. Numbers on the right indicate the size of the PCR fragment. For studies of PACAP mRNA regulation in follicles, RNA extracts were prepared from bovine preovulatory follicles obtained 0, 6, 12, 18 and 24 h after hCG treatment, and samples (100 ng) were analyzed by a semiquantitative RT-PCR/Southern blot. (B) Representative results of PACAP and GAPDH mRNA levels are presented from one follicle per time point. (D) The PACAP signal was normalized to the corresponding GAPDH level, and results are presented as a ratio of PACAP to GAPDH. Mean $\pm$ S.E.M.; $n=4$ distinct follicles (each from a different animal) per time point; except $n=2$ at 6 h post-hCG. Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

Cloning of the VPAC-2 cDNA and regulation of PAC-1 and VPAC-2 mRNA in granulosa cells

A fragment of bovine VPAC-2 cDNA was isolated using RNA extracts obtained from granulosa cells 12 h post-FSK, and primers designed from highly conserved regions identified after sequence alignment of human, mouse and rat VPAC-2 homologs. Results from RT-PCR showed that the bovine VPAC-2 DNA fragment of 589 bp was obtained (Fig. 6A), and encodes for a 196 amino acid peptide, corresponding to the human VPAC-2 region of 149–344 amino acid residues. The predicted amino acid sequence of bovine VPAC-2 is highly similar to human (87%), rat (84%) and mouse (85%) homologs (Fig. 6B).

To examine the regulation of PAC-1 and VPAC-2 in granulosa cells, total RNA extracts isolated from granulosa cells, cultured in the absence or presence of FSK for various times, were analyzed by RT-PCR using pairs of primers specific for bovine PAC-1 (Genbank Accession number: NM_175715) and bovine VPAC-2 fragment obtained from the present study. Results showed that the expression of PAC-1 mRNA was very low or undetectable at 0 h, markedly increased at 6 and 12 h, and decreased to basal levels at 24 h post-FSK (Fig. 6C and D), whereas levels of VPAC-2 mRNA were low at 0 h, highly increased at 6, and returned to basal levels from 12 to 24 h post-FSK (Fig. 6C and E). A fragment of 684 bp for bovine VPAC-1 cDNA was also isolated (data not shown) using RNA extracts from intestine, and primers designed from highly conserved regions identified after sequence alignment of human, mouse and rat VPAC-1 homologs. However, RT-PCR analyses did not reveal the presence of VPAC-1 mRNA expression in bovine granulosa cells (data not shown).

Discussion

This study is the first to describe the molecular cloning of bovine PACAP cDNA, and the regulation of PACAP as well as its two receptors in bovine preovulatory follicles prior to ovulation. The present study is of interest to assess the importance of PACAP in species with a long ovulatory process, including cows, mares or primates, since there have been no reports on the regulation of PACAP in follicles of large monoovulatory species and previous studies have indicated that several genes, involved in steroidogenesis and ovulation, are differently regulated in preovulatory follicles of these species compared to rodents (Sirois 1994, Duffy & Stouffer 2001, Joyce *et al.* 2001, Boerboom *et al.* 2003). Likewise, the current study identified other key differences. Indeed, the gonadotropin-dependent induction of PACAP transcripts in bovine preovulatory follicles was highly and transiently increased at 6 and 12 h post-hCG that differs from rodents in which the levels of PACAP were transiently and markedly induced from 3 to 9 h after hCG in preovulatory follicles, with a maximum induction 6 h post-hCG (Ko *et al.* 1999, Lee *et al.* 1999). Moreover, our data showed that both granulosa and theca cells equally contribute to a high induction of PACAP, differing with its increase in rodents, which is mostly observed in granulosa and cumulus cells of large preovulatory follicles (Gras *et al.* 1996, Ko *et al.* 1999, Lee *et al.* 1999). Taken together, these results suggest that PACAP may have important functions in both follicular cell populations during the ovulatory process. The present study also indicated that high levels of PACAP mRNA were found in the uterus, intestine, pituitary and testis, suggesting that PACAP may play important roles in these tissues.

Figure 3 Localization of PACAP mRNA in bovine preovulatory follicles. Bovine preovulatory follicles were isolated from 0, 6, 12, 18 and 24 h post-hCG. Granulosa and theca cells were prepared from each follicle by the dissection, and used to extract total RNA, as described in Materials and Methods. Samples (100 ng) were analyzed for PACAP and GAPDH mRNA by a semiquantitative RT-PCR/Southern blot. Representative results of PACAP and GAPDH mRNA expression levels are presented from one granulosa (A) and one theca (C) sample per time point. The PACAP signal was normalized to the corresponding GAPDH level, and results are presented as a ratio of PACAP to GAPDH for granulosa (B) and theca (D) cells. $n=2$ distinct follicles (each from a different animal) per time point. Bars marked with an asterisk are significantly different from 0 h post-hCG ($P<0.05$).

