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Bradykinin is not involved in angiotensin converting enzyme modulation of ovarian steroidogenesis and prostaglandin production in frog *Rana esculenta*

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ABSTRACT

Angiotensin converting enzyme (ACE) was demonstrated to modulate the production of 17β-estradiol, progesterone and prostaglandin E_2 (PGE_2) in frog ovary of *Rana esculenta*. However, the activity was not mediated by angiotensin II (Ang II). In an attempt to identify the peptide involved in the pathway modulated by ACE, bradykinin, another physiological substrate of ACE, was chosen and incubated in the presence of the membrane suspension purified from the frog ovary homogenate. The hydrolytic products were analysed by reverse-phase high-pressure liquid chromatography (HPLC) analysis and the results showed that bradykinin was metabolized by membrane suspension. The presence of the protease inhibitors in the incubation mixture indicated ACE and neutral endopeptidase as being responsible for the bradykinin hydrolysis. Frog ovary was incubated *in vitro* in the presence of bradykinin (10 μM), bradykinin receptor antagonist NPC 567 (1 mg mL^{-1}), bradykinin fragment (1–7) (10 μM), ACE (2.5 mU mL^{-1}), captopril (0.1 mM) and lisinopril (0.1 mM). The results showed no modulating activity by bradykinin on ovarian 17β-estradiol and PGE_2 production, thus demonstrating that it was not involved in the ACE-modulated pathway.

Keywords 17β-estradiol, neutral endopeptidase 24.11, prostaglandin E_2.

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Angiotensin converting enzyme (ACE, peptidyl-dipeptidase A, EC 3.4.15.1) is a glycosylated integral membrane protein located on the luminal surface of the cell membrane. Its predominant physiological function is in cardiovascular homeostasis through cleavage of the C-terminal dipeptide from angiotensin I (Ang I) to produce the potent vasoconstrictor, angiotensin II (Ang II). Angiotensin converting enzyme also inactivates the vasodilator, bradykinin, by sequential cleavage of two C-terminal dipeptides (Yang *et al.* 1970), and it can also hydrolyse a wide range of other endogenous bioactive peptides (Rieger *et al.* 1993, Hooper & Turner 1987). However, its role in mammalian ovary is controversial. Although, Ang II has been demonstrated to be an important mediator in the mechanism of ovulation in rats (Pellicer *et al.* 1988) and in production of estradiol and prostaglandins in perfused rabbit ovaries (Yoshimura *et al.* 1993), ACE inhibitors have no effect on ovulation and ovarian steroidogenesis in perfused rat ovary (Peterson *et al.* 1993).

We used an amphibian model to study the influence of ACE and Ang II on ovarian steroidogenesis and prostaglandin production. In the water frog *Rana esculenta*, 17β-estradiol, progesterone, and prostaglandin E_2 (PGE_2) production was modulated by ovary ACE; on the other hand, Ang II modulated the production of progesterone and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), whereas androgen production was not influenced (Bramucci *et al.* 1997). In the frog testis, ACE caused a decrease of 17β-estradiol production and an increase of androgen production, while Ang II showed an opposite effect, decreasing androgen production and increasing 17β-estradiol. The determination of aromatase activity showed a positive regulation by Ang II and a negative one by ACE. Angiotensin II also modulated the production of $PGF_{2\alpha}$ (Miano *et al.* 1999). The data suggest the existence of two pathways independently regulated

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by ACE and Ang II modulating ovarian and testicular steroidogenesis and prostaglandin production.

