Characterization and expression of different pituitary adenylate cyclase-activating polypeptide/vasoactive intestinal polypeptide receptors in rat ovarian follicles

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Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a bioactive peptide transiently expressed in preovulatory follicles. PACAP acts by interacting with three types of PACAP receptors. PACAP type I receptor (PAC₁-R), which binds specifically to both PACAPs and vasoactive intestinal polypeptide (VIP), although with lower affinity, and two VIP receptors, VPAC₁-R and VPAC₂-R, which bind to PACAP and VIP with equal affinity. In the present study, we showed the expression of all three receptors in whole ovaries obtained from juvenile and gonadotropin-treated immature rats. A more detailed analysis on cells from preovulatory follicles showed that PAC₁-R and VPAC₂-R were expressed in granulosa cells, whereas only VIP receptors were expressed in theca/interstitial (TI) cells and fully grown oocytes presented only PAC₁-R. The distribution of the

VIP receptors was confirmed by immunofluorescence. HCG treatment induced stimulation of PAC₁-R in granulosa cells and VPAC₂-R in TI cells. The presence of functional PACAP/VIP receptors was also supported by metabolic studies. We further evaluated the presence of PACAP and VIP receptors by testing the effect of these peptides on apoptosis in granulosa cells cultured, isolated or in whole follicles. Treatment of follicles with PACAP and VIP dose-dependently inhibited apoptosis, while only PACAP significantly inhibited isolated granulosa cells. These results demonstrate a different expression of PACAP/VIP receptors in the various follicle compartments and suggest a possible role for PACAP and VIP on granulosa and TI cells, both during follicle development and ovulation.

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Introduction

Pituitary adenylate cyclase-activating polypeptide (PACAP), originally isolated from ovine hypothalamus, exists in two forms, PACAP-27 and PACAP-38, which share the same N-terminal 27 amino acids and are derived from tissue-specific proteolytic processing of the 176-amino acid precursor protein (Arimura 1992a). The name reflects their potent stimulation of cAMP production in anterior pituitary cells (Miyata *et al.* 1989). On the basis of sequence similarity, PACAP belongs to the secretin–glucagon–vasoactive intestinal polypeptide (VIP) family of peptides (Kimura *et al.* 1990). PACAP and VIP act by binding to three types of G-protein-coupled PACAP/VIP receptors. PAC₁-R binds specifically to both PACAPs and VIP, although with very low affinity; VPAC₁-R and VPAC₂-R bind to PACAP and VIP with equal affinity (Lutz *et al.* 1993). It has been demonstrated that these three receptors are coupled with the adenylate cyclase pathway, and PAC₁-R is also coupled with phospholipase C

(PLC) (Spengler *et al.* 1993). Moreover, as a result of alternative splicing, PAC₁-R exists in five different splice variant forms, which either contain or lack each of the two alternative exons, named hip and hop (Spengler *et al.* 1993).

PACAPs, VIP, and their receptors are expressed not only in the central nervous system, but also in various organs and peripheral tissues, such as lung, testis, adrenal gland, and ovary (Gottschall *et al.* 1990, Arimura 1992a,b), which suggests that they may not play a neuroendocrine role alone. Interestingly, PACAP stimulates various ovarian functions, including steroidogenesis, cAMP accumulation, and plasminogen activator (PA) production in rat granulosa cells (Zhong & Kasson 1994, Heindel *et al.* 1996, Apa *et al.* 2002), accelerates meiotic maturation in cumulus-enclosed rat oocytes (Apa *et al.* 1997), and inhibits apoptosis in preovulatory follicles (Lee *et al.* 1999), thus indicating that it plays an important role in the female reproductive system. We have also shown a direct action of PACAP on denuded oocytes (Apa *et al.* 1997).

Here, we describe the characterization and the signal transduction pathway of the three PACAP/VIP receptors in the various cellular compartments of the ovarian follicle. We reveal that PACAP receptors are present on the surface of denuded germinal vesicle (GV) oocytes, thus demonstrating a direct action of the peptide on these cells.

Materials and Methods

Materials

The materials used in the present study were obtained from the following sources: equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) from Intervet (Livorno, Italy); [125 I]cAMP and [3 H]inositol-1,4,5-trisphosphate from NEN, Perkin Elmer Life Science (Monza, Italy); PACAP and VIP from Calbiochem (San Diego, CA, USA); PACAP/VIP receptor antagonists PACAP 6–38 (H-2734), and a hybrid of neurotensin (6–11) and VIP (7–28) (H-9935) from Bachem (Bubendorf, Suisse); Mebstain Apoptosis Kit Direct from MBL International (Woburn, MA, USA); the antibodies to VPAC₁ H-130 (sc-30019) and VPAC₂ H-50 (sc-30020) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA); Dulbecco's modified Eagle medium (DMEM), minimum essential medium with Earl's salts (MEM), Hank's balanced salt solution and fetal calf serum (FCS) from Gibco (Grand Island, NY, USA); dibutyryl cAMP (dbcAMP), cAMP, VIP receptor antagonist (D-P-Chloro-Phe6, Leu17)-VIP, and all other reagents from Sigma. Highly purified ovine follicle-stimulating hormone (NIIDK-o-FSH-19-SIAFP, BIO) was kindly provided by Dr Parlow (National Hormone and Pituitary Program of the NIH).

