Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and Its Receptors in the Zebrafish Ovary: Evidence for Potentially Dual Roles of PACAP in Controlling Final Oocyte Maturation

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide originally purified from ovine hypothalamus for its potent activity to stimulate cAMP production. However, its presence and action have also been demonstrated in various peripheral tissues including the ovary. In the zebrafish, two forms of PACAP (PACAP38-1, adcyap1a; and PACAP38-2, adcyap1b) and three PACAP receptors (PAC1-R, adcyap1r1; VPAC1-R, vipr1; and VPAC2-R, vipr2) were all expressed in the ovary. Interestingly, although both follicle cells and oocytes express adcyap1b, the expression of adcyap1a was restricted to the oocytes only. Among the three receptors, adcyap1r1 and vipr2 were expressed in the oocytes, whereas the expression of vipr1 was exclusively located in the follicle cells. Temporal expression analysis of PACAP ligands and receptors during folliculogenesis suggested that PACAP might play differential roles in regulating follicle growth and maturation through different receptors. The two receptors that are expressed in the oocyte (adcyap1r1 and vipr2) showed a significant increase in expression at the transition from the primary growth (PG) stage to previtellogenic (PV) stage and their levels maintained high during follicle growth. However, when the follicle development approached full-grown (FG) stage, these two receptors both decreased significantly in expression. In contrast, vipr1, the receptor expressed in the follicle cells, showed little change in expression at the PG-PV transition and afterwards during follicle growth; however, its expression surged dramatically at the FG stage prior to oocyte maturation. Based on these results, we hypothesized that PACAP might play dual roles in regulating follicle growth and maturation through different receptors located in different compartments. PACAP may stimulate oocyte growth but block its maturation in early follicles by acting directly on the oocyte via PAC1-R and VPAC2-R, whose expression is dominant in growth phase; however, PACAP may promote oocyte maturation in the maturation phase via VPAC1-R on the follicle cells, whose expression surges in FG follicles prior to maturation and is consistently high in the follicles undergoing final maturation. This hypothesis was further supported by the observation that PACAP promoted maturation of follicle-enclosed oocytes but suppressed spontaneous maturation of denuded oocytes in vitro. This study provides strong evidence for a PACAP-mediated signaling network in the zebrafish ovarian follicle, which may play roles in orchestrating follicle growth and maturation via different types of receptors located in different compartments of the follicle.

follicular development, follicular maturation, folliculogenesis, ovary, pituitary adenylate cyclase-activating polypeptide (PACAP), zebrafish

INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) was first isolated from ovine hypothalamus for its ability to stimulate cAMP production in cultured rat pituitary cells [1]. PACAP is a member of the secretin-glucagon-vasoactive intestinal peptide (VIP) family for its 68% amino sequence identity with VIP in humans [2]. The peptide exists in two amidated forms, PACAP38 and PACAP27. Both forms of PACAP are derived from the same gene and mRNA precursor with similar adenylate cyclase (AC)-stimulating activity [3]. Three PACAP receptors (PAC1-R, VPAC1-R, and VPAC2-R) have been identified in vertebrates [4]. They are all G protein-coupled receptors (GPCRs) belonging to family B of the GPCR superfamily [5]. PACAP receptors are classified into two classes based on their relative affinity for PACAP and VIP. The type I receptors (PAC1-R) have a higher binding affinity for PACAP than VIP, whereas the type II receptors (VPAC1-R and VPAC2-R) have similar affinity for both PACAP and VIP. Both types of receptors can stimulate AC activity to increase intracellular cAMP levels [6].

In addition to the central nervous system, PACAP and its receptors are also expressed in a variety of peripheral tissues including testis and ovary [6]. The widespread distribution of PACAP and its receptors suggests diverse physiological functions for this peptide in different tissues. PACAP and PAC1-R receptor knockout female mice both exhibited impaired fertility [7, 8], indicating roles of the PACAP family in reproduction. There have been lines of evidence that PACAP regulates reproduction by acting at different levels of the hypothalamic-pituitary-gonadal axis [9-13]. In the rat ovary, PACAP is transiently expressed in the granulosa cells of preovulatory follicles [14, 15], whereas its expression in the mouse preovulatory granulosa cells increases transiently in response to human chorionic gonadotropin (hCG) treatment [16]. PACAP mRNA and PACAP immunoreactivity were also found in the theca cells of some immature antral and preantral follicles as well as the interstitial glandular tissues around some preantral or primordial follicles [14, 17]. As for PACAP receptors, all three receptors are expressed in the preovulatory follicles of rat [18-20] and mouse [16]; however, they exhibit distinct distributions in different cells. PAC1-R and VPAC1-R were expressed in the granulosa cells and theca/interstitial cells, respectively, whereas VPAC2-R expression could be detected in both [16, 18]. The germ cells

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...expression patterns of PACAP and its receptors in follicles suggest potential roles for PACAP in both early follicle development and later meiotic oocyte maturation. This is supported by a series of in vitro experiments. PACAP promotes proliferation of mouse primordial germ cells [21], facilitates recruitment of immature rat follicles [22], increases preantral rat follicle growth, and inhibits follicular apoptosis in both rat and mouse ovaries [9, 16, 17]. However, PACAP has also been found to display inhibitory effects on primordial to primary follicle transition, granulosa cell proliferation, and growth of isolated rat preantral follicles [23]. In the late stage of folliculogenesis, PACAP accelerates final oocyte maturation in both mammals and fish [24, 25]; however, PACAP suppresses maturation of denuded oocytes in the rat [25].