Bradykinin in mammals is reported to be involved in the ovulatory process. It induced ovulation in the absence of gonadotropin in a dose related fashion, but did not induce maturation of follicular oocytes in perfused rabbit ovary. Furthermore bradykinin stimulated PGE_2 and $PGF_{2\alpha}$ production (Yoshimura *et al.* 1988). In perfused rat ovary, it partially stimulated oocyte maturation in the pre-ovulatory follicles, potentiated the ovulation rate and increased the tissue levels of PGE_2 and prostacyclin (Hellberg *et al.* 1991). In non-mammalian vertebrates, bradykinin has received less attention. The skin of amphibians has proved to be a remarkably rich store-house of regulatory peptides, including bradykinin. Bradykinin-related peptides have been purified and sequenced from skin extracts of a wide range of frogs (Anastasi *et al.* 1965, Conlon & Aronsson 1997) and with an amino acid sequence identical to that of the mammalian peptide (Simmarco *et al.* 1990). The physiological role played by bioactive peptides in amphibian skin is not entirely clear but there is strong evidence that they are an important component of the organism's survival strategy, protecting it against ingestion by predators. The existence of a bradykinin generating system in the amphibian blood has yet to be established. Recently, a peptide has been isolated from trypsin-treated amphiuma plasma that was identified as [Phe^1, Ile^2, Leu^5]-bradykinin (Li *et al.* 1998). The biological properties of this peptide have not yet been determined and it is unclear whether it is derived from a kininogen precursor protein. However, activity of mammalian bradykinin has been reported to inhibit the vasopressor-induced increase in transepithelial osmotic water flux in the isolated urinary bladder of the toad *Bufo marinus paracnemis* (Furlando 1972). In literature, no data are available about the influence of bradykinin on amphibian reproduction.

In an attempt to identify the peptide involved in the pathway modulated by ACE, we studied the role of bradykinin on 17β-estradiol and PGE_2 production by the ovary of water frog *R. esculenta* in the pre-reproductive period. The results showed an involvement of ACE and neutral endopeptidase of the ovary in the hydrolysis of bradykinin, but no modulating activity of bradykinin on 17β-estradiol and PGE_2 production.

MATERIALS AND METHODS

Chemicals

Bradykinin (Arg–Pro–Pro–Gly–Phe–Ser–Pro–Phe–Arg), bradykinin fragment 1–7, angiotensin converting enzyme (rabbit lung), captopril, lisinopril, *N*-(α-ramnopyranosyloxy-hydroxyphosphinyl)-Leu–Trp (Phosphoramidon), Dulbecco's modified Eagle medium (DME), penicillin G, streptomycin, 17β-estradiol, and PGE_2 were purchased from Sigma (St Louis, MO, USA). D-Arg[Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin (NPC 567) was from Calbiochem–Novabiochem (Läufelfinger, Switzerland). Acetonitrile was from J.T. Baker (Deventer, the Netherlands), trifluoroacetic acid (TFA) was from Fluka (Buchs, Switzerland). The high-pressure liquid chromatography (HPLC) column was Supelcosil LC-318 from Supelco (Bellefonte, PA, USA). Multiwell tissue culture plates were from Becton Dickinson and Co. (Lincoln Park, NJ, USA). 17β-Estradiol and PGE_2 antisera were purchased from Sigma. [2,4,6,7–^{3}H]–17β Estradiol, and [5,6,8,11, 12,14, 15(*n*)-^{3}H]-prostaglandin E_2 were purchased from Amersham Int. (Buckinghamshire, UK).

Preparation of tissue membranes

Adult female frogs, *R. esculenta*, were collected in Colfiorito pond (Umbria, Italy; 870 m above sea level) in April (pre-reproductive period). All procedures described below were carried out at 4 °C. Three adult female frogs were killed by decapitation. Ovaries were removed and pooled, and tissues were weighed, finely minced with scissors, and homogenized in 10 volumes of ice-cold phosphate buffer (50 mM, pH 8.3) with a Braun homogenizer set at the highest speed for 5 min. The homogenate was filtered through cotton gauze and then centrifuged at 1000 × *g* for 20 min. Tissue membranes were isolated by ultra-centrifugation of supernatant at 100 000 × *g* at 4 °C for 60 min. The resulting pellet was resuspended in 0.5 or 1 mL of phosphate buffer and used in the incubation mixture. Protein content was evaluated by the method of Bradford (1976) with bovine serum albumin as a standard.