Animals

Immature female Wistar rats were purchased from Charles River (Como, Italy). They were housed in groups, maintained in controlled temperature (25 °C) and light (12 h light/day) conditions, and given a regular supply of food and water and allowed to feed *ad libitum*. Animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals. Experimental protocols were approved by the University 'La Sapienza' Committee for Animal Care and Use. Animals aged from 3 to 25 days were killed by cervical dislocation and the ovaries collected for further analysis. Twenty-five-day-old rats were either killed (T₀, untreated rats) or injected subcutaneously with 10 IU eCG. After 48 h, the latter group was either killed (eCG-rats) or injected with 10 IU hCG (hCG-rats) and killed after 6 h by cervical dislocation.

Granulosa and theca/interstitial cell cultures

Granulosa cell cultures were prepared from eCG-treated rats as previously described (Canipari & Strickland 1985). Briefly,

the largest follicles from each ovary were punctured with a 25-gauge needle under stereomicroscope and gently pressed to release the granulosa cells. These cells were collected and cultured at a density $1.5 \times 10^5/200 \mu\text{l}$ in MEM supplemented with 0.1% BSA, 2 mM glutamine and antibiotics (100 mM penicillin and 100 $\mu\text{g/ml}$ streptomycin). Viability was estimated by the trypan blue dye exclusion method.

According to the procedure of Hwang *et al.* (1996), with minor modifications, theca/interstitial (TI) cells were obtained by digestion of the residual ovarian tissues from untreated rats, after granulosa cell isolation. Briefly, the residuals of the largest follicles were cut into small pieces, washed, and digested with collagenase in a two-step procedure. To obtain TI cells, the tissue was first digested in DMEM containing collagenase (1 mg/ml) and DNase (0.5 $\mu\text{g/ml}$) at 37 °C for 30 min in order to eliminate the adhering granulosa cells, then washed and digested in DMEM containing 4 mg/ml collagenase and 0.5 $\mu\text{g/ml}$ DNase for 45 min at 37 °C. The cells were plated in growth medium (DMEM supplemented with 10% FCS, 5 mM glutamine, and antibiotics) at a density 2×10^5 cells in 6 cm Petri dishes and incubated until use. Viability was estimated by the trypan blue dye exclusion method. All incubations were carried out at 37 °C in a 5% CO₂ atmosphere.

Early antral follicles between 320 and 400 μm diameter were mechanically dissected from ovaries of 19-day-old rats as previously described (Cecconi *et al.* 2004). Groups of 25 follicles were incubated on stainless steel grids in α MEM supplemented with 0.3% BSA in the absence or presence of increasing concentrations of PACAP or FSH (100 ng/ml) for 24 h at 37 °C. In additional experiments follicles were incubated with PACAP and VIP in the presence of 10^{-6} M PACAP and VIP antagonists.

RNA extraction and RT-PCR

After the animals had been killed, ovaries were removed aseptically, freed from adherent tissues and stored, ready for RNA extraction, at -80°C .

Total RNA from whole ovaries, granulosa cells, and TI cells was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi 1987). The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis. Total RNA (1–2 μg) was reverse transcribed, in a final volume of 20 μl , using the SuperScript II kit (Gibco) according to the manufacturer's instructions. The PCRs were carried out using Taq DNA polymerase (Roche) according to the manufacturer's instructions.

For each primer set, the number of cycles for the PCR was chosen in the exponential phase of amplification, using the annealing temperature provided. For each sample, 10 μl PCR product was submitted to electrophoresis on agarose gel (1.5%) and stained with ethidium bromide. Amplified products were analysed using AIDA software (Advanced Image Data Analyzer, 2.11) and mRNA levels normalized against the expression of ribosomal protein s16 mRNA.

Controls for DNA contamination were performed with gene-specific primers on RNA without reverse transcriptase treatment.

The primers used to amplify PAC₁-R, VPAC₁-R, VPAC₂-R, the ribosomal protein S16 (S16), and the follicle-stimulating hormone receptor (FSH-R) are shown in Table 1. Primers for PAC₁-R were chosen in a region that allowed the detection of all splice variants.

RT-PCR on single oocytes

According to the method of Fiorenza & Mangia (1998), mRNA from rat oocytes was amplified by PCR with slight modifications. Briefly, GV oocytes, obtained by puncturing ovarian follicles of eCG-treated rats, were mechanically isolated from the surrounding cumulus cells and either immediately lysed for RNA extraction or allowed to mature for 6 h in DMEM supplemented with 0.23 mM sodium pyruvate and 5% FCS (Met I-oocytes), then treated for RNA isolation. Ovulated metaphase II-oocytes (Met II) were obtained from the oviducts of eCG-hCG-primed animals, 15 h after hCG injection. The oocytes were freed from cumulus cells (CCs) by hyaluronidase treatment.

In order to remove the zona pellucida (ZP), the oocytes were incubated at room temperature for 1–2 min in protein-free Hepes-buffered medium M2 (Quinn *et al.* 1982) containing 0.5% Pronase E (Sigma) (Canipari *et al.* 1988). Groups of five oocytes were transferred to a 0.5 ml tube containing 2 µl H₂O supplemented with 1 U/µl RNasin Ribonuclease Inhibitor (Promega). Tubes were rapidly frozen on dry ice and stored at –80 °C until used. Immediately before the assay, the oocytes were diluted by adding 2 µl diethylpyrocarbonate (DEPC)-treated water. Total RNA was extracted by rapidly thawing and freezing the tube twice. The lysate was then digested with 40 U/ml RNase-free bovine pancreatic DNase I at 37 °C for 7 min to eliminate the genomic DNA, then heated at 65 °C for 5 min to inactivate the DNase I and denature the RNA. Total RNA was reverse transcribed using the Sensiscript Reverse Transcriptase Kit (Qiagen S.p.a., GmbH, Germany), according to the

manufacturer's instructions. Controls were performed by omitting the Sensiscript enzyme during RT. The PCR step was performed by adding 15 pmole of each specific primer and 2.5 U Taq DNA polymerase (Roche) in a final volume of 60 µl. The primers used to amplify PAC₁-R and S16 are shown in Table 2. VPAC₁-R and VPAC₂-R were the same as those used for the granulosa and TI cells (Table 1).

