

Pituitary adenylate cyclase-activating polypeptide acts synergistically with relaxin in modulating ovarian cell function in rats

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Abstract

The interactive effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and relaxin on the secretion of gelatinases, involved in matrix remodeling, in ovarian theca-interstitial cells and granulosa cells, were investigated in gonadotropin-primed immature rats. The gelatinases secreted from cultured cells were analyzed using gelatin zymography and scanning densitometry. We have previously shown that relaxin stimulated the secretion of a 71 kDa gelatinase, identified as a type IV collagenase (matrix metalloproteinase 2), in rat theca-interstitial cells. This study has demonstrated that PACAP27 and PACAP38, with similar potency, dose-dependently enhanced relaxin-induced secretion of 71 kDa gelatinase, whereas PACAP alone had no effect. In rat granulosa cells, both PACAP27 and PACAP38 alone dose-dependently increased the secretion of a 63 kDa gelatinase. In addition, this study has shown that cAMP signaling pathway mediators act similarly to that of PACAP on gelatinase secretion in rat ovarian cells. Cholera toxin, forskolin and 8-bromoadenosine cAMP

augmented relaxin-induced secretion of 71 kDa gelatinase in theca-interstitial cells, and alone they had no effect. These mediators also increased the secretion of 63 kDa gelatinase in granulosa cells. It is well known that the increase in cellular cAMP level is associated with the morphological rounding-up phenomenon in granulosa cells. This study has shown that PACAP and cAMP pathway mediators, but not relaxin, could cause such changes in cell shape in granulosa cells as well as in theca-interstitial cells. In conclusion, this study provides original findings that PACAP acts synergistically with relaxin in stimulating the secretion of gelatinases in rat ovarian theca-interstitial cells and granulosa cells. This supports the idea that relaxin and PACAP may serve as ovarian physiological mediators of gonadotropin function in facilitating the ovulatory process. In addition, PACAP appears to act through the cAMP signaling pathway to affect biological functions in ovarian cells, whereas relaxin does not.

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Introduction

During each mammalian reproductive cycle, ovarian follicles develop, mature, ovulate, transform into corpora lutea and possibly become atretic at various stages of development. All these changes are accompanied by the remodeling of the extracellular matrix surrounding the follicles. The ovulatory process, triggered by the gonadotropins (Richards *et al.* 1987, LeMaire 1989, Irianni & Hodgen 1992, Espey & Lipner 1994), is probably the extreme example of matrix remodeling in the ovary. It is generally believed that remodeling of extracellular matrix involves at least two important enzyme systems, the matrix metalloproteinase (MMP) system and the serine proteinase plasminogen activator system (Seller & Murphy 1981, Harris *et al.* 1984, Saksela 1985, Matrisian 1990). These

proteinases have been shown to play important roles in the ovulatory process (Reich *et al.* 1985, Palotie *et al.* 1987, Brannstrom *et al.* 1988, Tsafirri *et al.* 1989, Butler *et al.* 1991, Curry *et al.* 1992).

Besides gonadotropin, several factors are indicated as playing a role in ovulation, including prostaglandins, steroids, histamine and gonadotropin-releasing hormone (GnRH) (Tsafirri *et al.* 1987, Richards & Hedin 1988, LeMaire 1989, Tonetta & DiZerega 1989, Espey & Lipner 1994, Richards 1994). An ovarian peptide hormone, relaxin, was recently shown to facilitate ovulation in the rat. Human relaxin can induce ovulation in the *in vitro* perfused rat ovary (Brannstrom & MacLennan 1993). Furthermore, administration of monoclonal antibody specific for rat relaxin partially suppressed gonadotropin-induced ovulation in rats, and this was reversed by

concomitant treatment with porcine relaxin (Hwang *et al.* 1996). Limited studies suggest that this effect of relaxin may be mediated through regulating the remodeling of extracellular matrix. Relaxin increases the secreted activity of plasminogen activator, collagenase (MMP1) and proteoglycanase in rat ovarian granulosa cells (Too *et al.* 1982, 1984). In addition, relaxin increases the secretion of different major gelatinases from rat granulosa cells and theca-interstitial cells including a type IV collagenase (MMP2) (Hwang *et al.* 1996).