Experimental protocol

To study the ovarian steroidogenesis and prostaglandin production, pre-reproductive female frogs were captured and killed in the field by decapitation. The ovaries were removed, placed in cold DME containing 10 mM Hepes, 0.1 mg mL^{-1} penicillin G, and 0.1 mg mL^{-1} streptomycin, and then transferred to the laboratory, where they were divided into equal-sized fragments, pooled and equally distributed over incubation wells (about 1 g $well^{-1}$) each containing 2 mL of incubation medium (Gobbetti & Zerani 1995). Each set of incubation wells was divided into six experimental groups (each consisting of four wells): (1) medium alone; (2) medium plus 2.5 mU mL^{-1} rabbit lung ACE; (3) medium plus 0.1 mM captopril; (4) medium plus 0.1 mM lisinopril; (5) medium plus 0.1 mM captopril plus 2.5 mU mL^{-1} rabbit lung ACE; (6) medium plus 0.1 mM lisinopril plus 2.5 mU mL^{-1} rabbit lung ACE. Bradykinin (10 μM, final concentration),

bradykinin antagonist NPC 567 (1 μg mL^{-1} final concentration), bradykinin plus NPC 567 (10 μM and 1 μg mL^{-1}, final concentration), and bradykinin fragment (1–7) (10 μM, final concentration), were added to a second, third, fourth, and fifth incubation set, respectively. Culture plates were wrapped in aluminium foil and incubated at room temperature. Incubation medium was removed after 6 h and stored at –20 °C until hormone assays. Ovarian tissues were homogenized in amphibian saline, and protein contents were determined using the method of Bradford (1976). The control experiment was repeated with incubation media without ovarian tissue.

Hydrolysis of bradykinin by homogenate and membrane suspension of ovary

Homogenate (32 μg) or membrane suspension (3.6 μg) of ovary was added to 100 μM bradykinin solution in 50 mM Tris–HCl buffer, pH 7.4, containing 150 mM NaCl in a total volume of 20 μL. The solution was incubated at 37 °C at different times. The enzymatic reaction was stopped by adding 2 μL of 5% TFA and centrifuged. Metabolite separations were performed on a Beckman HPLC System Gold Nouveau using a 5-μm Supelcosil LC-318 (25 cm × 4.6 mm i.d) column, protected with a 5-μm Supelcosil LC-318 guard column (2 cm × 4.6 mm i.d). The elution was performed with a linear gradient from 0 to 30% of 0.1% TFA in water and 0.1% TFA in acetonitrile at a flow rate of 1 mL min^{-1}. Eluate absorbance was monitored by UV absorbance at 214 nm. Fractions, corresponding to peaks present in the elution profile, were collected and subjected to amino acid analysis. Metabolite peaks were hydrolysed for 24 h at 110 °C in 6 N HCl. The hydrolysates were dried in a vacuum desiccator and then derivatized following the procedure of Stocchi (Stocchi *et al.* 1989). The enzymes involved in the bradykinin hydrolysis were identified by incubating the mixture in the presence of 10 μM captopril, and 10 μM phosphoramidon.

Determination of 17β-estradiol and PGE$_2$

Concentrations of 17β-estradiol and PGE$_2$ were measured in incubation media by radioimmunoassay as described previously (D'Istria *et al.* 1974, Gobbetti & Zerani 1995). Intra- and interassay coefficients of variation and minimum detectable doses were: 17β-estradiol, 5%, 7%, 8 pg; PGE$_2$, 8%, 12%, 12 pg.

Statistical analysis

An analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan 1955, Sokal & Rohlf 1981) was used to analyse the data.

RESULTS

To prove that frog ACE is responsible for the bradykinin hydrolysing activity, a partial purification of the enzyme was carried out from frog ovary tissue using the procedure of Smiley & Doig (1994). The method consists in the ultracentrifugation of frog ovary homogenate and recovery of the particulate fraction, where ACE is bound to the membranes. Aliquots of the different purification steps were analysed for the presence of ACE activity following the hydrolysis of a synthetic ACE substrate *N*-[3-(2-furyl) acryloyl] L-phenylalanyl glycyl glycine (FAPGG), as reported by Miano *et al.* (1997). The recovery of ACE activity in the pellet was 31% of the total homogenate activity. The purified membrane suspension was used to determine bradykinin hydrolysis incubating it at 37 °C at different times. The hydrolytic products were analysed by reverse-phase HPLC following the procedure described in Materials and Methods. Figure 1 shows the chromatographic elution profile of bradykinin at 0 min (Fig. 1a) and 10 min (Fig. 1b) of incubation in the presence of purified ovary membranes. Four metabolites, corresponding to the peaks labelled I–IV in Figure 1b, appeared and eluted with retention times of 5.04, 11.72, 20.45, and 21.10 min, respectively. Bradykinin eluted with a retention time of 25.84 min. The amino acid composition of metabolites was: peak I corresponding to the dipeptide Ser–Pro, peak II to the dipeptide Phe–Arg, peak III to the bradykinin fragment (1–5), Arg–Pro–Pro–Gly–Phe, and peak IV to the bradykinin fragment (1–7), Arg–Pro–Pro–Gly–Phe–Ser–Pro. Figure 2 shows the effects of the proteinase inhibitors on bradykinin hydrolysis by ovary membrane suspension, which was incubated with 2 nmol of