Calcium measurement

Cytoplasmic Ca²⁺ concentration ([Ca²⁺]_i) was measured by dual wavelength fluorescence of single cells loaded with the Ca²⁺-sensitive intracellular indicator fura-2-acetoxymethyl-ester (AM), as described by Panizza *et al.* (1995), with slight modifications.

Zona-free oocytes, 5–10, washed and transferred to M2 (without BSA) on glass coverslips that had been precoated with concanavalin A (Con A, 0.2 mg/ml in PBS), forming the base of the chamber for the Ca²⁺ measurement (McGuinness *et al.* 1996). Oocytes were then loaded with 3 µM fura-2/AM in serum-free, but otherwise complete, MEM at 37 °C in 5% CO₂ for 60 min. Coverslips were then rinsed twice with Krebs–Henseleit–Hepes buffer (KHH: 140.7 mM Na⁺, 5.3 mM K⁺, 132.4 mM Cl[–], 0.98 mM PO₄^{2–}, 1.25 mM Ca²⁺, 0.81 mM Mg²⁺, 20.3 mM HEPES, and 5.5 mM glucose). [Ca²⁺]_i-dependent fluorescence was measured by means of an AR-CM microfluorimeter (Spex Industries, Edison, NJ, USA) connected with a Diaphot TMD inverted microscope equipped with a CF ×40 fluor objective (Nikon Corp., Tokyo, Japan). Recordings were performed at 340 and 380 nm excitation wavelengths. Emission, collected by a photomultiplier carrying a 510 nm cut-off filter, from 340 to 380 nm and a real-time 340:380 nm ratio was recorded by an ASEM Desk 2010 computer (ASEM S.p.A., Buia, Italy). Calibration of the signal was obtained at the end of each experiment by maximally increasing intracellular Ca²⁺-dependent fura-2 fluorescence with 5 µM Ca²⁺-ionophore ionomycin, followed by the recording of minimal fluorescence after the addition of 7.5 mM EGTA and 60 mM Tris–HCl, pH 10.5 [Ca²⁺]_i was calculated as previously described (Gryniewicz *et al.* 1985).

Table 1 Sequence of selected oligonucleotides used as RT-PCR primers

Gene	Primer	PCR conditions	bp	Gene bank accession number
PAC ₁ -R	Fw 5'-GGACCCTTCTCAGCCTGT-3' Rw 5'-CACATGATCCGGTCTTGA-3'	94 °C 30 s, 60 °C 1 min, 72 °C 1 min	343	Z23279
VPAC ₁ -R	Fw 5'-TCCGAGCGGAAGTACTTCTG-3' Rw 5'-ACCTGGGCCTTGAAGTTGTC-3'	94 °C 30 s, 57 °C 45 s, 72 °C 30 s	359	NM012685
VPAC ₂ -R	Fw 5'-CACTAGTGATGGGTGGTCCGG-3' Rw 5'-GCCAGTAGAAGTTCGCCATG-3'	94 °C 30 s, 57 °C 45 s, 72 °C 30 s	399	Z25885
FSHR	Fw 5'-CTTGTCGACCTGGATTGGAGACCTG-3' Rw 5'-CTTGAGCTCCTTAATGCCTGTGTTGG-3'	94 °C 30 s, 60 °C 45 s, 72 °C 30 s	209	L02842
S16	Fw 5'-CGTTACCTTGATGAGCCCAT-3' Rw 5'-TCCAAGGTCCGCTGCAGTC-3'	94 °C 30 s, 60 °C 30 s, 72 °C 30 s	117	X17665

Fw, forward primer; Rw, reverse primer.

Table 2 Sequence of selected oligonucleotides used as RT-PCR primers

	Primer	PCR conditions	bp	Gene bank accession number
Gene				
PAC ₁ -R	Fw 5'-CAGCACTTCCCTCGCCACCC-3' Rw 5'-GGTGTCCCCAGCCGATGATG-3'	94 °C 30 s, 55 °C 30 s, 72 °C 45 s	348	Z23279
S16	Fw 5'-AGGAGAGATTGCTGGTGTGGA-3' Rw 5'-AGCTTTTGAGATAGACTGCCCG-3'	94 °C 30 s, 55 °C 30 s, 72 °C 45 s	95	M11408

Fw, forward primer; Rw, reverse primer.

Assay of cAMP

Cultured TI cells were incubated for 1 h in serum-free medium and then for 2 h in the presence of various hormones. At the end of the incubation period, the samples were processed as previously described (Apa *et al.* 1997), with slight modifications. Stimulation was stopped by means of ice-cold 10% trichloroacetic acid (TCA), and the cells were scraped and sonicated twice for 5 s at 5 W. The lysate was centrifuged and the supernatant extracted thrice with two volumes of water-saturated diethyl ether. The amount of cAMP was measured by RIA (Steiner *et al.* 1972). Samples were acetylated before the assay, according to the procedure of Harper & Brooker (1975). The RIA had a sensitivity of 2–4 fmol cAMP, an intraassay coefficient of variation of 5%, and an interassay coefficient of variation of 10–3%. The standard curve was calculated using a log-linear curve fit with %B/B₀ (y-axis) against cAMP. The values were normalized to the milligrams of proteins present in the sample. Protein content was measured by the method of Lowry *et al.* (1951) using BSA as standard.