Ovulation appears to be triggered by the co-operative action of several ovarian factors at the preovulatory period. Interestingly, pituitary adenylate cyclase-activating polypeptide (PACAP) has recently been suggested to play a role in ovarian physiology. PACAP, a member of the vasoactive intestinal peptide (VIP)/secretin/glucagon/growth hormone-releasing hormone family, was originally isolated from the ovine hypothalamus for its ability to stimulate cAMP production in rat pituitary cells (Miyata *et al.* 1989, 1990). PACAP can activate ovarian function through stimulating basal and GnRH-induced secretion of luteinizing hormone (LH) (Culler & Paschall 1991, Hart *et al.* 1992). Moreover, PACAP may play an autocrine/paracrine role in the ovary. First, PACAP stimulates steroidogenesis and increases cAMP levels in cultured rat granulosa cells (Zhong & Kasson 1994, Heindel *et al.* 1996, Kotani *et al.* 1998). Secondly, PACAP was recently shown to accelerate meiotic maturation in follicle- and cumulus-enclosed rat oocytes (Apa *et al.* 1997). In addition, PACAP was transiently expressed during the periovulatory period in the rat ovary (Gras *et al.* 1996). It is thus of great interest to understand whether PACAP plays a role in modulating the remodeling of extracellular matrix involved in ovulation. To that end, the purpose of this study was to investigate the effect of PACAP and its interaction with relaxin on modulating the secretion of gelatinases involved in matrix remodeling in the rat ovary.

Materials and Methods

Materials

Porcine relaxin was generously provided by Dr O D Sherwood (University of Illinois, Urbana-Champaign, IL, USA). PACAP27 and PACAP38 were obtained from Peninsula Laboratories, Inc. (Belmont, CA, USA). Equine chorionic gonadotropin (eCG), bovine insulin, lactalbumin hydrolysate, 8-bromoadenosine cAMP (8-Br-cAMP), forskolin and cholera toxin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's minimal essential medium (DMEM)/F-12 medium, penicillin and streptomycin were from Atlanta Biologicals (Norcross, GA, USA). Fetal bovine serum was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA).

Animals

Immature Sprague-Dawley-derived rats (25–27 days) were obtained from the Animal Center at National Yang-Ming University (Taipei, Taiwan). Rats were maintained under controlled temperature (20–23 °C) and light conditions (14 h light:10 h darkness). Food (Lab Diet from PMI Feeds, Inc., St Louis, MO, USA) and water were available *ad libitum*.

Cell culture and treatment

Isolation of ovarian granulosa cells and theca-interstitial cells from eCG-treated immature rats was performed as previously described (Too *et al.* 1984, Liu & Hsueh 1986) with slight modifications (Hwang *et al.* 1996). Ovarian cells were plated in 24-well plates (Falcon Labware, Lincoln Park, NJ, USA) at approximately 5×10^5 viable cells per well in 500 μ l DMEM/F-12 medium containing 10% fetal bovine serum, 2 μ g/ml bovine insulin, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were allowed to attach and grow to confluence for 1 to 2 days at 37 °C, 5% CO₂–95% air. Cultured cells were then washed and incubated in 500 μ l serum-free medium (DMEM/F12 containing 0.1% lactalbumin hydrolysate, 100 U/ml penicillin and 100 μ g/ml streptomycin) overnight before the beginning of treatment. This was to avoid the interference of serum proteinase and inhibitor activities during the analysis of gelatinase activity.

Ovarian cells were treated with various doses of PACAP27 and PACAP38 once alone, or in combination with porcine relaxin, in 500 μ l serum-free medium for 24 h at 37 °C, 5% CO₂–95% air. In granulosa cell cultures, relaxin (5 μ g in 10 μ l) was given three times a day at 4-h intervals as previously described (Hwang *et al.* 1996). The final concentration of relaxin was expressed as 10 μ g/ml. This treatment regimen was used based on other researchers' preliminary work (Too *et al.* 1984) and our own (Hwang *et al.* 1996), which showed that multiple relaxin treatment has a better stimulatory effect than single treatment on proteinase secretion in rat granulosa cells. In theca-interstitial cell cultures, relaxin was given only once and incubated for 24 h. Our preliminary work showed that relaxin (10 μ g/ml) given one or three times a day induced a similar magnitude of response on gelatinase secretion from theca-interstitial cells. In order to determine whether cAMP signaling pathway mediators could mimic PACAP effects on gelatinase secretion, ovarian cells were treated with cAMP signaling pathway mediators once alone, or in combination with porcine relaxin, in 500 μ l serum-free medium for 24 h at 37 °C, 5% CO₂–95% air. cAMP signaling pathway mediators include cholera toxin (an activator of stimulatory G protein), forskolin (an activator of adenylate cyclase) and 8-Br-cAMP (a cAMP analog). In every experiment, each treatment group was processed in triplicate. Cells were observed occasionally for morphological changes during