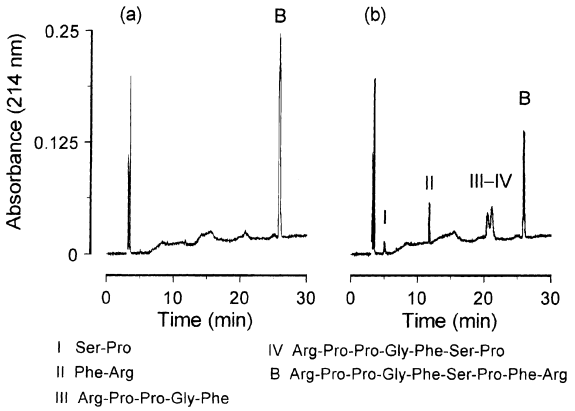

Figure 1 Elution profile of bradykinin incubated with frog ovary membrane suspension. Two nanomoles of bradykinin were incubated at 37 °C for 0 min (a) and 10 min (b) with 3.6 μg of ovary membrane suspension in a total volume of 20 μL in the condition described in Materials and Methods. B indicates the peak corresponding to bradykinin.

bradykinin for 10 min in the presence of a specific ACE inhibitor, 10 μM captopril (Fig. 2b). Comparison with the elution profile obtained without ACE inhibitor shows the disappearance of peaks I and III, metabolites derived from hydrolysis of the bound Phe–Ser of bradykinin fragment (1–7), while the peak II and peak IV areas were decreased by 64%. These results suggest that ACE is responsible for bradykinin hydrolysis, but another proteinase was present on the frog ovary membrane involved in bradykinin digestion. In literature, it has been reported that another dipeptidase, neutral endopeptidase 24.11 (NEP; EC 3.4.24.11), can inactivate bradykinin by removing the COOH terminal Phe–Arg dipeptide (Gafford *et al.* 1983). The incubation of the ovary membrane suspension with a specific inhibitor of NEP, 10 μM phosphoramidon, gave the same elution profile as digested bradykinin with a 34% decrease of hydrolysis activity (Fig. 2c). When captopril and phosphoramidon were incubated at the same time with ovary membrane suspension (Fig. 2d), bradykinin hydrolysis was reduced by 4.9%, whereas at 60 min the reduction was 27.3%. Incubation of bradykinin with 32 μg of ovary tissue homogenate showed 74.9% hydrolysis after 20 min, while the presence of captopril and phosphoramidon decreased the hydrolysis by 34.3%, proving that ACE and NEP were the main proteolytic enzymes involved in bradykinin hydrolysis *in vitro*. These results prove the capacity of frog ovary ACE to hydrolyse bradykinin *in vitro*.

To study the role of bradykinin in 17β-estradiol and PGE_2 production, frog ovary tissue in pre-reproductive period was incubated *in vitro* in the presence of rabbit lung ACE, captopril, lisinopril, bradykinin, bradykinin receptor antagonist NPC 567, and bradykinin fragment (1–7). The production of 17β-estradiol and PGE_2 was determined. Figure 3a shows 17β-estradiol production, expressed as picograms per milligram of protein, by frog ovary incubated *in vitro*. A 17β-estradiol basal value of 166.5 ± 11 pg mg^{-1} was inhibited by adding rabbit lung ACE at a final concentration of 2.5 mU mL^{-1}. Treatment with specific ACE inhibitors, 0.1 mM captopril and 0.1 mM lisinopril, increased the production of 17β-estradiol by 137 and 79%, respectively, compared with the basal value. The data confirmed the results obtained in a previous report (Bramucci *et al.* 1997). 17β-Estradiol was not affected by the addition of 10 μM bradykinin (Fig. 3b) or 1 μg mL^{-1} NPC 567 (Fig. 3c), or 10 μM bradykinin fragment (1–7) (Fig. 3e) to the incubation system with or without the presence of rabbit lung ACE and/or ACE inhibitors. Figure 4 shows PGE2 production, expressed as picograms per milligram of protein, by frog ovary incubated *in vitro*. The data show the same pattern as that seen in 17β-estradiol production, confirming the influence of ACE activity on PGE_2 production and no activity by bradykinin (Fig. 4b), NPC 567 (Fig. 4c) and bradykinin fragment (1–7) (Fig. 4e).