Phosphoinositide turnover

In order to measure phosphoinositide degradation, TI and granulosa cells were incubated for 48 h with 4 µCi/ml myo-[2-³H]inositol in DMEM supplemented with 5% FBS. After labeling, cells were rinsed thrice with Hank's balanced salt solution and preincubated for 20 min with 20 mM LiCl. PACAP and VIP treatment were started a few minutes after LiCl addition, and stopped 30 min later by rapidly placing the culture plates on ice and replacing the medium with ice-cold 10% TCA. TCA was cleared away by extraction with 1 volume trichlorotrifluoroethane and trioctilamine (3:1). The samples were separated by ion exchange chromatography on Dowex 1×8–200 (Sigma) and eluted with ammonium phormiate solution at different concentrations. Samples were counted using a β-counter (Beckman Coulter, Inc., NY, USA).

Immunofluorescence

Frozen sections (7 µm) obtained from ovaries of 23-day-old rats were mounted on Polysine-TM slides (Menzel-Glaser, Braunschweig, Germany), fixed in 4% formaldehyde, treated with 10% normal goat serum to minimize non-specific binding, and incubated for 20 h at 4 °C with antibodies to

VPAC₁-R (1:200) or VPAC₂-R (1:200). The sections were extensively washed with PBS and incubated for 1 h at room temperature with a goat anti-rabbit IgG (Molecular Probes; 1:1000). As a negative control, the primary antibody was omitted and substituted with rabbit immunoglobulin G. Samples were analyzed using a Zeiss Axioplan fluorescence microscope (Carl Zeiss SpA, Milano, Italy).

Morphological analysis of granulosa cell apoptosis

Granulosa cell apoptosis was evaluated as previously described (Cecconi *et al.* 2004). Briefly, early antral follicles were mechanically dissected from 19-day-old untreated rats and cultured as described previously. Granulosa cells were released in the medium immediately before or after 24 h of follicle culture. Cells from single follicles were fixed for 15 min in 4% paraformaldehyde/PBS and cytocentrifuged onto a glass slide at 200 g for 10 min. The samples were washed thrice with PBS and the chromatin was stained using the TUNEL (TdT-mediated dUTP-X nick end labeling) method according to the manufacturer's instructions (Mebstain Apoptosis Kit Direct, MBL International, Woburn, MA, USA). Apoptotic cells were identified and counted in three or more randomly selected fields with at least 100 cells each.

Statistical analysis

Data are expressed as the mean ± s.e.m. from at least three independent experiments. Statistical analysis was performed using ANOVA followed by the Tukey–Kramer test for comparisons of multiple groups or paired Student's *t*-test for comparison of data derived from two groups. Values with *P* < 0.05 were considered statistically significant.

Results

Expression of PACAP receptors in the ovary

In order to determine the expression of different PACAP receptors in rat ovary, total RNA was extracted from whole ovaries obtained from untreated, eCG-, and hCG-treated rats. Semi-quantitative RT-PCR (25 cycles of amplification) showed the presence of PAC₁-R in untreated and hCG-treated rats and very low levels in eCG-treated rats, as previously demonstrated (Park *et al.* 2000), but no signal for the presence of the other two

Figure 1 Expression of the different PACAP/VIP receptor subtypes in whole rat ovaries detected by RT-PCR. Total RNA from untreated, eCG-, or hCG-treated immature rat whole ovaries was subjected to RT-PCR for 25, 30, and 35 cycles of amplification using primers specific for PAC₁-R, VPAC₁-R, VPAC₂-R, and S16 as indicated in the Materials and Methods section. The expression of S16 was used as an internal standard. An aliquot of each PCR product was electrophoresed onto 1.5% agarose gel and stained with ethidium bromide. No signal was detected in the negative controls for either set of primers. The figure is representative of three independent experiments carried out with independent animals.

receptors (Fig. 1). Positive signals for VPAC₁-R and VPAC₂-R were observed only after increasing cycles of amplification, thus suggesting a lower level of expression for these two genes. VPAC₂-R was evidenced after 30 cycles and apparently not modulated by gonadotropin treatment (Fig. 1). VPAC₁-R was seen only after 35 cycles and decreased slightly after gonadotropin stimulation (Fig. 1).

In order to investigate the expression of the three receptors in the different ovarian compartments, semi-quantitative RT-PCR experiments were performed on isolated granulosa and TI cells obtained from ovaries of eCG- and hCG-treated rats. A positive signal for PAC₁-R was observed in granulosa cells, after 25 cycles of amplification, and was stimulated by hCG (Fig. 2). This receptor was not observed in TI cells also at 35 cycles of amplification (data not shown). Positive signals for VIP receptors were observed after further amplification confirming lower levels of expression. VPAC₁-R was observed only in TI cells, and VPAC₂-R was observed in both granulosa and TI cells; VPAC₂-R was slightly stimulated by hCG in TI cells (Fig. 2).