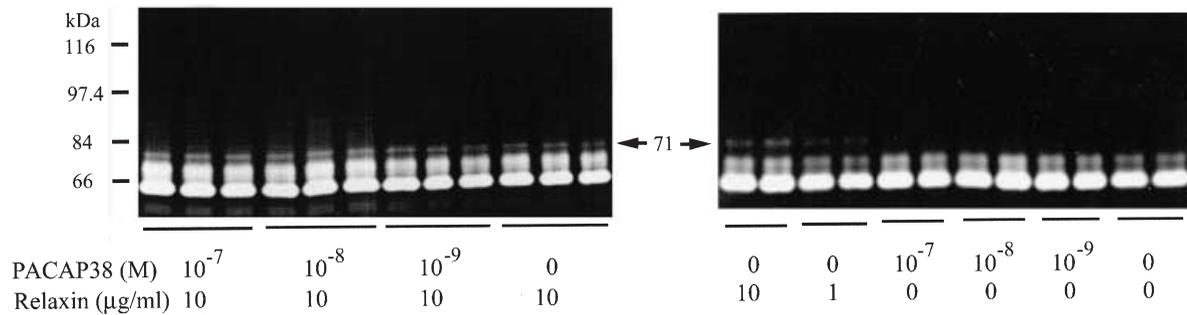


Figure 1 Effect of PACAP and relaxin on the secretion of gelatinase in rat theca-interstitial cells: representative gelatin zymograms of conditioned media from cells treated with PACAP38 and/or relaxin for 24 h of culture. Each treatment group in one experiment was carried out in triplicate.

the 24-h treatment period. At the end of incubation, medium samples were collected, cleared by centrifugation and stored at -70°C until the performance of gelatin zymography. Cells were detached with 0.25% trypsin/1 mM EDTA and counted using a hemocytometer. There was no significant difference in the cell numbers among different treatment groups.

Zymography analysis

Gelatin zymography was performed as previously described (Hwang *et al.* 1996) with slight modifications. In brief, medium samples were electrophoresed on a 7.5% or 10% SDS-polyacrylamide gel (14 cm \times 10 cm) containing 0.1% gelatin from porcine skin. The volume of each medium sample analyzed was normalized based on cell number. Electrophoresis was run in 192 mM glycine, 25 mM Tris (pH 8.0) and 0.1% SDS at 15 mA/gel during stacking and at 20 mA/gel during separation. At the end of the run, gels were washed in 2.5% Triton X-100 for about 40 min with a change of solution, and in reaction buffer (50 mM Tris-HCl (pH 8.0) containing 5 mM CaCl_2 and 0.02% NaN_3) for 15 min. Gels were incubated in reaction buffer at 37°C for 16–18 h, then stained with 0.25% Coomassie brilliant blue R-250 in 10% acetic acid–30% ethanol, and destained in the same solution without dye. Quantification of gelatinases was achieved by computerized image analysis using a two-dimensional laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Statistics

Data were analyzed using analysis of variance and Duncan's multiple range test. Student's *t*-test was used for comparison between two treatment groups.

Results

Effects of PACAP and relaxin on gelatinase secretion in rat theca-interstitial cells

The purpose of this study was to investigate the interactive effect of PACAP and relaxin on the secretion of gelatinases

in rat ovarian theca-interstitial and granulosa cells. Both natural amidated peptides, PACAP38 and PACAP27, were used to determine their relative biological potency. The gelatinase activity of conditioned media was analyzed using gelatin zymography and quantified by two-dimensional scanning densitometry. Representative gelatin zymograms on the effect of PACAP and relaxin are shown in Fig. 1. Our previous work showed that relaxin dose-dependently (1 and 10 $\mu\text{g/ml}$) induced the secretion of a major gelatinase of 71 kDa in cultured rat ovarian theca-interstitial cells (Hwang *et al.* 1996), which is also seen in this study (Figs 1 and 2). The 71 kDa gelatinase was identified as a type IV collagenase (MMP2) (Hwang *et al.* 1996). This study clearly demonstrated that PACAP27 and PACAP38 dose-dependently (10^{-9} to 10^{-7} M) enhanced relaxin-induced secretion of 71 kDa gelatinase at both doses of relaxin, 1 and 10 $\mu\text{g/ml}$ (Fig. 2), whereas both PACAPs alone had no effect (Fig. 1). The potency of PACAP27 on enhancing the effect of relaxin was similar to that of PACAP38 (Fig. 2). Also, PACAP27 and PACAP38 each achieved its maximal effect at a similar dose, around 10^{-8} M in the presence of 10 $\mu\text{g/ml}$ relaxin (Fig. 2). In addition, we observed clear cell shrinkage and a rounding phenomenon in theca-interstitial cells treated with both PACAP38 and PACAP27 at doses of 10^{-8} to 10^{-7} M. Representative photographs of control and PACAP-treated theca-interstitial cells are shown in Fig. 3A and B respectively. We also noticed that relaxin treatment alone did not induce any significant morphological change as compared with the control group; in addition, the effects of PACAP on cell shape appeared to be similar with or without the presence of relaxin (data not shown).