Other activities reported for PACAP in the ovary include increasing cAMP production in cultured rat granulosa cells [26, 27], stimulating progesterone and estradiol (E2) accumulation [26–28], and modulating plasminogen activator expression (increasing tissue-type but decreasing urokinase-type) in rat granulosa cells [29].

The ovarian PACAP system seems to be controlled by pituitary gonadotropins. Injection of hCG significantly increased PACAP expression in the preovulatory granulosa cells in the mouse ovary [16] and a similar effect was observed in cultured bovine and rat preovulatory follicles [9, 30]. This regulation appears to be evolutionarily conserved across vertebrates, as hCG also significantly increased PACAP (PACAP38-2) expression in cultured zebrafish follicle cells [24]. PACAP is therefore likely a downstream mediator or modulator of pituitary gonadotropins in controlling ovarian function [24, 30].

As a well-conserved peptide, PACAP has been well characterized in fish [24, 31–36]. Similar to the situation in mammals, PACAP and its receptors are also expressed in a variety of tissues, including the nervous system, pituitary, and gonads (ovary and testis) [24, 32, 33, 37]. The functions of PACAP as a hypophysiotropic factor are well studied in fish [32]. In the grass carp, PACAP stimulates the secretion of both growth hormone (GH) and luteinizing hormone in the pituitary [37, 38]. In the goldfish, PACAP was detected in the pituitary by immunohistochemical staining and its regulatory effect on GH secretion was demonstrated in vitro in perfused pituitary cells [39]. In the zebrafish, treatment with PACAP for 72 h significantly stimulated the mRNA level of GH and suppressed that of both gonadotropin β subunits in primary pituitary cell culture [40]. Unlike in mammals, in which PACAP is a weak stimulator of GH release, it is a potent GH-releasing factor in fish [32, 39, 41]. Compared to its well-characterized hypophysiotropic activities as a neuropeptide in fish, the information on PACAP expression and function in peripheral tissues such as gonads is rather limited.

In the zebrafish, two forms of PACAP (PACAP27-1 and PACAP27-2) encoded by two separate genes (adcyap1a and adcyap1b) [24, 33] as well as all three PACAP receptors (PAC1-R, adcyap1r1; VPAC1-R, vipr1; and VPAC2-R, vipr2) have been reported [24, 42]. Interestingly, a recent study showed that zebrafish PAC1-R also responded to VIP, albeit to a lesser extent, and all three PACAP receptors expressed in the COS cells could also be activated by zebrafish peptide histidine-isoleucine to different extents in terms of cAMP accumulation, with the response of VPAC2-R being the strongest and that of PAC1-R the weakest (VPAC2-R > VPAC1-R > PAC1-R) [43]. As the study only tested zebrafish PACAP of 27 amino acids (PACAP27-1 and PACAP27-2), which activated the receptors in a reversed order (PAC1-R > VPAC1-R > VPAC2-R), it would be of interest to see how PACAP27-1 and PACAP38-2 behave in the same assay in future studies. Similar to the situation in mammals, all PACAP ligands and receptors are expressed in the zebrafish ovary. This raises a question on the physiological importance of the system in fish ovarian function. We have previously reported that the expression of PACAP38-2 (adcyap1b) is highly responsive to gonadotropin (hCG) stimulation in cultured zebrafish follicle cells, and PACAP38-2 significantly stimulates follistatin (fstd) expression and promotes oocyte maturation in intact follicles in vitro [24]. Other than these studies, our understanding of the physiological relevance of the PACAP system in zebrafish ovary remains largely unknown. To provide further insights into the roles of PACAP in zebrafish ovarian function, this study was undertaken to systematically characterize PACAP genes and its receptors in the ovarian follicle and during folliculogenesis. Our results suggest that PACAP may play dual roles in orchestrating folliculogenesis by promoting follicle growth and inhibiting maturation in early stage and vice versa in late stage via different receptors in different follicle compartments.

