Role of calcium/calmodulin-dependent protein kinase II in gonadotrophin-induced ovulation in *in vitro* perfused rabbit ovaries

K. Kugu, A. M. Dharmarajan, S. Preutthipan and E. E. Wallach*

Department of Gynecology and Obstetrics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA

The objectives of these experiments were to determine (i) the role of calcium/calmodulin-dependent protein kinase II-mediated signal transduction in hCG-induced ovulation and (ii) whether there is an association between arachidonic acid metabolites, nitric oxide and calcium/calmodulin-dependent protein kinase II in the overall scheme of ovulation induction. Ovarian arteries were cannulated *in situ*, and the ovaries were excised and perfused *in vitro*. Ovulatory efficiency ([number of ovulated follicles/number of mature follicles > 1.5 mm] × 100) was calculated for each experiment. Calcium/calmodulin-dependent protein kinase II substrate induced ovulation in the absence of gonadotrophin (calcium/calmodulin-dependent protein kinase II substrate: 66.3%; control: 0%). In the next experiment, perfusion medium of the experimental ovary was supplemented with KN 62, a potent inhibitor of calcium/calmodulin-dependent protein kinase II, while the contralateral ovary served as control. Ovulations were induced in both ovaries with hCG (50 iu (150 ml)⁻¹) and perfusion was continued for 8 h. In the third experiment, ovaries were perfused with prostaglandin F_{2α} (PGF_{2α}) with and without KN 62, while the contralateral ovary was perfused with medium alone. KN 62 reduced the ovulatory efficiency of hCG-treated ovaries *in vitro* during perfusion (hCG + 10⁻⁷ mol KN 62 l⁻¹: 32.9%; hCG: 80.9%). Furthermore, it significantly reduced the ovulatory efficiency of PGF_{2α}-treated ovaries (PGF_{2α} + KN 62 = 21.5%; PGF_{2α} = 59.9%). In the final experiment, *N*-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase, reduced ovulation calcium/calmodulin-dependent protein kinase II substrate, indicating an interaction between ovarian nitric oxide synthesis and calcium/calmodulin-dependent protein kinase II. These findings suggest that the calcium/calmodulin-dependent protein kinase II signal transduction system plays a significant role in hCG-induced ovulation. Furthermore, the data demonstrate an interaction between the arachidonic acid metabolites, nitric oxide and calcium/calmodulin-dependent protein kinase II pathway.

Introduction

Many tissues, including ovarian tissue, possess at least two major classes of secondary messengers: the cAMP-dependent protein kinase system and the protein kinase C system. The cAMP-dependent protein kinase pathway is widely believed to be the regulator of steroidogenesis in follicular cells (LeMaire and Marsh, 1975; Davis *et al.*, 1986; Adashi *et al.*, 1990) and cAMP has also been implicated in oocyte maturation (Strickland and Beers, 1976; Holmes *et al.*, 1986; Brännström *et al.*, 1987; Hosoi *et al.*, 1989). Workers in our laboratory have found that high concentrations of cAMP inhibited hCG-induced ovulation in a dose-dependent fashion, possibly by decreasing prostaglandin (PG) production (Yoshimura *et al.*, 1994). Less is known about the role of protein kinase C-mediated signal transduction in the activation of tissue

plasminogen activator and follicle rupture during ovulation. The protein kinase C pathway may be important during the periovulatory interval, both alone and in combination with the cAMP pathway (Kawai and Clark, 1985; Leung and Wang, 1989). It is known that phorbol-12,13-dibutyrate, a phorbol ester known to stimulate protein kinase C, leads to ovulation in a dose-dependent manner in the *in vitro* perfused rabbit ovary in the absence of gonadotrophins and that the calcium-dependent protein kinase C pathway is involved in gonadotrophin-dependent protein kinase II-mediated follicular rupture (Kaufman *et al.*, 1992).