DISCUSSION

The present study was undertaken to determine which peptide is involved in the modulatory activity of ACE

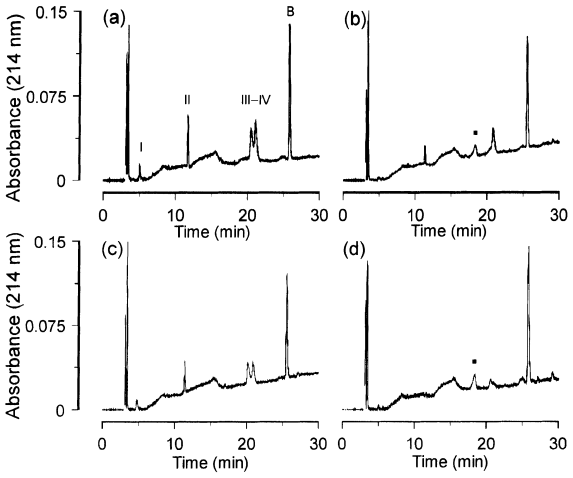

Figure 2 Elution profile of bradykinin incubated with frog ovary membrane suspension and proteinase inhibitors. (a) The same as in Figure 1; (b) incubation mixture in presence of 10 μM captopril; (c) incubation mixture in presence of 10 μM phosphoramidon; (d) incubation mixture in presence of 10 μM captopril and 10 μM phosphoramidon. The point and B indicate peaks corresponding to captopril and bradykinin, respectively.

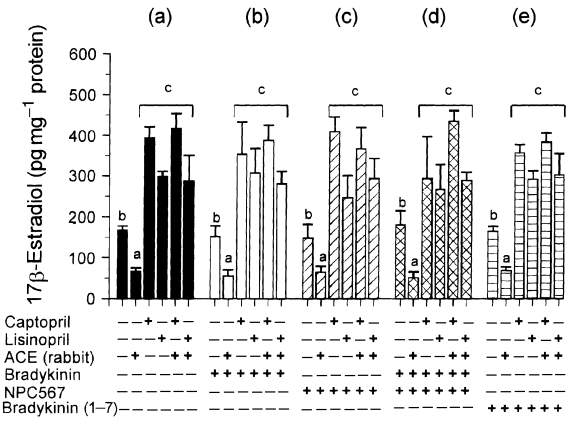

Figure 3 17β-Estradiol production by *Rana esculenta* ovary, in pre-reproductive period, incubated *in vitro* with following substances: rabbit lung angiotensin converting enzyme (ACE), 2.5 mU mL^{-1}; captopril, 0.1 mM; lisinopril, 0.1 mM; bradykinin, 10 μM; bradykinin antagonist NPC 567, 1 μg mL^{-1}; and bradykinin fragment 1–7, 10 μM. Each mean refers to 4 determinations ± SD. Groups with different letters are significantly different ($P < 0.01$, Duncan's multiple range test).