**MATERIALS AND METHODS**

**Animals and Chemicals**

Zebrafish (Danio rerio) were purchased from local fish stores and maintained in flow-through aquaria at 28 ± 1°C on a photoperiod of 14L:10D, with lights-on at 0900 h (previously 0800 h in our laboratory). The fish were fed twice a day with the commercial tropical fish food Otohime S1 (Marubeni Nissin Feed Co., Tokyo, Japan) and frozen Artemia once a day. All experiments performed were under the license from the Government of the Hong Kong Special Administrative Region and endorsed by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong.

Unless otherwise specified, all common chemicals were obtained from Sigma-Aldrich (St. Louis, MO), GE Healthcare (Waukesha, WI), Merck (Whitehouse Station, NJ), and USB Corporation (Cleveland, OH). The enzymes were purchased from Promega (Madison, WI) and culture medium from Gibco Invitrogen (Carlsbad, CA). 17α, 20β-dihydroxy-4-pregnene-3-one (DHP) and 17β-E2 were purchased from Sigma-Aldrich and 3-isobutyl-1-methylxanthine (IBMX) from Merck. Zebrafish PACAP-2 was generously provided by Dr. Anderson O.L. Wong (School of Biological Sciences, The University of Hong Kong) and its synthesis and characterization have been described in our previous report [24]. The peptide was first dissolved in water as stock and then diluted to the desired concentrations with medium before use.

**Isolation of Ovarian Follicles**

Zebrafish were anesthetized by ice shock and decapitated before dissection. For isolation of ovarian follicles, the two ovaries were carefully removed from 10–20 female zebrafish and placed in a 100-mm Petri dish containing 60% Leibovitz L-15 medium. The follicles of different stages were manually separated with fine forceps and grouped into six developmental stages based on both diameter and morphology: primary growth (PG; stage I, ≤100 μm), previtellogenic (PV; stage II, 100–250 μm), early vitellogenic (EV; stage III, 250–350 μm), middle vitellogenic (MV; stage III, 350–450 μm), late vitellogenic (LV; stage III, 450–550 μm) and full-grown (FG; stage III, ≥550 μm). The staging of the follicles was based on our previous study [44] with minor modifications. First, the defining size of FG follicles was reduced from 150 μm to 100 μm and less. Second, an additional stage of LV was added to better illustrate the developmental profiles of gene expression during folliculogenesis.

To study temporal expression profiles of PACAP and its receptors during the periovulatory period in vivo, we obtained follicles of different maturational stages from fish sampled at different times before lights-on at 0900 h (0530, 0630, 0730, and 0830 h). Three types of follicles were collected: immature FG follicles without germinal vesicle breakdown [GVBD(−)], mature but nonovulated follicles [GVBD(+)], and ovulated eggs without follicle layer. The experimental setup and fish grouping were based on our recent report [45]. At each time point, 10 female zebrafish from each group were sampled and the ovaries from all fish were pooled together and quickly dispersed in 60% L-15.
medium for follicle isolation and collection. The GVBD(–) and GVBD(+) follicles and ovulated eggs were obtained from fish sampled at 0530 h and 0630, 0730, and 0830 h, respectively, for RNA extraction. Both GVBD(–) and GVBD(+) follicles were collected at 0730 h to compare immature and mature follicles at the same time.

**Separation of Oocyte and Follicle Layer**

To examine spatial distribution of PACAP and its receptors in different compartments of the follicle and study the effects of PACAP on intact follicles and denuded oocytes without follicle layer, we carefully separated the somatic follicle layer from the FG oocytes using fine forceps according to the manufacturer’s protocol and our previous report [50]. Reverse transcription (RT) was performed at 37°C for 2 h in a total volume of 10 μl reaction solution containing 0.5 μg oligo (dT), 1 mM dithiothreitol, and 100 U M-MLV reverse transcriptase (Invitrogen). For quantitative analysis, the total RNA was quantified and 3 μl total RNA was used for PCR amplification.

**Follicle Incubation and Oocyte Maturation Assay**

To obtain spontaneously matured follicles in vitro, the immature FG follicles (≥650 μm) were isolated and incubated as previously reported [48]. In brief, immature FG follicles were randomly placed into a 24-well plate (about 40 follicles per well) and incubated in Cortland medium M199 supplemented with 10% fetal calf serum (HyClone, Logan, UT) at 28°C in 5% CO₂. The incubation lasted for 6 days, during which the follicle cells proliferated significantly. The proliferated follicle cells were harvested by trypsinization and plated in 24-well plates for drug treatment and RNA extraction.

**Primary Follicle Cell Culture**

The primary culture of zebrafish ovarian follicle cells was performed according to our previous report [49]. Briefly, the follicles of MV and earlier stages were isolated from about 20 female zebrafish, washed, and incubated in medium M199 supplemented with 10% fetal calf serum (HyClone, Logan, UT) at 28°C in 5% CO₂. The incubation lasted for 6 days, during which the follicle cells proliferated significantly. The proliferated follicle cells were harvested by trypsinization and plated in 24-well plates for drug treatment and RNA extraction.