The close proximity of granulosa and TI cells in the ovarian follicle makes it difficult to separate these cellular types. To exclude contamination by granulosa cells in TI cell preparations and to validate the expression of VPAC₁-R in the TI, but not in the granulosa cells, we performed similar experiments on enzymatically isolated TI cells. We first investigated the expression of FSH-R, which is expected to be present in granulosa cells but not in TI cells. In granulosa cells from eCG- and hCG-treated rats, mRNA encoding FSH-R was present (Fig. 3). A faint band was also present in TI cells obtained by means of mechanical dissociation of the follicles and was presumably due to granulosa cell contamination. FSH-R mRNA was not expressed in *in vitro* cultured

Figure 2 Expression of the different PACAP/VIP receptor subtypes in isolated rat granulosa and theca/interstitial cells detected by RT-PCR. (A) Granulosa (Gr) and theca/interstitial (TI) cells were obtained from ovaries of eCG- and hCG-treated immature rats. Equal amount of total RNA extracted from the cells was subjected to RT-PCR. Specific primers for PAC₁-R, VPAC₁-R, VPAC₂-R, and S16 were used for different cycles of amplification. The expression of S16 was used as an internal standard. The bands obtained after 30 cycles of amplification were analyzed with a chemiluminescence detection system (Raytest). PACAP/VIP receptor values were normalized by their respective S16 values and represent the mean $\pm$ S.E.M. of three to five independent experiments carried out with independent animals. (B) granulosa cells, and (C) TI cells. a.u., arbitrary units. * $P < 0.005$ vs respective eCG.

Figure 3 Expression of FSH receptors in granulosa and theca/interstitial cells. Granulosa (GR) and theca/interstitial (TI) cells were obtained as described in Fig. 2. *In vitro* cultured TI cells (TI *in vitro*) were obtained from the residuals of the largest follicles after granulosa cells had been isolated from ovaries of 25-day-old untreated rats. Total RNA was extracted and subjected to RT-PCR for 30 cycles of amplification. The figure is representative of the three independent experiments carried out with independent animals.

TI cells, suggesting the absence or very low contamination by granulosa cells (Fig. 3). As already observed in *ex vivo* TI cell preparations after 35 cycles of amplification, a positive signal for VPAC₁-R and VPAC₂-R was identified in cultured TI cells, although none was present for PAC₁-R. VPAC₂-R was the predominant form in TI cells (Fig. 4).

In order to investigate the developmental changes in the expression of the three different receptors, RT-PCR was performed on total RNA extracted from whole ovaries obtained from rats of different ages. As shown in Fig. 5, all the receptors were expressed in 3-day-old rats although higher numbers of cycles of amplification were needed to detect VIP receptors suggesting lower level of expression for these two genes also in immature ovaries. PAC₁-R markedly increased after the 12th day of age (Fig. 5A), VPAC₁-R significantly

Figure 4 Expression of different molecular forms of PACAP/VIP receptor subtypes as detected by RT-PCR in cultured theca/interstitial cells. Total RNA was subjected to RT-PCR for 35 cycles of amplification using primers specific for PAC₁-R, VPAC₁-R, VPAC₂-R, and S16, as indicated in the Materials and Methods section. An aliquot of each PCR product and DNA molecular weight markers (Promega 100 bp DNA ladder) were electrophoresed onto 1.5% agarose gel and stained with ethidium bromide. No signal was detected in the negative control for either set of primers. The figure is representative of four independent experiments carried out with independent animals.

decreased with increasing age (Fig. 5B), while VPAC₂-R remained constant through time (Fig. 5C).

Immunolocalization of PACAP/VIP receptors

To localize the VIP receptors, immunofluorescence analysis was performed on ovarian sections obtained from 23-day-old rats. The results showed that VPAC₁-R was predominantly found in association with the blood vessel wall and in the stroma near follicles (Fig. 6). We observed a strong immunoreactivity at the level of the ovarian hilus (Fig. 6A), where the ovarian arteries enter the ovary (Hossain & O'Shea 1983). Consistent with mRNA expression pattern, VPAC₂-R was detected ubiquitously in the ovary (Fig. 7) and, as already shown (Bajo *et al.* 2000), a positive signal was observed in the oviduct (Fig. 7A).

Expression of PACAP receptors in fully grown oocytes

As previously shown (Apa *et al.* 2002), PACAP was able to act directly on denuded oocytes, delaying meiotic maturation by modulating their intracellular cAMP levels. Therefore, we investigated oocytes for the expression of PACAP receptors. In order to eliminate any possible contamination by granulosa cell remnants, the ZP was first removed from denuded oocytes by pronase treatment (Canipari *et al.* 1988), then RNA extraction and RT-PCR were performed as described in the Materials and Methods. A positive signal for the expression of PAC₁-R was detected in GV oocytes after 40 cycles followed by a second amplification for 30 cycles on an aliquot of the first amplification. No signal was detected for VPAC₁-R and VPAC₂-R under the same conditions (Fig. 8). No signal for PAC₁-R was observed in *in vivo* matured Met I- and Met II-oocytes (data not shown).

These results are supported, in single zona-free GV oocytes, by the observation of a transient spike of $[Ca^{2+}]$ triggered by the addition of 10^{-6} M PACAP, which peaked 150 s after the stimulus (427 ± 28 nM), followed by a lower sustained phase (106 ± 7 nM), which lasted more than 800 s (Fig. 9). No response was detected, in similar conditions, in Met I- and

Figure 6 VPAC₁-R immunoreactivity in 23-day-old rat ovaries. (A) Low-power photomicrograph showing immunofluorescence at the ovarian hilus (arrows). (B) Higher magnification showing staining in the stroma near follicles. No positive signal is seen in the granulosa cells. Immunostaining (C) and phase contrast photomicrography (D) of a small ovarian artery. These are representative images of three independent experiments. Scale bar=100 μ m.

Met II-oocytes (data not shown). This result indicates that functional PAC₁-Rs are only present on the oocyte surface at the GV stage.