Effects of PACAP and relaxin on gelatinase secretion in rat granulosa cells

Differences between the types of gelatinases secreted in rat granulosa cells and theca-interstitial cells were observed (Figs 1 and 4). A representative gelatin zymogram

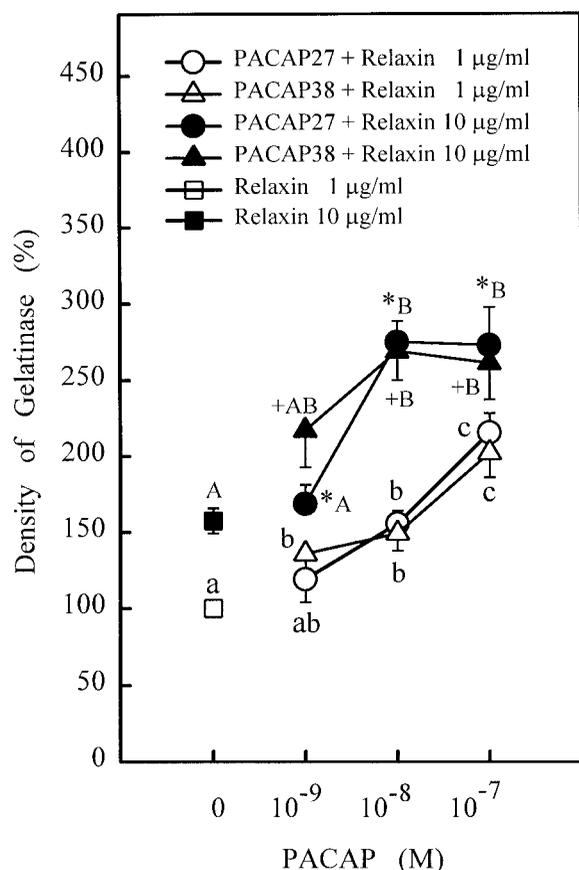


Figure 2 The modulatory effect of PACAP on relaxin-stimulated secretion of 71 kDa gelatinase in rat theca-interstitial cells. Cells were treated with PACAP27 (or PACAP38) in the presence of relaxin at 1 µg/ml or 10 µg/ml for 24 h of culture. Conditioned media were collected and analyzed for gelatinase activity using gelatin zymography and scanning densitometry. Each point represents the mean (\pm S.E.) of mean percentage density from triplicate samples of three or four separate experiments. Percentage of density was calculated using the mean density of the group treated with 1 µg/ml relaxin as 100%. Different lower-case and upper-case letters indicate significant differences among groups treated with various doses of PACAP27 (or PACAP38) in combination with 1 and 10 µg/ml relaxin respectively ($P < 0.05$); * and + indicate a significant difference between the two groups treated with 1 µg/ml and 10 µg/ml relaxin in the presence of same dose of PACAP27 and PACAP38 respectively ($P < 0.05$).

regarding the interactive effect of PACAP and relaxin on the secretion of gelatinases in rat granulosa cells is shown in Fig. 4. This study demonstrated that PACAP27 and PACAP38 alone could dose-dependently (10^{-9} to 10^{-6} M) increase the secretion of 63 kDa gelatinase in rat granulosa cells (Fig. 5A). The dose of PACAP27 and PACAP38 needed to obtain maximal effects were similar at around 10^{-7} M (Fig. 5A). The potency of PACAP27 was similar to that of PACAP38 at doses between 10^{-8} M and 10^{-6} M, while PACAP38 at 10^{-9} M exhibited

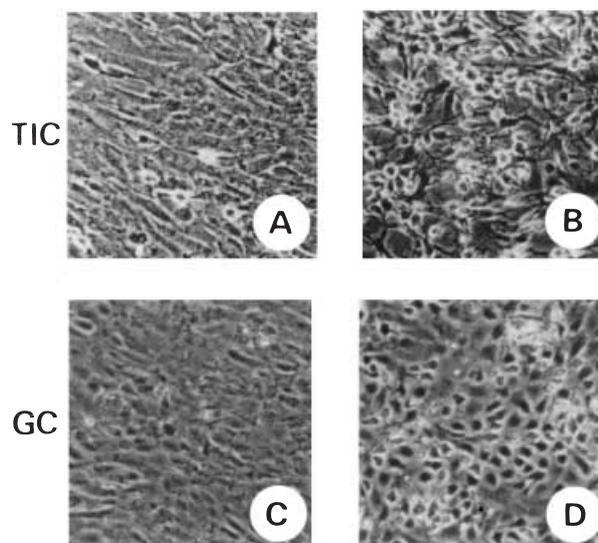


Figure 3 Effect of PACAP and relaxin on morphological change of ovarian theca-interstitial cells (TIC) and granulosa cells (GC). (A and C) show representative phase contrast images of cells of vehicle control groups. (B and D) show representative phase contrast images of cells treated with 10^{-7} M PACAP38 ($100\times$). Photographs were taken at around 3 h after treatment. Note that cells treated with 10 µg/ml relaxin alone and relaxin combined with PACAP exhibited morphology similar to that in the vehicle control group and the group treated with PACAP alone respectively (data not shown).