**RNA Extraction and Reverse Transcription**

Total RNA was extracted from follicle samples or cultured follicle cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol and our previous report [50]. Reverse transcription (RT) was performed at 37°C for 2 h in a total volume of 10 μl reaction solution containing 0.5 μg oligo (dT), 1× MMLV RT buffer, 0.5 mM each dNTP, 0.1 mM dithiothreitol, and 100 U M-MLV reverse transcriptase (Invitrogen). For quantitative analysis, the total RNA was quantified and 3 μg cDNA from each sample was used for RT reaction.

**Semiquantitative RT-PCR and Real-Time Quantitative PCR**

The primers for PCR were designed according to the sequences available in GenBank and synthesized by Integrated DNA Technologies (Coralville, IA; Table 1). Conventional semiquantitative RT-PCR was used to study spatial distribution of the PACAP family in the follicle of the FG stage and the cycle numbers used were 29, 33, 30, 34, 30, 36, 33, and 31 for glyceraldehyde 3-phosphate dehydrogenase (gapdh), genes encoding gonadotropin releasing hormone (lhcgr), growth differentiation factor 9 (gdf9), PACAP₁-R (adcyap1r1), PACAP₂-R (adcyap1b), PACAP₅-R (adcyap1r1), VPAC₁-R (vipr1), and VPAC₂-R (vipr2), respectively. PCR was performed in a total volume of 15 μl.
containing 5 µl of 1:15 diluted RT products and standard PCR reagents. The PCR products were then visualized in 1.5% agarose gel containing ethidium bromide.

The mRNA levels of the PACAP system in the zebrafish ovarian follicles at different developmental stages were quantified by real-time quantitative PCR (qPCR). The standards of real-time qPCR were prepared by PCR amplification of cDNA fragments with specific primers (Table 1). The amplicons were purified after gel electrophoresis with the PCR Purification Kit (Qiagen, Valencia, CA) and quantified by electrophoresis with the Mass Ruler DNA Marker (MBI Fermentas, Hanover, MD) and the software Quantity One (Bio-Rad, Hercules, CA). The quantified DNA molecule of each gene was used to construct a standard curve in each real-time qPCR assay. The real-time qPCR was performed on the iCycler iQ or CFX96 Real-Time PCR Detection System (Bio-Rad) according to our recent report [46]. The specificity of the detection was confirmed by both gel electrophoresis and melt-curve analysis.

Data Analysis

The mRNA level of each target gene was normalized to the internal control ef1a, and in some experiments was further normalized as percentage or fold change compared with control or reference group. All values were expressed as the mean ± SEM, and the data were statistically analyzed by one-way ANOVA, followed by Dunnett or Newman-Keuls test using Prism 5 on Macintosh OS X (GraphPad Software, San Diego, CA). All the results were confirmed by repeated experiments.

RESULTS

Spatial Distribution of PACAP System Within the Follicle

To understand the spatial distribution of the PACAP family within the zebrafish follicle, we separated the somatic follicle layer, which is supposed to include both granulosa and theca cells, from the oocyte at the FG stage, and analyzed the expression distribution of both PACAP ligands and receptors in these two compartments. The clean separation of these two compartments was monitored by detecting the expression of two marker genes lhcgr and gdf9 for follicle layer and oocyte, respectively [47, 51]. The exclusive expression of lhcgr in the somatic follicle cells and gdf9 in the oocytes demonstrated the purity of the two compartments separated (Fig. 1A). PACAP ligands and their receptors exhibited distinct spatial distribution patterns in the follicle. Both ligands (adcyap1a and adcyap1b) were detected in the oocyte, with adcyap1b being also equally detectable in the follicle layer. Among the three receptors, adcyap1r1 and vipr2 were exclusively expressed in the denuded oocytes together with gdf9, whereas vipr1 was exclusively expressed in the follicle layer together with lhcgr. Gapdh was used as the housekeeping gene in this experiment.

B) Expression of PACAP family members in cultured follicle cells. Most genes analyzed showed expected expression in these cells except vipr2, whose expression could not be detected in the freshly isolated follicle layer shown in A, but showed up in the cultured follicle cells.

C) Schematic representation of the spatial distribution of PACAP system in the follicle and its potential action modes.
compartments, the cultured follicle cells expressed *adcyap1b* with little expression of *adcyap1a*. Also, *vipr1* expression could be easily detected in these cells but not *adcyap1r1*. The only gene that exhibited discrepancy was *vipr2*, whose expression could not be detected in the freshly isolated follicle layer, but showed up in the cultured follicle cells (Fig. 1B).

