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.


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); [125I]cAMP and [3H]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, Swiss); Meibstain 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 (NIDDK-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. 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 (Campari & 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 × 10^3/200 μl in MEM supplemented with 2% BSA, 2 mM glutamine and antibiotics (100 mM penicillin and 100 μg/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 μg/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 μg/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 (Ceconi et al. 2004). Groups of 25 follicles were incubated on stainless steel grids in zMEM 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 PCR’s 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 ovulatory follicles of eCG-treated rats, were mechanically isolated from the surrounding cumulus cells and either immediately lysed for RNA extraction or allowed to mature isolated from the surrounding cumulus cells and either immediately lysed for RNA extraction or allowed to mature isolated from the surrounding cumulus cells and either immediately lysed for RNA extraction or allowed to mature isolated from the surrounding cumulus cells and either immediately lysed for RNA extraction or allowed to mature.

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 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 by rapidly thawing and freezing the tube containing 2

mL RNasin (Promega). Tubes were then rinsed twice with Krebs–Henseleit–HEPES, and 5.5 mM glucose). [Ca2+]i, was calculated as previously described by Paniccia et al. (1995), with slight modifications.

Cytoplasmic Ca2+ concentration ([Ca2+]i) was measured by dual wavelength fluorescence of single cells loaded with the Ca2+-sensitive intracellular indicator fura-2-acetoxyethyl-ester (AM), as described by Paniccia 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, 5% CO2 for 60 min. Coverslips were then rinsed twice with Krebs–Henseleit–Hepes buffer (KH2O: 140 mM Na+, 5.3 mM K+, 132.4 mM Cl–, 0.98 mM PO43–, 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 by Grynkiewicz et al. (1985).

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<th>PCR conditions</th>
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Fw, forward primer; Rw, reverse primer.

Table 1 Sequence of selected oligonucleotides used as RT-PCR primers
Table 2  Sequence of selected oligonucleotides used as RT-PCR primers

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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 trioctylamine (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% formalaldehyde, 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 (Ceconi 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 Apoposis 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
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 1](image1.png) 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.

![Figure 2](image2.png) 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 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).
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

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**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.

**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 VPAC$_2$-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 VPAC$_1$-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, VPAC$_2$-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 PAC$_1$-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 VPAC$_1$-R and VPAC$_2$-R under the same conditions (Fig. 8). No signal for PAC$_1$-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 ($427 \pm 28$ nM), followed by a lower sustained phase ($106 \pm 7$ nM), which lasted more than 800 s (Fig. 9). No response was detected, in similar conditions, in Met I- and Met II-oocytes (data not shown).

Figure 6 VPAC$_1$-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 $\mu$m.

Met II-oocytes (data not shown). This result indicates that functional PAC$_1$-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
present in granulosa and TI cells, we studied the effect of the two peptides on inositol monophosphate (IP_1), inositol bisphosphate (IP_2), and inositol trisphosphate (IP_3) production. Granulosa cells were cultured for 48 h in the presence of myo-[2-H]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 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 [Ca^{2+}]_i in a single denuded and zona-free oocyte. The output of a microspectrofluorometer was calibrated as [Ca^{2+}]_o, as described in the Materials and Methods section. Upper panel: representative trace of the effect of $10^{-6}$ M PACAP on the [Ca^{2+}]_o of a single oocyte. Lower panel: values represent the mean ± s.e.m. of [Ca^{2+}]_o 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.
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 in the absence of serum. At the end of incubation total cAMP was measured by RIA. The values represent the mean ± S.E.M. of three independent experiments each performed in duplicate. *P<0.001, †P<0.0001 vs control.

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 (4.8%). In granulosa cells obtained from follicles incubated in serum-free medium for 24 h, apoptosis increased to 22.3% and PACAP inhibited apoptosis in a dose-dependent manner (24, 17, 12, 10, 10, and 10% respectively). Levels of apoptosis similar to those obtained with FSH (14% 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% 1-7% and FSH significantly reduced it to 47% 1-5%. PACAP inhibited apoptosis in a dose-dependent manner (53, 50, 46, 46, 1-9%, 10, 10, and 10% 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^{-8} 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 PAC$_1$-R, while VPAC$_1$-R and VPAC$_2$-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 PAC$_1$-R, lower levels of mRNA for VPAC$_2$-R and no mRNA for VPAC$_1$-R. PAC$_1$-R was the only receptor found to be statistically modulated by gonadotropin.

As far as TI cells are concerned, indeed, we found that VPAC$_2$-R and VPAC$_1$-R were both expressed in the TI compartment, whereas PAC$_1$-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 PAC$_1$-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$^{2+}$ mobilization in the same oocytes. These data are consistent with the presence of a functional PAC$_1$ 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$^{2+}$ 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 PAC$_1$-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 PAC$_1$-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, VPAC$_2$-R was ubiquitously found in the ovary, while VPAC$_1$-R was found in the proximity of follicles, but not in the GCs. The localization of VPAC$_1$-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 PAC$_1$-R, VPAC$_1$-R and VPAC$_2$-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 PAC$_1$-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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