Effect of PACAP and VIP on granulosa and TI cell IPs and cAMP production

In order to determine the signal transduction pathway associated with the activation of PACAP and VIP receptors

Figure 5 Developmental expression of the different receptor subtypes in whole rat ovaries detected by RT-PCR. Total RNA, extracted from whole ovaries obtained from rats at the postnatal age indicated, was subjected to RT-PCR for 27 (A) or 35 (B and C) cycles of amplification using primers specific for PAC₁-R, VPAC₁-R, VPAC₂-R, and S16, as indicated in the Materials and Methods section. The expression of S16 was used as an internal standard. An aliquot of each PCR product was electrophoresed onto 1.5% agarose gel and stained with ethidium bromide. The bands obtained after amplification were analyzed with a chemiluminescence detection system (Raytest). PACAP/VIP receptor values are normalized by their respective S16 values and represent the mean $\pm$ S.E.M. of three to four independent experiments carried out with independent animals. The insets are representative RT-PCR. * $P < 0.05$, † $P < 0.001$ vs respective day 3.

Figure 7 VPAC₂-R immunoreactivity in 23-day-old rat ovaries. (A) Photomicrograph showing distribution of VPAC₂-R. As expected from RT-PCR experiments, this receptor is ubiquitously expressed in the ovary, white arrowhead shows the strong signal in the oviduct. (B) Higher magnification showing a stronger signal in the TI cell compartment (white arrowheads). These are representative images of three independent experiments. Scale bar = 100 μm.

Figure 8 Expression of PACAP receptors in denuded pronase-treated GV oocytes. RT-PCR was conducted as described in the Materials and Methods section. Briefly, S16 was amplified for 35 cycles, while PAC₁-R, VPAC₁-R, and VPAC₂-R were amplified for 40 cycles followed by a second amplification for 30 cycles on 2% of the volume obtained in the first amplification. The figure is representative of three independent experiments carried out with independent animals.

Figure 9 Effect of PACAP on [Ca²⁺]_i in a single denuded and zona-free oocyte. The output of a microspectrofluorometer was calibrated as [Ca²⁺]_i, as described in the Materials and Methods section. Upper panel: representative trace of the effect of 10⁻⁶ M PACAP on the [Ca²⁺]_i of a single oocyte. Lower panel: values represent the mean ± S.E.M. of [Ca²⁺]_i concentration of the spike (4.27 ± 0.28 mM), and of the stable, prolonged plateau (1.06 ± 0.07 mM) obtained in three independent experiments. *P < 0.0001.

present in granulosa and TI cells, we studied the effect of the two peptides on inositol monophosphate (IP₁), inositol bisphosphate (IP₂), and inositol trisphosphate (IP₃) production. Granulosa cells were cultured for 48 h in the presence of myo-[2-H³]inositol, pretreated for 10 min with LiCl, then stimulated with increasing concentrations of PACAP and VIP. Gonadotropin-releasing hormone (GnRH) was used as a positive control for IP production of granulosa cells (Anderson *et al.* 1996). Figure 10 shows that both PACAP-38 and PACAP-27 activate PLC, which in turn, catalyses the breakdown of polyphosphoinositides. Treatment with VIP did not significantly affect IP production in these cells, even at high concentrations (10⁻⁶ M). When the same experiment was performed on TI cells, cultured for

Figure 10 Effect of PACAP stimulation on IP production in granulosa cells. Granulosa cells, prepared as described in the Materials and Methods section, were cultured with 4 μ Ci myo-[2-³H]inositol for 48 h, pre-incubated with LiCl for 10 min, and stimulated with 10⁻⁷ M GnRH, with medium alone (C), or with increasing concentrations of PACAP-27, PACAP-38, and VIP. The reaction was interrupted after 30 min of incubation by replacing medium with ice-cold 10% TCA. Aliquots of all samples were analyzed by ionic-exchange chromatography on Dowex 1X8-200. The values represent the mean $\pm$ S.E.M. of three independent experiments each performed in duplicate. * P <0.05, † P <0.01 vs control.

48 h in the presence of PACAP and VIP, no effect was detected on IP production (data not shown).

To evaluate whether the action of PACAP and VIP on TI cells was mediated by cAMP production, isolated TI cells were cultured for 2 h in medium alone (control), FSH (100 ng/ml), hCG (100 mU/ml), or increasing concentrations of PACAP-38 and VIP (10⁻⁷ and 10⁻⁸ M) in the absence of serum. As expected, the addition of hCG significantly increased the levels of cAMP, while that of FSH had no effect. Both PACAP and VIP produced statistically significant increases in cAMP levels (Fig. 11).

Figure 11 Effect of PACAP and VIP on cAMP production by TI cells. Isolated TI cells, prepared as described in the Materials and Methods section, were cultured for 2 h in medium alone (control, C), FSH (100 ng/ml), hCG (100 mU/ml), or increasing concentrations of PACAP-38 and VIP in the absence of serum. At the end of incubation total cAMP was measured by RIA. The values represent the mean $\pm$ S.E.M. of three independent experiments each performed in duplicate. * P <0.001, † P <0.0001 vs control.