**Temporal Expression Profiles of the PACAP System During Folliculogenesis**

To provide clues to the roles of the PACAP family in follicle growth and maturation, we examined the expression profiles of PACAP family ligands and receptors during folliculogenesis. As controls, we included two gonadotropin receptors, *fshr* (follicle-stimulating hormone receptor) and *lhcgr* (luteinizing hormone receptor), in the analysis, as their distinct expression profiles during zebrafish folliculogenesis have been well established [52]. As expected, the expression of *fshr* increased dramatically during the PG-PV transition when the follicles are activated to enter the secondary growth phase involving vitellogenesis, peaked at the MV stage, and then decreased significantly at the LV and FG stages. In contrast, the expression of *lhcgr* was low with little change in early stages, increased progressively from the MV to the LV stage, and surged at the FG stage to its peak level. As housekeeping gene, the expression of *ef1a* maintained relatively stable during folliculogenesis (Fig. 2A). In comparison with *fshr* and *lhcgr*, the expression profiles of *adcyap1a*, one of the PACAP ligands, was similar to that of *fshr*. Its expression increased significantly during the PG-PV transition and continued to rise until the MV stage, followed by a decline at the LV and FG stages. However, the expression profile of *adcyap1b*, the second PACAP ligand, was different in that its expression steadily declined during the entire folliculogenesis, reaching the lowest level at the LV and FG stages (Fig. 2B). The three PACAP receptors also exhibited distinct temporal patterns of expression during folliculogenesis. The expression profiles of *adcyap1r1* and *vipr2*, the two receptors that are expressed in the oocyte, were similar to that of *adcyap1a*. Their expression quickly rose to the highest levels at the PG-PV transition and maintained high during most parts of vitellogenic growth until the MV stage; however, their levels both decreased significantly at the LV and FG stages prior to final oocyte maturation (Fig. 2C). In contrast, the follicle cell-expressed receptor *vipr1* showed little change in expression during most parts of follicle growth (in most experiments it exhibited a slight decline, with a small bounce often observed at the MV stage), reaching the lowest at the LV stage; however, its expression level surged dramatically to its highest level at the FG stage prior to final oocyte maturation (Fig. 2C).

It should be noted that the expression of *adcyap1b* was nearly 10 times more abundant than that of *adcyap1a*, and *vipr2* had the highest expression level among the three receptors during folliculogenesis (Fig. 2, B and C).

**Expression Profiles of PACAP System in the Periovulatory Period In Vivo**

We have previously reported that PACAP could induce final oocyte maturation or GVBD in vitro in FG follicles [24]. To provide further clues to the involvement of the PACAP system in final oocyte maturation, we analyzed the expression patterns of PACAP system during the periovulatory period in GVBD(−) and GVBD(+) follicles as well as ovulated eggs. Follicles of these stages were collected at different times before lights-on at 0900 h (0 h) as we recently reported [45]. We collected immature FG follicles [GVBD(−)] at 0530 h (−3.5 h) and 0630 h (−2.5 h), mature but nonovulated follicles [GVBD(+) at 0730 h (−1.5 h), and ovulated defollicled eggs at 0830 h (−0.5 h); Fig. 3B). The two PACAP ligands *adcyap1a* and *adcyap1b* both decreased their expression during and after maturation, with the level of *adcyap1a* expression reaching its lowest at 0830 h in the ovulated eggs. However, after reaching its lowest level at 0730 h, the expression of *adcyap1b* rebounded significantly in the ovulated eggs at 0830 h (Fig. 3A). As for the receptors, the oocyte-expressed *adcyap1r1* and *vipr2* showed a slight decrease in expression during maturation at 0630 and 0730 h, although the change of *adcyap1r1* was not significant. In

![FIG. 2. Temporal expression profiles of the PACAP system during folliculogenesis. A]('https://www.biolreprod.org/content/619/3/619/F2A)

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contrast, the follicle cell-expressed $\text{vipr1}$ dramatically increased its expression in GVBD follicles at 0730 h compared with immature FG follicles at 0530 and 0630 h. Its expression was not detectable in the ovulated eggs at 0830 h, reflecting the fact that its expression is restricted to the follicle layer (Fig. 3A).

**Expression Change of the PACAP System During In Vivo and In Vitro Maturation**

The result described above showed that the expression of the PACAP system underwent distinct changes during final oocyte maturation, with PACAP ligands ($\text{adcyap1a}$ and $\text{adcyap1b}$) and oocyte-expressed receptors ($\text{adcyap1r1}$ and $\text{vipr2}$) decreasing and follicle cell-expressed receptor ($\text{vipr1}$) increasing during GVBD, as observed in the matured follicles collected at 0730 h. To further assess the correlation between oocyte maturation and the expression changes, as well as the potential impact of sampling time on the result, we performed another analysis on PACAP family expression by comparing the follicles that failed to mature [GVBD(−)] and the mature GVBD(+) follicles at 0730 h. As shown in Figure 4, $\text{adcyap1a}$ decreased whereas $\text{adcyap1b}$ increased the expression in mature follicles, a pattern somehow very similar to that observed at 0830 h described above (Fig. 4A). On the other hand, although $\text{adcyap1r1}$ and $\text{vipr2}$ had slightly increased expression in the GVBD(+) follicles, which differed from the observation on follicles collected at different times, the expression of $\text{vipr1}$ exhibited a phenomenal increase in GVBD(+) follicles (Fig. 4B), in agreement with that described in Figure 3.