Protein kinase C-mediated signal transduction requires the coupling of cytoplasmic membrane receptors to G proteins that activate phospholipase C and, in turn, stimulate the hydrolysis of phosphatidylinositol-4,5-bisphosphate into 1,2-diacylglycerol and inositol-1,4,5-trisphosphate. Calcium and 1,2-diacylglycerol in combination activate protein kinase C. Inositol-trisphosphate can also produce an increase in intracellular calcium by

*Correspondence and reprint requests.

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liberating stores from endoplasmic reticulum. The increase in intracellular calcium and its subsequent binding to calmodulin is a major mechanism of signal transduction in response to hormonal stimulation or membrane depolarization (Rasmussen and Rasmussen, 1990). Calcium/calmodulin stimulates a wide variety of enzymes, including protein kinases such as the multifunctional calcium/calmodulin-dependent protein kinase II (Colbran and Soderling, 1990; Hanson and Schulman, 1992).

Using an *in vitro* perfusion model, we investigated specific substances that affect ovulation including eicosanoids (Wallach and Dharmarajan, 1992), proteolytic enzymes and inhibitors (Yoshimura *et al.*, 1987), bradykinin (Yoshimura *et al.*, 1988), oxygen free radical scavengers (Miyazaki *et al.*, 1991a), nitric oxide inhibitors (Hesla *et al.*, 1993), angiotensin II (Kuo *et al.*, 1991), epidermal growth factor (Endo *et al.*, 1992), and interleukin-1 β (Takehara *et al.*, 1994). The above agents may also increase intracellular calcium which, in turn, stimulates calcium/calmodulin-dependent protein kinase II and nitric oxide synthase. These observations suggest a role for calcium/calmodulin-dependent protein kinase II signal transduction in mediating gonadotrophin-induced follicular rupture.

The objectives of the study were to investigate: (i) the effect of calcium/calmodulin-dependent protein kinase II substrate, a stimulator of calcium/calmodulin-dependent protein kinase II, on ovulation and progesterone and prostaglandin production in the absence of gonadotrophins; (ii) the effect of specific calcium/calmodulin-dependent protein kinase II inhibitors on gonadotrophin-induced ovulation and progesterone and prostaglandin production; (iii) the effect of calcium/calmodulin-dependent protein kinase II inhibitor on PGF_{2 α} -induced ovulation; and (iv) the effect of *N*-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase which, in the presence of calcium/calmodulin-dependent protein kinase II, converts L-arginine to citrulline, a reaction of nitric oxide synthesis, on ovulation. The *in vitro* perfused rabbit ovary model was therefore used to determine the effects of these agents on ovarian function in a carefully controlled and monitored environment.

Materials and Methods

Animals

New Zealand White mature female rabbits, 3.0–4.5 kg, were used. All rabbits were caged individually for at least 3 weeks before use. Animals were given water and an unrestricted diet of rabbit chow. Rabbits were anaesthetized i.v. with 32 mg sodium pentobarbital kg⁻¹ body mass (Anpro Pharmaceutical, Arcadia, CA), treated with 120 iu heparin sulfate kg⁻¹ body mass (Invenex Laboratories, Chagrin Falls, OH) for anticoagulation, and then subjected to laparotomy. Ovaries were excluded from the study if they were immature or if two or more of the follicles were haemorrhagic at the time of laparotomy. Experimental ovaries were also excluded if the paired contralateral control unstimulated ovary ovulated, presumably as a result of an endogenous LH surge before ovariectomy. All studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In vitro perfusion