Effect of PACAP and VIP on granulosa cell apoptosis

In order to evaluate the presence of functional receptors in granulosa or TI cells, we studied the effect of PACAP and VIP on granulosa cell apoptosis. Cells, obtained from 22-day untreated rats, were cultured either isolated or in whole follicles. The presence of apoptotic cells was evaluated in granulosa cells after 24 h of culture in the presence of 100 ng/ml FSH, as a positive control, or increasing concentrations of PACAP (from 10⁻⁹ to 10⁻⁷ M). To this end granulosa cells were stained with Hoechst 33258 or with TUNEL at the end of culture. Granulosa cells obtained from early antral follicles immediately after isolation did not show detectable signs of apoptosis (4 $\pm$ 0.8%). In granulosa cells obtained from follicles incubated in serum-free medium for 24 h, apoptosis increased to 22.3 $\pm$ 1.5% and PACAP inhibited apoptosis in a dose-dependent manner (24 $\pm$ 1.5, 17 $\pm$ 1.2, 12 $\pm$ 0.9%, 10⁻⁹, 10⁻⁸, and 10⁻⁷ respectively). Levels of apoptosis similar to those obtained with FSH (14 $\pm$ 1.1%) were obtained with 10⁻⁷ M PACAP. As already shown (Flaws *et al.* 1995) also VIP inhibited granulosa cell apoptosis when cultured in whole follicles (Fig. 12B) suggesting the presence of VIP receptors in the whole follicles.

In isolated granulosa cells cultured in serum-free medium for 24 h, apoptosis increased to 55 $\pm$ 1.7% and FSH significantly reduced it to 47 $\pm$ 1.5%. PACAP inhibited apoptosis in a dose-dependent manner (53 $\pm$ 1.5, 50 $\pm$ 1.2, 46 $\pm$ 1.9%, 10⁻⁹, 10⁻⁸, and 10⁻⁷ respectively), while VIP

did not show any significant effect (data not shown) confirming the presence of PAC₁-R and of a low number of VIP receptors in isolated granulosa cells.

To further characterize the contribution of the different receptor subtypes to this inhibitory effect, follicles were incubated with PACAP (Fig. 12A) and VIP (Fig. 12B) in the presence of PACAP/VIP receptor antagonists. The antagonists utilized were: PACAP (6–38) a PAC₁-R, and to a lesser degree VPAC₂-R selective antagonist (D-P-Chloro-Phe6, Leu17), VIP, a moderately potent VPAC₁-R antagonist and a hybrid of neurotensin (6–11) and VIP (7–28), a moderately potent PAC₁-R antagonist and a weak antagonist at VPAC₂-R (Dickinson *et al.* 1997). As shown in Fig. 12A, all receptor antagonists significantly reversed PACAP inhibition of apoptosis, while PACAP (6–38) did not inhibit VIP action (Fig. 12B) supporting the presence of all three receptor subtypes in the follicle and the presence of PAC₁-R predominantly in the GC compartment.

To determine if PAC₁-R was effectively the predominant form present in granulosa cells, GCs isolated from early antral follicles, were incubated in the same conditions as described for whole follicles. PACAP and FSH prevented apoptosis (Fig. 12C), while VIP had no effect (data not shown). PACAP action was reversed by the addition of PACAP (6–38) and VIP (7–28), while (D-P-Chloro-Phe6, Leu17) VIP had no effect (Fig. 12C). These data are in accordance with the presence of PAC₁-R and the absence of VPAC₁-R in GCs. However, we cannot discriminate between the presence of PAC₁-R and VPAC₂-R due to the low selectivity of VIP (7–28).

Discussion

PACAP is known to bind to at least three types of receptors: PAC₁-R, which is specific for PACAP and has a low affinity for VIP, and the two VIP receptors VPAC₁-R and VPAC₂-R, which bind PACAP and VIP with similar affinities. Reports in the literature on which receptor is found in the ovary are somewhat contrasting. While there is a general agreement on the presence of PAC₁-R, there are some discrepancies regarding VIP receptors. Gras *et al.* (2000) found only VPAC₂-R in granulosa cells, whereas Ko & Park-Sarge (2000) found both VPAC₁-R and VPAC₂-R. No evidence is yet available on the presence of PACAP/VIP receptors in TI cells.

Figure 12 Percentage of apoptosis in (A) granulosa cells isolated from early antral follicles cultured for 24 h in the absence of serum or supplemented with FSH (100 ng/ml) or PACAP (10⁻⁷ M) with or without 10⁻⁶ M PAC₁ antagonist PACAP (6–38) (P₁) or 10⁻⁶ M VIP₁ antagonist (V₁) or PAC₁/VIP₂ antagonist, (P₁/V₂) or all antagonists together; (B) granulosa cells isolated as shown in (A), but cultured in the presence of VIP (10⁻⁷ M). (C) Granulosa cells isolated from early antral follicles and cultured for 24 h in the absence of serum or supplemented with FSH (100 ng/ml) or PACAP (10⁻⁷ M) with or without PACAP/VIP receptor antagonists. Values represent the mean ± s.e.m. of three to six independent experiments. *P<0.05, †P<0.01, ‡P<0.001.

We used the RT-PCR approach to determine the expression of PACAP/VIP receptors in the whole ovary. All three forms were found at all ages examined, though in different amounts. We found that the predominant form expressed in the rat ovary is PAC₁-R, while VPAC₁-R and VPAC₂-R are less abundant.

In the preovulatory follicles, we analyzed granulosa cells, oocytes, and TI cells separately for the presence of VIP/PACAP receptors. In accordance with the data published by Gras *et al.* (2000) in isolated granulosa cells, we observed abundant mRNA transcripts for PAC₁-R, lower levels of mRNA for VPAC₂-R and no mRNA for VPAC₁-R. PAC₁-R was the only receptor found to be statistically modulated by gonadotropin.

As far as TI cells are concerned, indeed, we found that VPAC₂-R and VPAC₁-R were both expressed in the TI compartment, whereas PAC₁-R was not. This is in agreement with our previous demonstration that PACAP and VIP are equally effective in stimulating granulosa cells cultured within the whole follicle (Apa *et al.* 1997, 2002), which thus suggests the presence of VIP receptors in TI cells.