We also performed an experiment to analyze the expression changes of the PACAP system during spontaneous oocyte maturation in vitro. After the immature FG follicles had been incubated at 28°C for 10 h, some follicles had undergone spontaneous maturation or GVBD, and these follicles were separated from the immature ones. As shown in Figure 5, most genes examined did not show significant changes in expression during in vitro maturation, except $\text{adcyap1a}$, whose expression again decreased (Fig. 5A). The most striking difference was the
expression of vipr1. Although this follicle cell-expressed receptor dramatically increased expression in vivo during maturation, it only displayed a slight, nonsignificant increase during in vitro maturation (Fig. 5B).

**Effects of PACAP on Final Maturation of Intact Follicles and Denuded Oocytes**

As oocyte maturation in fish is accompanied by a decrease in cAMP level in the oocyte but an increase in the follicle cells [53], the expression of two PACAP receptors in the oocyte (adcyap1r1 and vipr2) and one in the follicle cells (vipr1) suggests that the activation of these receptors in the two compartments by PACAP may lead to different responses of oocytes during maturation. To provide critical evidence for this, we examined the effects of PACAP on final oocyte maturation using denuded oocytes without a follicle layer and intact FG follicles. In agreement with our previous report [24], PACAP significantly increased maturation rate in intact follicles in a dose-dependent manner; however, its potency was not as high as that of DHP, the most important maturation-inducing hormone in teleosts (Fig. 6A). Interestingly, removal of follicle layer led to a striking increase in spontaneous maturation rate. In contrast to its stimulatory effect on intact follicles, the addition of PACAP significantly reduced, albeit did not completely abolish, the surged spontaneous maturation (Fig. 6B). As a control, we also tested the effect of E2, which was recently reported to act directly on the oocyte to inhibit its maturation [54]. As expected, E2 also reduced the maturation rate with potency similar to that of PACAP. The combination of PACAP and E2 caused a further, but not complete, reduction in spontaneous maturation (Fig. 6B). Inclusion of IBMX in the medium before treatment effectively suppressed spontaneous maturation, an indication of the importance of cAMP in controlling final maturation (Fig. 6C).

**DISCUSSION**

In the zebrafish, two forms of PACAP (adcyp1a and adcyp1b) and three PACAP receptors (adcyp1r1, vipr1, and vipr2) have been reported, and they are all expressed in the ovary [24, 33, 42], suggesting that PACAP may be involved in controlling folliculogenesis in a paracrine or autocrine manner.

To provide clues to the importance of the PACAP system in the function of zebrafish ovary, we first investigated the spatial distribution of both PACAP ligands and the three potential receptors in the somatic follicle layer and denuded oocytes. Interestingly, both PACAP ligands were expressed in the oocyte, with adcyp1b expression also detectable in the follicle layer. In contrast, PACAP in mammalian ovary is primarily expressed in the granulosa cells of preovulatory follicles [14, 16]. In rat primordial and small preantral follicles, PACAP immunoactivity could also be observed in the thecal/interstitial cells [14, 17]. On the other hand, all three PACAP receptors (PAC1-R, VPAC1-R, and VPAC2-R) in mammals were expressed in the somatic follicle cells, with PAC1-R and
was used as the positive control. Values are the mean of PACAP at different doses for 12 h before scoring GVBD follicles. DHP
The intact follicles were isolated and incubated in the absence or presence
of PACAP at different doses for 12 h before scoring GVBD follicles. DHP was
used as the positive control. Values are the mean ± SEM (n = 3) from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 vs.
control.

B) PACAP suppression of GVBD in denuded oocytes. The denuded oocytes were incubated in the presence or absence of PACAP (10 nM) for 6 h, followed by scoring of GVBD follicles or oocytes. E2 was
used as the positive control. Because the preparation of denuded oocytes
was performed in Ca2+/Mg2+-free medium, the same medium was used for
the intact follicles to ensure no effect of the medium on the maturation rate. Values are the mean ± SEM (n = 3) from three independent experiments. *P < 0.05.

C) Denuded oocytes in the presence or absence of
IBMX (0.1 mM). IBMX was added to the medium during preparation of the
denuded oocytes to ensure no spontaneous maturation before treatment.