The cannulation procedure and perfusion apparatus were described by Lambertsen, Jr *et al.* (1976), Kobayashi *et al.* (1981), and Dharmarajan *et al.* (1988). Each ovarian artery was isolated and cannulated *in situ* after ligation of the major anastomotic connections. The ovary with its cannulated vascular pedicle and supportive connective tissue was removed. The number of mature follicles (> 1.5 mm in diameter) observed on the surface of each ovary was recorded, and the ovary was immediately placed in the perfusion chamber. The perfusion system consists of a chamber containing the ovary, an oxygenator, a reservoir, and a pulsatile roller pump that maintains perfusate flow at 1.5 ml min⁻¹, the approximate blood flow to the rabbit ovary (Ahrén *et al.*, 1971). The oxygenator was gassed with 95% O₂:5% CO₂. Ovaries were perfused for 10.5 h at 37°C in 150 ml medium 199 (Gibco, Grand Island, NY) supplemented with 200 iu heparin sulfate l⁻¹, 20 iu insulin l⁻¹ (Novo Nordisk Pharmaceuticals Inc., Princeton, NJ), 50 mg streptomycin sulfate l⁻¹ (Sigma Chemical Co., St Louis, MO) and 75 mg penicillin G l⁻¹ (Sigma Chemical Co.). Perfusate samples were collected from the arterial cannula at time zero and at 1, 2, 4, 6, and 8 h after the administration of either hCG (Organon Inc., West Orange, NJ) or calcium/calmodulin-dependent protein kinase II substrate (Research Biochemicals International, Natick, MA) and replaced by an equal volume of fresh medium. Samples were stored at -20°C for later determination of progesterone and prostaglandin concentrations. The ovary was monitored for fresh ovulation points every 15 min. A follicle was considered to be ruptured when the cumulus containing an ovum was observed protruding from the ovarian surface. Ovulatory efficiency was calculated for each ovary using the following formula: [number of ovulated follicles/number of mature follicles] × 100 (Lambertsen, Jr *et al.*, 1976; Kobayashi *et al.*, 1981).

Experimental design

Effect of calcium/calmodulin-dependent protein kinase II substrate on ovulation and progesterone and PGF_{2 α} production and influence of calcium/calmodulin-dependent protein kinase II inhibitor on calcium/calmodulin-dependent protein kinase II substrate-induced ovulation. These experiments were designed to investigate the effect of calcium/calmodulin-dependent protein kinase II stimulation on ovulation and progesterone and PGF_{2 α} production, in the absence of hCG. Ten rabbits were used for these experiments. One milligram of calcium/calmodulin-dependent protein kinase II substrate was dissolved in 1.5 ml distilled water as the stock solution, yielding a concentration of 4 × 10⁻⁴ mol l⁻¹. Ovaries were placed in the perfusion chambers at -0.5 h, and a bolus injection of 0.125 ml of 4 × 10⁻⁴ mol calcium/calmodulin-dependent protein kinase II substrate l⁻¹ or vehicle alone was administered via an arterial cannula at 0 h. Six ovaries were treated with calcium/calmodulin-dependent protein kinase II substrate and observations were compared with those of contralateral control ovaries. Another four rabbits were used for experiments investigating the influence of the calcium/calmodulin-dependent protein kinase II inhibitor, KN 62 (Calbiochem, La Jolla, CA). One milligram of KN 62 was

dissolved in 27.6 ml of 10% (v/v) dimethyl sulfoxide as the stock solution. Either KN 62 or vehicle alone was dissolved in 150 ml of perfusion medium at a concentration of 10^{-6} mol KN 62 l⁻¹. Ovaries were placed in the perfusion chambers at -0.5 h, and bolus injections of 0.125 ml of 4×10^{-4} mol calcium/calmodulin-dependent protein kinase II substrate l⁻¹ were administered via arterial cannula at 0 h. Four ovaries were perfused with the medium containing KN 62 and observations were compared with those of contralateral ovaries. Samples were collected for determination of progesterone and PGF_{2α} concentrations, and ovulatory efficiencies were calculated as described above.