Ovulation is a complex process triggered by LH surge, during which there is increased expression of several genes thought to be involved in a rupture of the follicle and the release of the oocyte, including PGHS-2, mPGES, membrane type 1-metalloproteinase, early growth factor-1, and PACAP (Richards *et al.* 2002, Sirois *et al.* 2004, Sayasith *et al.* 2006). Many studies have suggested the role of PACAP as a regulator of ovarian functions in the rodent ovary (Lee *et al.* 1999, Park *et al.* 2000, Cecconi *et al.* 2004). Indeed, it has been reported that incubation of preovulatory follicles or granulosa cells with PACAP markedly stimulates cAMP production and amplifies FSH stimulation of cAMP, suggesting that PACAP may act as a mediator for LH during ovulation (Tornell *et al.* 1988, Heindel *et al.* 1996, Cecconi *et al.* 2004). It is known that the preovulatory LH surge induces follicular cAMP production, causing a transient increase of several enzymes and receptors, such as PGHS-2, PR and ADAMTS-1, and production of PGE2 (Richards *et al.* 2002, Sirois *et al.* 2004). Furthermore, previous studies have shown that PACAP increases the production of progesterone and PGE2 in the ovary of crested newt, *Triturus cristatus* (Zhong & Kasson 1994, Gobbetti *et al.* 1997, Gras *et al.* 1999, Ko *et al.* 1999). In the present work, we used primary granulosa cell cultures that have been previously established as a valuable *in vitro* model to study the regulation of several bovine genes, including PGHS-2 and cPLA2, and the production of PGE2 recapitulating results observed

in vivo (Liu *et al.* 1999, Sayasith *et al.* 2004, Diouf *et al.* 2006). A similar *in vitro* model was employed in rodents to study the gonadotropin-dependent regulation of PACAP and its role in the suppression of follicle apoptosis (Lee *et al.* 1999, Park *et al.* 2000). As the increase in cAMP by LH or FSK leads to the activation of several signaling mechanisms, including PKA and guanine nucleotide-exchange factors (also known as exchange protein directly activated by cAMP or EPAC) signaling pathways (Gonzalez-Robayna *et al.* 2000, Chin & Abayasekara 2004, Li *et al.* 2006), the present study indicates that PKA activation in granulosa cells by FSK changes the expression of PACAP, with a transient increase in the transcript at 12 h post-hCG as observed *in vivo* after hCG. Interestingly, we provide new findings to demonstrate that overexpression of PACAP increases the abundance of PGHS-2, mPGES and EP2 mRNA's in bovine granulosa cell cultures, and these responses were amplified by FSK. As the expression of PGHS-2 and EP2 is required for ovulation, our data suggest the involvement of PACAP in ovulatory process with an important role in the prostaglandin biosynthesis pathway, including PGE2 production. In addition, the follicular induction of PGHS-2 (Sirois 1994) and PGES (Filion *et al.* 2001) occurs much later (18–24 h post-hCG) than that of PACAP (6–12 h post-hCG), thereby supporting the concept that PACAP may be involved in the increase of PGHS-2 and mPGES mRNA in bovine preovulatory follicles.

It has been reported that LH/gonadotropin stimulation selectively induces PR, ADAMTS-1 and progesterone production in granulosa cells of preovulatory follicles; and there is evidence to indicate that progesterone,

acting through its nuclear receptor (PR), plays an essential role in the regulation of ovulation (Tsafiri *et al.* 1987, Espey *et al.* 1990). Furthermore, mice deficient for PR not only fail to undergo the expected induction of ADAMTS-1 but also fail to ovulate (Lydon *et al.* 1995, Conneely *et al.* 2000, Robker *et al.* 2000). Reduction in ADAMTS-1 expression was also observed when the preovulatory synthesis of progesterone was inhibited with epistane (Espey *et al.* 2000), suggesting that ADAMTS-1 is a downstream target for LH-induced expression of progesterone and PR, and may be involved in proteolytic cascades culminating in the rupture of the follicle. Of note, mice deficient for ADAMTS-1 display impaired ovulation with morphologically abnormal in ovaries (Shindo *et al.* 2000). The present study reports for the first time that overexpression of PACAP causes a marked increase of PR and ADAMTS-1 mRNA in granulosa cells. This increase was supported by an induction of PR in granulosa cell cultures after treatment with FSK, in which the levels of PR mRNA were greatly increased from 12 and 24 h post-FSK (data not shown) as compared to those of PACAP (Fig. 4A and B).