FIG. 6. Inverse effects of PACAP on the maturation of intact follicles and
denuded oocytes. A) PACAP stimulation of GVBD in intact FG follicles.
The intact follicles were isolated and incubated in the absence or presence
of PACAP at different doses for 12 h before scoring GVBD follicles. DHP
was used as the positive control. Values are the mean ± SEM (n = 3) from a
representative experiment. *P < 0.05, **P < 0.01, ***P < 0.001 vs.
control. B) PACAP suppression of GVBD in denuded oocytes. The
denuded oocytes were incubated in the presence or absence of PACAP (10
nM) for 6 h, followed by scoring of GVBD follicles or oocytes. E2 was
used as the positive control. Because the preparation of denuded oocytes
was performed in Ca2+/Mg2+-free medium, the same medium was used for
the intact follicles to ensure no effect of the medium on the maturation rate. Values are the mean ± SEM (n = 3) from three independent experiments. *P < 0.05.
C) Denuded oocytes in the presence or absence of
IBMX (0.1 mM). IBMX was added to the medium during preparation of the
denuded oocytes to ensure no spontaneous maturation before treatment.

The PACAP receptors also displayed interesting patterns of expression in follicle development. The two oocyte-expressed receptors, adcyap1r1 and vipr2, showed similar expression profiles in parallel to that of the ligand adcyap1a; they both increased expression at PG-PV transition and maintained high
VPAC1-R in granulosa cells and theca/interstitial cells, respectively, whereas VPAC2-R was in both [16, 18]. The
oocytes could also be the target of PACAP, as PAC1-R
expression was detectable in these germ cells [18–20]. In the
zebrafish ovarian follicle, however, two PACAP receptors
(PAC1-R, adcyap1r1, and VPAC2-R, vipr2) were expressed in
the oocyte, whereas the somatic follicle cells expressed
VPAC1-R (vipr1). Interestingly, although it could not be
detected in freshly isolated follicle layers, the expression of
VIPRA2 showed up in cultured zebrafish follicle cells. This
is probably because its expression level in the fresh follicle layer
was too low to detect, but the mRNA became more
concentrated in pure follicle cell culture. The discrepancy also
raises the possibility that the cultured follicle cells may have functionally deviated from their original physiological state in
the follicle after incubation in vitro for more than 1 week. The
distribution of PACAP system within the follicle appears to be
different between zebrafish and mammals; however, it seems to
hold true in both animals that PACAP can act on both follicle
cells and oocytes that express different receptors.

The evidence from mammalian models on PACAP expression at different stages of follicle development suggests
that PACAP may function in both early folliculogenesis and
final stage toward oocyte maturation. Indeed, recent studies in
the rat have shown that PACAP stimulates proliferation of
primordial germ cells and increases the recruitment of
immature follicles [17, 21, 22]. The involvement of PACAP
in final oocyte maturation and ovulation is evidenced by the
observations that PACAP exerts positive effects on several
parameters related to follicle maturation and ovulation [6] and
that the production of PACAP and its receptors is stimulated by
gonadotropin in rat preovulatory follicles [20]. To provide
clues to potential roles of the PACAP system in zebrafish
folliculogenesis, we analyzed the temporal expression profiles
of PACAP ligands and receptors during follicle growth and
maturation. PACAP ligands and receptors displayed dynamic
changes in their expression during folliculogenesis. The
expression of adcyap1a exhibited a significant increase and
adcyap1b showed a decrease during the transition from PG to
PV stage, which marks the recruitment of the follicles from the
gonadotropin-independent PG phase to the gonadotropin-
dependent secondary growth phase. The significant changes
in PACAP expression at the PG-PV transition suggest a role
for the peptides in early folliculogenesis, in particular at the
activation of follicles or the initiation of vitellogenesis. After
the PG-PV transition, the expression of adcyap1a continued to
rise and remained high during most parts of vitellogenic growth
until the MV stage, but the level decreased significantly at the
LV stage and reached the lowest point at the FG stage prior to
oocyte maturation. Although our previous nonquantitative gel
electrophoresis showed a seemingly increased expression of
adcyap1b from the PV to the MV stage [24], real-time qPCR
analysis in this study revealed a steady decrease in adcyap1b
expression during the entire process of follicle growth with the
lowest level reached at the FG stage, which is different from
that of adcyap1a. The differential expression of adcyap1a and
adcyap1b during folliculogenesis implies distinct roles for
these two forms of PACAP in zebrafish follicle growth and
maturation. Future studies using synthetic zfPACAP38-1 and
zfPACAP38-2 promise to provide insights into this interesting
issue.
levels in vitellogenic growth until the MV stage, but decreased quickly before maturation. This expression pattern, together with their localization in the oocyte, has led us to hypothesize that there may be a strong PACAP signaling towards the oocyte during early follicle growth, which weakens when the follicle approaches the end of growth for final maturation. In contrast to the oocyte-expressed receptors, the follicle cell-expressed receptor vipr1 had a relatively stable and often a declining expression during follicle growth, reaching the lowest point at the LV stage; however, its expression level surged dramatically at the FG stage when the oocyte-expressed receptors dropped to their lowest point. This strongly implicates vipr1 in the process of final oocyte maturation. In support of this speculation is the evidence that vipr1 expression significantly increased in the follicles undergoing maturation in vivo at 0730 h as compared to the immature follicles collected at earlier times or those from the same time point. It is interesting to note that vipr1 expression did not increase significantly in the follicles undergoing maturation in vitro. One explanation for this discrepancy is that the increased expression of vipr1 in vivo might be induced by certain endocrine hormones acting on the follicle cells, and such endocrine environment was lacking in vitro. It would be interesting in the future to investigate how vipr1 expression is regulated in the follicle cells.