stronger effects than that of PACAP27 (Fig. 5A). In addition, relaxin together with either 10^{-8} M PACAP27 or PACAP38 significantly increased the secretion of a 63 kDa gelatinase as compared with 10^{-8} M PACAP or 10 µg/ml relaxin treatment alone (Fig. 5B and C). However, relaxin with 10^{-6} M of either PACAP27 or PACAP38 did not further stimulate the secretion of gelatinase as compared with 10^{-6} M PACAP treatment alone (Fig. 5B and C). PACAP also acted synergistically with relaxin in stimulating the secretion of a 92 kDa gelatinase (Fig. 4); it was not quantified, however, because of smearing of the band. PACAP (10^{-8} to 10^{-6} M) also induced morphological changes, cell shrinkage and rounding in granulosa cells, similar to those seen in theca-interstitial cells. Representative photographs of control and PACAP-treated granulosa cells are shown in Fig. 3C and D respectively. Similar to that seen in theca-interstitial cells, relaxin treatment alone did not induce any significant morphological change in granulosa cells as compared with the control group; in addition, the effects of PACAP on granulosa cell shape appear similar in the absence or presence of relaxin (data not shown).

Effects of cAMP signaling pathway mediators on gelatinase secretion in rat theca-interstitial and granulosa cells

PACAP is named for its ability to increase cAMP levels in target cells, including rat pituitary and granulosa