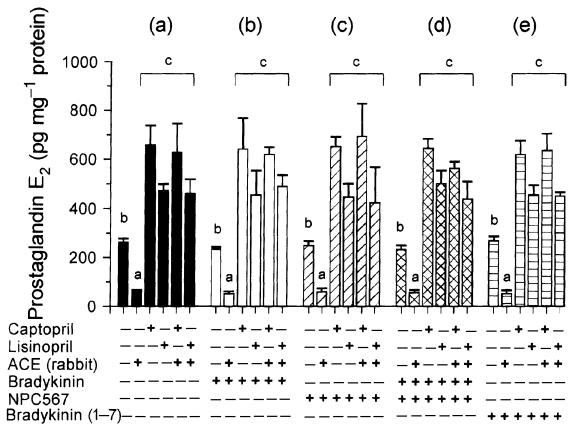

Figure 4 PGE_2 production by pre-reproductive *Rana esculenta* ovary incubated *in vitro* with following substances: rabbit lung angiotensin converting enzyme (ACE), 2.5 mU mL^{-1}; captopril, 0.1 mM; lisinopril, 0.1 mM; bradykinin, 10 μM; bradykinin antagonist NPC 567, 1 μg mL^{-1}; and bradykinin fragment 1–7, 10 μM. Each mean refers to 4 determinations ± SD. Groups with different letters are significantly different ($P < 0.01$, Duncan's multiple range test).

on 17β-estradiol and PGE_2 production in frog ovary, because in a previous report (Bramucci *et al.* 1997) we observed that steroidogenesis and prostaglandin production in frog ovary was modulated independently by ACE and Ang II. Between the peptides that present intraovarian regulatory activity, bradykinin was chosen, because it is a physiological substrate of ACE, it is inactivated by ACE and is involved in the ovulatory processes. The synthetic bradykinin was incubated *in vitro* in the presence of frog ovary membrane suspension to determine the capacity of the ovary tissue to metabolize the peptide. Two proteinases seem responsible for bradykinin hydrolysis, ACE and NEP. These data are confirmed also in the homogenate of ovary tissues where the presence of the inhibitors of ACE and NEP activity, captopril and phosphoramidon, protects the bradykinin from hydrolysis.

Recently, the NEP has been localized in the granulosa cells of follicles of rabbit ovaries (Zappulla & DesGroseillers 2001), at all stages of maturation, with the exception of atretic follicles. The same distribution was reported for ACE in rat ovaries with the difference that ACE is also present in atretic follicles, and the level of ACE is reduced in pre-ovulatory ones (Daud *et al.* 1990). No data are available about localization of ACE and NEP in frog ovary tissue. The colocalization of ACE and NEP suggests an important role for these proteins in processes such as follicle maturation, ovulation, and/or regulation of ovary blood flow, by modulating the physiological function of biological peptides.

The influence of bradykinin on 17β-estradiol and PGE_2 production proved to be negative in our experimental model. Incubation of frog ovary with bradykinin in the presence of ACE inhibitors also showed no effect on the modulation of 17β-estradiol and PGE_2, excluding the involvement of the peptide in modulating activity of ACE on steroidogenesis and prostaglandin production in frog ovary. The data were confirmed by incubation with NPC 567, a bradykinin receptor antagonist. Some suppositions can be advanced to explain the lack of effects of bradykinin on ovarian steroidogenesis. First, the synthetic bradykinin used in the experiment does not have the identical amino acid sequence of the frog bradykinin, but in literature the isolation of a bradykinin from *R. esculenta* has been reported with the same sequence as that used in out experiment (Simmarco *et al.* 1990). Furthermore, synthetic bradykinin has a physiological effect on the frog capillaries (Williams & Huxley 1993). A second hypothesis regards the phase of the reproductive period. In mammals, bradykinin has a stimulatory effect on the ovulatory process inducing a simi-inflammatory reaction that culminates with oocyte release. Probably the pre-ovulatory period of the *R. esculenta* breeding cycle is not in conformity with the mammalian pre-ovulatory follicles phase.

From the data obtained, it appears that bradykinin was not involved in the ACE-modulated pathway. It is still opened the question regarding the mechanism of action of ACE. Intraovarian regulatory peptides such as substance P and gonadotropin-releasing hormone have been demonstrated to play important roles in the regulation of ovarian function, also in the frog ovary of *R. esculenta* (Gobbetti & Zerani 1995). Met- and Leu-enkephalin-like peptides have been localized in the ovary of frog *R. esculenta* (Pestarino *et al.* 1992). It has been suggested that ACE activity inactivates these peptide hormones (Ehlers & Riordan 1989), which in turn may have a modulating action on the estradiol and prostaglandin production. Probably, the mechanism of action of ACE has to be looked for in these peptides involved in the ovary process.

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