In agreement with functional (Apa *et al.* 1997) and binding data (Gras *et al.* 2000) reported in the literature, the present study provides direct evidence of the presence of PAC₁-R and the absence of VIP receptors on GV oocytes. After having previously shown that PACAP, but not VIP, increases intracellular cAMP levels in denuded GV oocytes (Apa *et al.* 1997), we now demonstrate that 10^{-6} M PACAP stimulates Ca^{++} mobilization in the same oocytes. These data are consistent with the presence of a functional PAC₁ receptor in fully grown GV oocytes. PACAP receptors are already present in primordial germ cells in fetal gonads; moreover, PACAP has been shown to stimulate primordial germ cell proliferation via cAMP production (Pesce *et al.* 1996) and inhibit meiotic resumption in fully grown oocytes (Apa *et al.* 1997). However, after resumption of meiotic maturation, we observed a decrease in the levels of mRNA for these receptors, followed by a decrease in the number of functional receptors on the cell surface, as measured by an inability to stimulate Ca^{++} mobilization. Therefore, at the time of PACAP production in preovulatory follicles, 3–6 h after the LH surge (Gras *et al.* 1996, Koh *et al.* 2000), this peptide is no longer able to directly interfere with oocyte maturation.

The presence and the distribution of functional PACAP/VIP receptors in granulosa and TI cells have been supported by metabolic studies. In isolated granulosa cells, PACAP stimulated both cAMP production (Apa *et al.* 1997) and phosphoinositide (IP) turnover (this paper), while VIP stimulated cAMP production alone at high concentrations in the presence of a phosphodiesterase inhibitor (IBMX) (Apa *et al.* 1997). These data demonstrate the presence of functional PAC₁-R, the only one associated with the activation of both adenylate cyclase and PLC and of low number of VIP receptors. Conversely, in TI cells both PACAP and VIP stimulated cAMP but not IP breakdown, which is consistent

with the presence of VIP receptors and the absence of PAC₁-R.

Furthermore, here we demonstrate that PACAP and VIP both were able to prevent granulosa cell apoptosis in serum-free medium cultured follicles as already shown (Flaws *et al.* 1995, Lee *et al.* 1999), while only PACAP efficiently prevented apoptosis in isolated granulosa cells. These results together with the data obtained with the receptor antagonists further support the presence of functional VIP receptors on TI cells.

However, since TI cells are a very heterogeneous population we characterized VIP receptor localization in this compartment by immunofluorescence. In accordance with RT-PCR data, VPAC₂-R was ubiquitously found in the ovary, while VPAC₁-R was found in the proximity of follicles, but not in the GCs. The localization of VPAC₁-R suggests an association with the smooth muscle cells located in the theca externa (Ko *et al.* 2006). A strong signal was found also in the blood vessel wall.

VIP has been detected in neonatal rat ovaries as early as 2 days after birth; VIP-containing nerve fibres are present in rat ovaries around blood vessels, as well as around follicles at different stages of development, in close proximity of the theca cell layers and occasionally between primordial follicles (Ahmed *et al.* 1986). The contemporary presence of VIP and its receptors (this paper) around preantral and antral follicles suggests a possible role of VIP in both growing and preovulatory follicles. Indeed, VIP has been shown to suppress granulosa cell apoptosis, to promote follicle survival in *in vitro* growing preantral rat follicles (Flaws *et al.* 1995), and to stimulate ovarian steroidogenesis (Davoren & Hsueh 1985, Ahmed *et al.* 1986). Moreover, it has been shown that it exerts a relaxant effect on the rabbit ovarian artery (Jorgensen 1991). Therefore, the localization of VIP and its receptors in association with blood vessels suggests that this neuropeptide might be involved in the regulation of ovarian blood flow. The increased ovarian stromal blood flow may lead to a greater delivery of gonadotropins to the granulosa cells of the developing follicles (Redmer & Reynolds 1996).

In conclusion, the different pattern of distribution of PAC₁-R, VPAC₁-R and VPAC₂-R suggests distinct functional roles. The presence of VIP receptors around blood vessels, suggests an important role for this peptide in the blood flow regulation. In fact, perifollicular vascular expansion associated with increased rates of blood flow are developmentally important for the generation of a normal follicle and competent oocyte (Van Blerkom 2000).

The temporal and spatial distribution of PACAP, after the luteinizing hormone (LH) surge, suggests that the action of PACAP via PAC₁-R may be restricted to a specific developmental window. The fact that PACAP has been shown to induce genes related to ovulation and luteinization, and to mediate some of the effects of LH on granulosa cell differentiation at the time of ovulation (Gras *et al.* 1999, Lee *et al.* 1999, Park *et al.* 2000), suggests that PACAP is involved in the preovulatory follicle and that it may serve as an ovarian

physiological mediator of gonadotropin at the time of the ovulatory process. However, the presence of PACAP around growing follicles in cyclic rats, points to an additional role of this peptide in the regulation of growing follicle development at least in the adult ovary after the first LH surge. Indeed, we have recently demonstrated an inhibitory effect of PACAP on mouse preantral follicle growth and differentiation (Cecconi *et al.* 2004).

Indeed, activation of follicle development is under the influence of both stimulatory and inhibitory regulation (Skinner 2005). Abnormal regulation of primordial follicle development can affect the reproductive capacity of the female and menopausal onset, as shown in anti-Mullerian hormone null females (Durlinger *et al.* 1999). Further studies are needed to evaluate the role of PACAP and VIP in ovarian physiology.

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