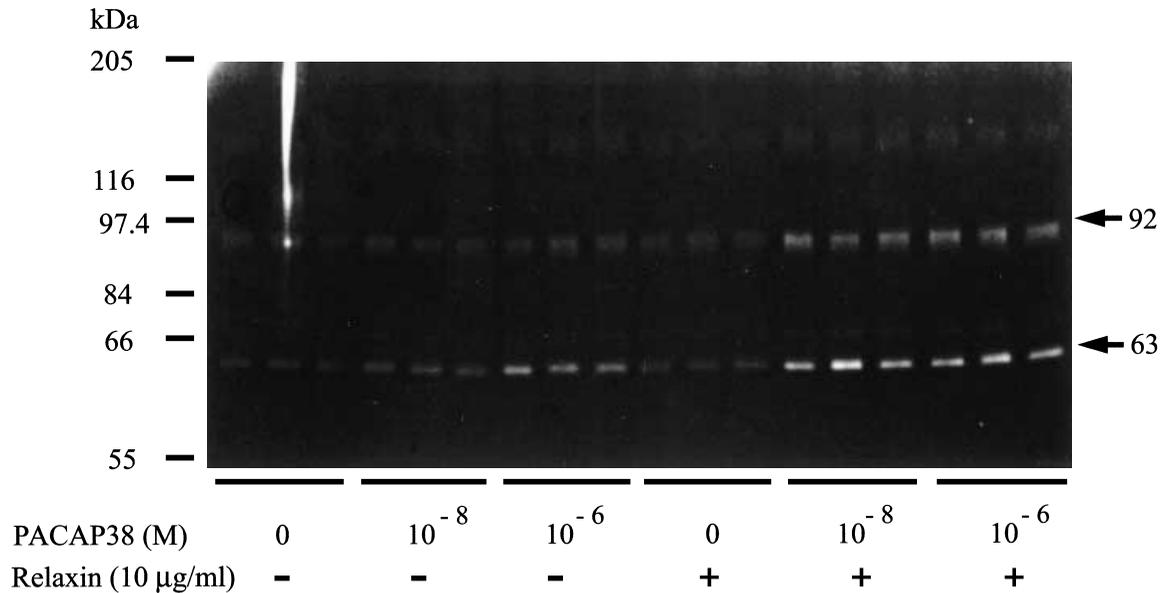


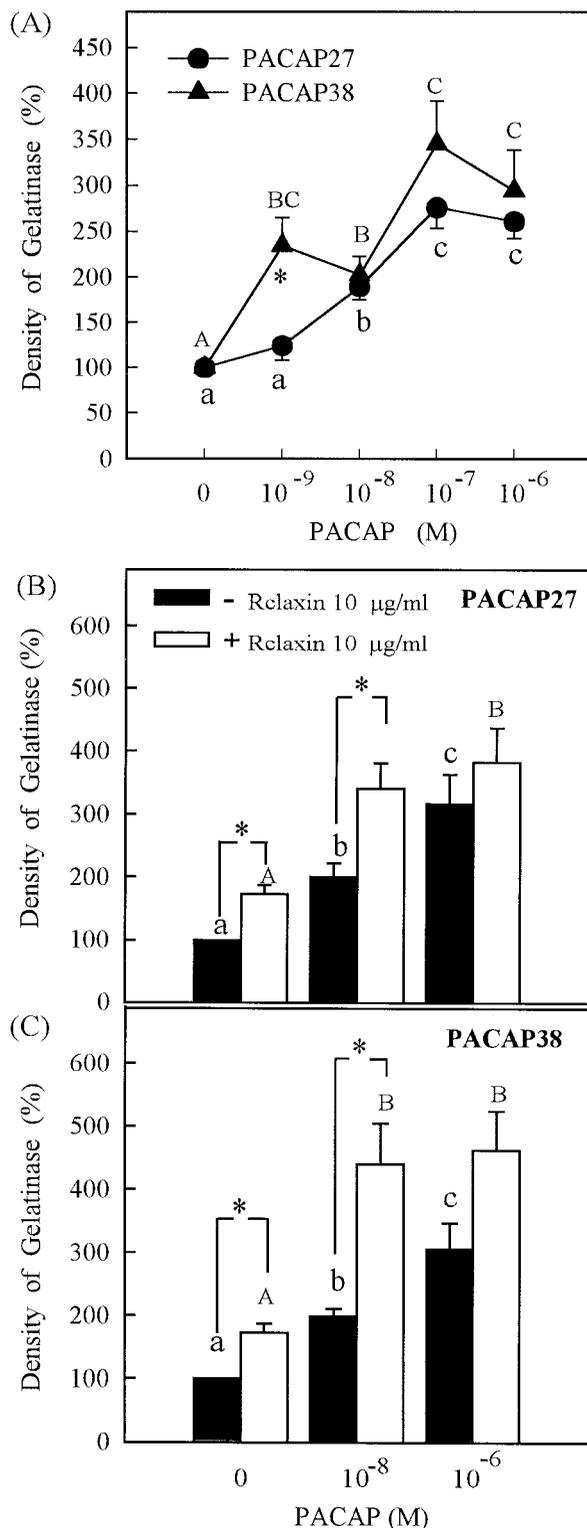
Figure 4 Effect of PACAP and relaxin on the secretion of gelatinases in rat granulosa cells: a representative gelatin zymogram of conditioned media from cells treated with PACAP38 in the absence or presence of relaxin for 24 h of culture. Each treatment group in one experiment was carried out in triplicate.

cells (Miyata 1989, 1990, Hart *et al.* 1992, Zhong & Kasson 1994, Heindel *et al.* 1996). In view of this, cAMP signaling pathway mediators (cholera toxin, activator of stimulatory G protein; forskolin, activator of adenylate cyclase; 8-Br-cAMP, cAMP analog) were used to determine whether these mediators could mimic PACAP effects on gelatinase secretion in rat ovarian cells. This study shows that cAMP signaling pathway mediators act similarly to those of PACAP on gelatinase secretion in rat ovarian cells. In cultured theca-interstitial cells, cholera toxin (10 ng/ml), forskolin (1, 10 μ M) and 8-Br-cAMP (0.2–1.0 mM) alone had no effect on the secretion of 71 kDa gelatinase, while these mediators could augment the relaxin-induced secretion of 71 kDa gelatinase (Table 1). In granulosa cell culture, cholera toxin (1, 10 ng/ml), forskolin (1, 10 μ M) and 8-Br-cAMP (0.2–1.0 mM) alone exhibited a stimulatory effect on the secretion of 63 kDa gelatinase (Fig. 6). In addition, cAMP signaling pathway mediators (cholera toxin, 10 ng/ml; forskolin, 10 μ M; 8-Br-cAMP, 0.5 and 1.0 mM), like PACAP, caused shrinkage and rounding of ovarian cells (data not shown).

Discussion

It is well known that gonadotropins trigger ovulation (Richards *et al.* 1987, LeMaire 1989, Irianni & Hodgen 1992, Epsey & Lipner 1994), yet through what mechanisms gonadotropins act in the ovary remains unclear. Our studies and other researchers' work together suggest

that both relaxin and PACAP may serve as ovarian physiological mediators of gonadotropin function in facilitating the ovulatory process. First, relaxin immunoactivity and mRNA are present in the rat ovary throughout the estrous cycle with peak levels at the periovulatory period (Sherwood & Rutherford 1981, Crish *et al.* 1986). Also, PACAP is expressed transiently during the periovulatory period in the rat ovary (Gras *et al.* 1996). Secondly, human chorionic gonadotropin stimulates ovarian expression of PACAP and PACAP receptor in rats (Lee *et al.* 1999, Park *et al.* 2000). Also, LH increases the release of relaxin in porcine preovulatory granulosa cells (Loeken *et al.* 1983). Thirdly, relaxin can induce ovulation in the rat both *in vivo* and *in vitro* (Brannstrom & MacLennan 1993, Hwang *et al.* 1996). The ovulatory process involves the remodeling of extracellular matrix which is executed by MMPs and plasminogen activators. Several studies have demonstrated that administration of inhibitors of MMPs and antibodies to plasminogen activator suppresses ovulation in rats (Reich *et al.* 1985, Brannstrom *et al.* 1988, Tsafirri *et al.* 1989, Butler *et al.* 1991). Also, the activities of plasminogen activator and MMPs, including interstitial collagenase, type IV collagenase, gelatinase and proteoglycanase, increases during the periovulatory period (Palotie *et al.* 1987, Curry *et al.* 1992). Our previous work and that of others show that relaxin increases the secreted activity of plasminogen activator and MMPs, such as proteoglycanase, interstitial collagenase (MMP1) and gelatinases, in rat ovarian granulosa cells (Too *et al.* 1982, 1984, Hwang *et al.* 1996). In addition, relaxin increases the secretion of