Luteinization is a differentiation process of granulosa cells characterized by a high and constitutive expression of P450_{scc}, changes of transcriptional regulation of *PGHS-2* gene from being PKA dependent to PKC dependent, and an increase in cell volume with an appearance of lipid droplets (Morris & Richards 1995, Wu & Wiltbank 2002). The present study indicates that expression of several genes was increased in PACAP-transfected cells. As the transfected cells did not display luteinized cell phenotypes, but weakly express P450_{arom} mRNA (a marker of non-luteinized state of granulosa cells), the observed increase in gene expression would appear to be a direct effect of PACAP expression as opposed to a change in differentiation

Figure 4 PKA pathway-dependent regulation of PACAP transcripts in granulosa cells. Primary granulosa cells were isolated from bovine preovulatory follicles, as described in Materials and Methods, and cultured in the presence or absence of forskolin (FSK; 10 μ M) for various times. Total RNA extracts were prepared and used for analyzing the content of PACAP and GAPDH mRNA by a semiquantitative RT-PCR/Southern blot. (A) Representative results of PACAP and GAPDH mRNA signals are presented from one sample per time point. (B) Results are presented as a ratio of PACAP to GAPDH. Mean $\pm$ S.E.M. ($n=3$ independent experiments). Bars marked with an asterisk are significantly different from 0 h post-FSK ($P<0.05$). (C) Granulosa cells were cultured in serum-free medium in the absence of forskolin (control) or transiently transfected with the vector expressing catalytic subunit of protein kinase A (cPKA), or treated with forskolin (FSK, 10 μ M) either alone (—), in the presence of PKA inhibitor (H89, 10 μ M) or after transient transfection with the vector expressing the PKA inhibitory polypeptide (PKI). After 24 h incubation, RNA extracts were prepared and changes in PACAP and GAPDH (internal control) mRNA were analyzed by RT-PCR as described in Materials and Methods. Representative results are presented from one sample per treatment. (D) Results are presented as a ratio of PACAP to GAPDH signal ($n=3$ independent experiments).

Figure 5 Effect of PACAP overexpression on the expression of PGHS-2, PGES, EP2, ADAMTS-1, P450arom and P450scc transcripts in primary granulosa cells. Granulosa cells, either controls (mock transfected) or those transfected (PACAP) with the vector expressing bovine PACAP, were cultured in the absence (–) or presence (+) of forskolin (10 μ M) for 24 h. After incubation, RNA extracts were prepared and used (100 ng sample per reaction) to analyze the content of bovine PR, PGHS-2, PGES, EP2, ADAMTS-1, P450scc, P450arom and GAPDH mRNA by RT-PCR reactions, as described in Materials and Methods. Representative results of each gene (A–G; at the top of each panel) and GAPDH (H) mRNA levels are presented from one sample per treatment, and results presented as a ratio of PACAP to GAPDH signal for each treatment ($n=3$ independent experiments) were also depicted. Numbers on the right indicate the size of each PCR fragment in bp.

status of the granulosa cells. Further supporting this conclusion, levels of the transcript for P450arom, P450scc and other genes, including PGHS-2, were markedly increased after treatment with FSK (PKA activator), which is also observed in differentiating follicles *in vivo* and in cultures of non-luteinized granulosa cells isolated from small follicles (Xu *et al.* 1995, Bao *et al.* 1997, Sahmi *et al.* 2004). Additionally, levels of P450arom and P450scc mRNA were not affected by PACAP transfection as compared to untransfected cells. Together, these suggest that granulosa cells were maintained in a non-luteinized state in cultures, and represent a valuable *in vitro* model to study the regulation of genes involved in the ovulatory process *in vivo*.