The distinct spatial distribution of PACAP receptors within the follicle and their contrasting temporal expression profiles during follicle growth and final oocyte maturation have led us to hypothesize that the ovarian PACAP ligands may play dual roles during follicle development by acting on the oocytes and follicle cells, respectively, at different stages. In early stages when PACAP ligands, particularly adcyap1a, and the two oocyte-associated receptors (adcyap1rl and vipr2) have high expression, PACAP may predominantly act on the growing oocytes to promote their growth while suppressing precocious maturation as a maturation-inhibiting factor. This action may be mediated by increasing the intracellular level of cAMP, which is the key second messenger that blocks oocyte maturation in both mammals [55] and fish [53, 56, 57]. When follicles approach the final stage of growth, the expression of adcyap1rl and vipr2 significantly decreases, which would signal the cessation of follicle growth and relieve the oocyte from the inhibition by PACAP, therefore preparing it for the final maturation induced by pituitary gonadotropins and DHP, the maturation-inducing hormone in the zebrafish. Whereas adcyap1rl and vipr2 decrease their expression in the oocyte, the expression of vipr1 in the follicle cells surges prior to maturation, suggesting a shift of PACAP target from the oocyte to the follicle layer, leading to a decreased cAMP level in the oocyte but an increased level in the follicle cells. The increased cAMP level in the follicle cells may mimic the action of pituitary gonadotropins to promote oocyte maturation. In support of this is our previous finding that PACAP promotes oocyte maturation in FG follicles and adcyap1b (zfPACAP38-2) is highly stimulated by hCG in zebrafish follicle cells [24]. Because both PACAP and gonadotropins use cAMP as the major signaling messenger, the strong response of adcyap1b to hCG has led to the hypothesis that PACAP may serve as a local amplifier of pituitary gonadotropins in stimulating oocyte maturation.

To provide further evidence for the hypothesis on dual roles of PACAP in controlling oocyte maturation during folliculogenesis, we performed a critical experiment using both intact FG follicles and denuded oocytes. In agreement with our previous report [24], PACAP significantly promoted final oocyte maturation in intact follicles with a follicle layer. In contrast, when applied to denuded oocytes, PACAP significantly reduced the spontaneous maturation rate. These results suggest that in the presence of follicle layer expressing vipr1, PACAP may exert a stimulatory effect on oocyte maturation through this receptor, mimicking the action of gonadotropins. The inhibitory effect of PACAP showed up after removal of the follicle layer, which is likely mediated by the receptors expressed in the oocyte. Our observation was surprisingly similar to a previous report in the rat that PACAP could stimulate meiotic resumption of follicle- and cumulus-enclosed follicles, but suppress that of denuded oocytes [25]. The similar phenomenon in both zebrafish and rat suggests that this could be a conserved function for PACAP in vertebrate ovary. What is interesting to note is that in agreement with a recent report by Pang and Thomas [54], removal of the somatic follicle layer alone in the zebrafish dramatically increased the rate of spontaneous maturation, supporting the notion that the surrounding follicle cells may exert a tonic inhibitory effect on oocyte maturation [54]. The factors that mediate such tonic inhibition are not fully understood. PACAP could be one of such factors that prevent oocytes from precocious maturation in the early developmental stage. However, as PACAP only partially suppressed the spontaneous maturation of the denuded oocytes, it is conceivable that the tonic inhibition may also involve other mechanisms within the follicle. One major factor could be E2, which has recently been well demonstrated to inhibit oocyte maturation via a membrane receptor, OPER (previously GPR30), in the zebrafish [54]. E2 also exhibited an inhibitory effect on oocyte maturation in the present study, with an activity similar to that of PACAP. The combination of PACAP and E2 led to a further decline in spontaneous maturation; however, the inhibition was not complete, implying the involvement of other factors. Our preliminary data suggested that the Kit system might also play a similar role in the process (Yao and Ge, unpublished results). Further studies are warranted to elucidate the details of the underlying mechanisms.

In summary, as suggested in mammals, the PACAP system may play important roles not only at the early stage of folliculogenesis to promote oocyte growth while maintaining meiotic arrest but also in late stage to promote final oocyte maturation. PACAP may likely exert these contrasting effects via different receptors located in different compartments of the follicle (Fig. 1C). Together with the evidence in mammals, the dual roles of PACAP in controlling final oocyte maturation may be a conserved mechanism across vertebrates.

REFERENCES


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