71 kDa gelatinase, identified as a type IV collagenase (MMP2) in rat ovarian theca-interstitial cells (Hwang *et al.* 1996). The present study has further shown that PACAP, like relaxin, can stimulate the secretion of 63 kDa gelatinase in rat granulosa cells. We have previously identified this 63 kDa gelatinase as a metalloproteinase (Hwang *et al.* 1996). However, we have not been able to identify this gelatinase as an MMP1 or an active MMP2 using immunoblotting technique (authors' unpublished data); thus its specific identity awaits further characterization. Furthermore, the present study demonstrates that relaxin acts synergistically with PACAP in stimulating the secretion of 63 kDa gelatinase and 71 kDa gelatinase (MMP2) in rat granulosa cells and theca-interstitial cells respectively. Type IV collagenases, including MMP2, can degrade type IV collagen in the basement membrane underlying granulosa cell layers and germinal epithelium in the ovary, while interstitial collagenase and proteoglycanase can cause the restructure of stromal tissue enriched with types I and III collagens and proteoglycans. These present studies therefore suggest that relaxin and PACAP may play an important physiological role in facilitating ovulation through the modulation of extracellular matrix remodeling in the ovary.

Relaxin receptor has not been purified or cloned although its ligand relaxin has been studied for several decades. On the other hand, PACAP has been purified quite recently (Miyata *et al.* 1989, 1990), and PACAP receptors have already been cloned. PACAP receptors are classified into two major types with different ligand preferences: type I (PACAP >> VIP) and type II (PACAP \cong VIP) (Gottschall *et al.* 1990, Rawlings 1994, Rawlings & Hezareh 1996). PACAP type I receptors are further classified into type IA (PACAP38 \cong PACAP27) and type IB (PACAP38 > PACAP27) (Arimura & Shioda 1995, Rawlings & Hezareh 1996). The present study has demonstrated that PACAP27 and PACAP38, with similar potency, enhance the relaxin-induced secretion of 71 kDa gelatinase in rat ovarian theca-interstitial cells. Also, earlier findings indicate the presence of PACAP type I and type II receptors in the rat ovary (Gottschall *et al.* 1990, Scalfaferrri *et al.* 1996). Our study therefore suggests that

Figure 5 The interactive effect of PACAP and relaxin on the secretion of 63 kDa gelatinase in rat granulosa cells. Cells were treated with (A) various doses of PACAP27 or PACAP38 alone, or (B and C) with relaxin at 10 µg/ml in combination with PACAP27 or PACAP38 for 24 h of culture. Conditioned media were collected and analyzed for gelatinase activity using gelatin zymography and scanning densitometry. Each point represents the mean (\pm S.E.) of mean percentage density from triplicate samples of four or five separate experiments. Percentage of density was calculated using the mean density of the vehicle control as 100%. Different lower-case and upper-case letters indicate significant difference among groups with the same symbol (or bar type) ($P < 0.05$). * $P < 0.05$ significant difference between two groups treated with the same dose of PACAP.

Table 1 The interactive effect of cAMP signaling pathway mediators and relaxin on the secretion of 71 kDa gelatinase in rat theca-interstitial cells. Data represent the mean (\pm S.E.) percentage of density from triplicate samples of two to four separate experiments

Treatment	Relative density (%)			
	Vehicle control	Cholera toxin (10 ng/ml)	Forskolin (1 μ M)	8-Br-cAMP (0.2 mM)
- Relaxin	ND	ND	ND	ND
+Relaxin	100 \pm 3.4	238 \pm 16.9*	208 \pm 22.2*	223 \pm 6.8*

Cells were treated with cAMP signaling pathway mediators in the absence or presence of relaxin 10 μ g/ml for 24 h of culture. Conditioned media were collected and analyzed for gelatinase activity using gelatin zymography and scanning densitometry. ND indicates that density was too weak to be accurately determined.