Collectively, our results suggest an important role of PACAP as an autocrine/paracrine regulator in preovulatory follicles. To mediate its biological action, PACAP must be processed and secreted from cells to bind cell surface PACAP receptors. There are at least three types of PACAP receptors, referred to as PAC-1, VPAC-1 and VPAC-2. However, the distribution of PACAP receptor types in the ovary among species is variable. Some reports have suggested that either VPAC-1 or PAC-1 alone is found in the rat ovary (Gottschall *et al.* 1990, Scaldaferrri *et al.* 1996), whereas others have indicated that PAC-1 and VPAC-2 mRNA, alone or together, are present in rodent granulosa cells (Gras *et al.* 2000, Ko & Park-Sarge 2000, Koh *et al.* 2000, Park *et al.* 2000, Apa *et al.* 2002). Our data indicate that PAC-1 and VPAC-2 were found in bovine granulosa cells and that these are modulated during the ovulatory process. Indeed, treatment with FSK regulated the expression of both receptors in these cells and elevated levels of transcripts for both receptors concurred with an elevated PACAP expression

Figure 6 Partial nucleotide sequence of bovine VPAC-2 cDNA, its deduced amino acid sequence in comparison to other mammalian homologs, and forskolin-dependent regulation of PAC-1 and VPAC-2 transcripts in granulosa cells. (A) The partial sequence of bovine VPAC-2 was obtained by the RT-PCR, as described in Materials and Methods, and is composed of an open reading frame of 589 bp. Numbers on the right refer to the last nucleotide on that line. (B) The amino acids of bovine VPAC-2 (bov) were deduced from its partial nucleotide sequence; and aligned with human (hum), rat and mouse (mou) homologs. Identical residues are noted by a printed period and numbers in parentheses refer to the degree of identity (percentage) of amino acid residues when comparisons are done with bovine VPAC-2. (C–E) Primary granulosa cells isolated from bovine preovulatory follicles, as described in Materials and Methods, were cultured in serum-free medium in the presence or absence of forskolin for 0 to 24 h. Total RNA extracts were prepared and used to analyze the content of mRNA for PAC-1, VPAC-2 and GAPDH by RT-PCR. (C) Representative results are presented from one sample per time point. Numbers on the right indicate the size of the PCR fragment in bp. (D and E) The PAC-1 and VPAC-2 signals were normalized to the corresponding GAPDH level respectively; and results are presented as a ratio of PAC-1 or VPAC-2 to GAPDH. Mean $\pm$ S.E.M.; $n=3$ independent experiments.

at 6–12 h post-FSK. However, it is not known whether VPAC-2 or PAC-1 acts alone or together, or to which receptor PACAP binds to exert its biological effects in granulosa cells. More studies are needed for the better understanding of the relative function of the two PACAP receptors.

Information on molecular control of PACAP expression is limited. Previous studies and the present work identified high/ovulatory levels of gonadotropins as a physiological regulator of PACAP in preovulatory follicles (Gras *et al.* 1996, Lee *et al.* 1999, Park *et al.* 2000, 2001). Results from granulosa cell cultures also indicated that the expression of PACAP transcripts is increased by the treatment with LH, FSH, GnRH, FSK and cAMP analogs (Lee *et al.* 1999, Park *et al.* 2001, Wang *et al.* 2003). The present study confirms that the expression of PACAP was significantly stimulated by FSK treatment or cPKA overexpression, but inhibited by PKA inhibitor H89 treatment or PKI overexpression, suggesting that activation of adenylyl cyclase/cAMP/PKA induces PACAP expression in bovine granulosa cells. In rodents, PR activation is required for PACAP expression in granulosa cells, since treatment with PR antagonist ZK98299 inhibits the effect of hCG on PACAP expression (Ko *et al.* 1999). Significantly, a potential consensus PR response element has been reported for PACAP promoter (Park *et al.* 2000), therefore, it will be of interest to understand the importance of the latter element in the regulation of PACAP by PR during the ovulatory process.

In summary, this study is the first to report the molecular cloning of bovine PACAP and describes the induction of PACAP mRNA in the bovine follicle during the ovulatory process. This induction occurred in both granulosa and theca cells after hCG, and a similar induction pattern was obtained in primary granulosa cells cultured with FSK. Moreover, FSK treatment also induced a concomitant increase of PAC-1 and VPAC-2, as observed with PACAP. Interestingly, overexpression of PACAP in granulosa cells stimulated the transcript expression of genes known to be involved in ovulation, such as PGHS-2, mPGES, EP2, PR and ADAMTS-1, suggesting its potential involvement in the ovulatory process. Further, studies are needed to elucidate the molecular mechanisms of the involvement of PACAP in the regulation of genes implicated in ovulation. As the regulation of these genes occurred in preovulatory follicles can be reproduced in primary granulosa cell cultures, these cultures represent a valuable model to address these issues.

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