* $P < 0.01$ compared with the relaxin-treated control group.

rat ovarian theca-interstitial cells may contain PACAP type IA and/or type II receptors. It also demonstrates that both PACAP27 and PACAP38 can increase the secretion of 63 kDa gelatinase in rat granulosa cells. In addition, the effect of PACAP38 was significantly stronger than PACAP27 at lower dose of 10^{-9} M, while at the higher doses of 10^{-8} to 10^{-6} M both PACAPs exhibited similar potency. At the moment, we do not have a clear explanation for this; however, it might be possible that granulosa cells contain different types of PACAP receptors with varying PACAP ligand affinity. A recent study has shown the presence of PACAP type IA receptor in rat granulosa cells (Kotani *et al.* 1998). In addition, the possible presence of PACAP type IB receptor in rat granulosa cells is supported by the work of Heindel *et al.* (1996) who showed that PACAP38 was more effective

than PACAP27 in stimulating steroidogenesis and cAMP accumulation in rat granulosa cells. The present study also shows that PACAPs at a sub-maximal dose of 10^{-8} M stimulate, synergistically with relaxin, the secretion of 63 kDa gelatinase in rat granulosa cells, while PACAPs at a maximal dose of 10^{-6} M alone or in combination with relaxin produced similar effects. These results suggest at least two possibilities. One possibility is that PACAP and relaxin may somehow act through a similar signaling mediator, such as cAMP, in stimulating gelatinase secretion in rat granulosa cells. Alternatively, PACAP and relaxin may act through a different signaling mediator in stimulating gelatinase secretion, and that PACAP at 10^{-6} M alone already stimulates the maximal response of granulosa cells. The latter may be the more likely possibility if the signaling mechanisms of PACAP and relaxin on the secretion of gelatinases in ovarian granulosa cells are consistent with those in theca-interstitial cells.

The nature of the relaxin receptor is currently unknown, and therefore the cellular signaling mechanism of relaxin remains unclear. Limited evidence suggests that relaxin may not act through the cAMP signaling pathway to exert certain biological functions in rat ovarian cells. We have previously shown that relaxin stimulates the secretion of 71 kDa gelatinase (MMP2) in theca-interstitial cells (Hwang *et al.* 1996). The present study further demonstrates that PACAP, known to increase cAMP in target cells (Miyata *et al.* 1989, 1990, Zhong & Kasson 1994, Heindel *et al.* 1996), and cAMP signaling pathway mediators (8-Br-cAMP, forskolin and cholera toxin) alone did not stimulate the secretion of 71 kDa gelatinase in theca-interstitial cells, while PACAP and cAMP mediators augment this effect of relaxin. In addition, PACAP, follicle-stimulating hormone (FSH) or cAMP analog can stimulate steroidogenesis in granulosa cells (Knecht *et al.* 1981, Richards *et al.* 1987, Richards 1994, Zhong & Kasson 1994, Heindel *et al.* 1996), whereas relaxin did not (Too *et al.* 1982). Moreover, the present study demonstrates that cell-rounding phenomena occurred both in

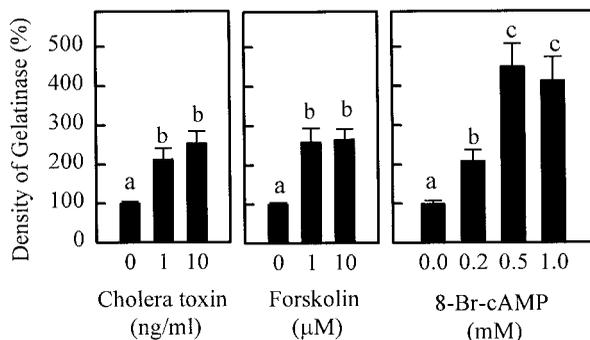


Figure 6 Effect of cAMP signaling pathway mediators on the secretion of 63 kDa gelatinase in rat granulosa cells. Cells were treated with various doses of cholera toxin, forskolin or 8-Br-cAMP for 24 h of culture. Conditioned media were collected and analyzed for gelatinase activity using gelatin zymography and scanning densitometry. Each point represents the mean (\pm S.E.) of mean percentage density from triplicate samples of two to four separate experiments. Percentage of density was calculated using the mean density of the vehicle control as 100%. Different lower-case letters indicate significant difference among the same mediator treatment groups ($P < 0.05$).

theca-interstitial cells and granulosa cells treated with PACAP or cAMP signaling pathway mediators, but not with relaxin. Earlier studies also showed that granulosa cells became rounded-up following treatment with FSH or cAMP analog (Lawrence *et al.* 1979, Amsterdam *et al.* 1981, Knecht *et al.* 1981). The present study did not observe any obvious correlation between the degree of increased secretion of 71 kDa gelatinase or 63 kDa gelatinase and the change of cell shape.

In conclusion, the present study provides original findings that PACAP acts synergistically with relaxin in stimulating the secretion of gelatinases in rat ovarian theca-interstitial cells and granulosa cells. This supports the idea that relaxin and PACAP may serve as ovarian physiological mediators of gonadotropin function in facilitating the ovulatory process. In addition, the signaling mechanism of relaxin receptor appears to be different from that of PACAP receptors (i.e. cAMP) in rat ovarian cells.

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