Effects of calcium/calmodulin-dependent protein kinase II inhibitors on hCG-induced ovulation and progesterone and PGF_{2α} production. These experiments were designed to investigate the effect of calcium/calmodulin-dependent protein kinase II inhibition on hCG-induced ovulation and progesterone and PGF_{2α} production. Sixteen rabbits were used for these experiments. The KN 62 was prepared as described above. One milligram of calcium/calmodulin-dependent protein kinase II inhibitor 281-302 (Research Biochemicals International) was dissolved in 1.5 ml of distilled water as the stock solution. Inhibitor or vehicle alone was dissolved in 150 ml of perfusion medium at a concentration of 10^{-9} , 10^{-8} , 10^{-7} or 10^{-6} mol KN 62 l⁻¹, or 10^{-6} mol inhibitor 281-302 l⁻¹ before the onset of the experiment. Ovaries were placed in the perfusion chambers at -0.5 h, and 50 iu hCG were administered via the arterial cannula at 0 h. Samples were collected for determination of progesterone and PGF_{2α} concentrations. Ovulatory efficiencies were calculated as described above.

Effect of calcium/calmodulin-dependent protein kinase II inhibitor (KN 62) on PGF_{2α}-induced ovulation. This experiment was designed to investigate the effect of calcium/calmodulin-dependent protein kinase II inhibition on PGF_{2α}-induced ovulation. Six rabbits were used. KN 62 was prepared as described above. Either KN 62 or vehicle alone was dissolved in 150 ml of perfusion medium supplemented with 100 ng PGF_{2α} ml⁻¹ (Sigma Chemical Co.) to yield a concentration of 10^{-6} mol KN 62 l⁻¹ before the onset of the experiment. Ovulatory efficiencies were calculated as described above.

Influence of nitric oxide synthase inhibitor on ovulation induced by either calcium/calmodulin-dependent protein kinase II substrate or hCG. This experiment was designed to investigate the interactions between synthesis of nitric oxide and ovulation induced by calcium/calmodulin-dependent protein kinase II substrate and hCG. N-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical Co.) was used as a nitric oxide synthase inhibitor. Ten rabbits were used for this experiment. L-NAME was dissolved into medium directly to yield a concentration of 10^{-5} mol l⁻¹ before the onset of the experiment. Ovaries were placed in the perfusion chambers with medium containing L-NAME and contralateral ovaries were placed in the perfusion chambers with medium alone at -0.5 h. Bolus injections of 0.125 ml 4×10^{-4} mol calcium/calmodulin-dependent protein kinase II substrate l⁻¹ were administered via arterial cannula at 0 h. In the second series of experiments, medium of one ovary

contained L-NAME while the contralateral ovary served as control. Both ovaries were stimulated at 0 h with 50 iu of hCG to induce ovulation. Ovulatory efficiencies were calculated as described above.

Progesterone and PGF_{2α} radioimmunoassays

Progesterone concentrations in perfusate samples were measured using a solid phase kit (Diagnostic Products Corp., Los Angeles, CA). Progesterone antibody is bound covalently to the inner surface of polypropylene assay tubes. All samples and standards were assayed in duplicate. The intra- and interassay coefficients of variation were 7.5% and 6.6%, respectively. Perfusate concentrations of PGF_{2α} were measured using a [³H]PGF_{2α} radioimmunoassay kit (INCSTAR Corp., Stillwater, MN). PGF_{2α} antibody crossreacts 1.2% with PGF_{2α} metabolite and less than 0.2% with other prostaglandin families. All samples and standards were assayed in duplicate.

Statistical analyses

Data are expressed as means ± SEM. Data for PGF_{2α} and progesterone concentrations were evaluated by analysis of variance, and $P < 0.05$ was considered significant. Comparison of ovulatory efficiencies was performed using chi-square analysis and $P < 0.05$ was considered significant. Dose-response studies were designed using a balanced block method; the order of the ovaries for each experiment was determined by a random number table.

Results

Effect of calcium/calmodulin-dependent protein kinase II substrate on ovulation and concentrations of progesterone and PGF_{2α} in the absence of hCG

Calcium/calmodulin-dependent protein kinase II substrate induced ovulation in the *in vitro* perfused rabbit ovary in the absence of gonadotrophin. Ovulatory efficiency varied significantly from $0.0 \pm 0.0\%$ for controls to $66.3 \pm 9.9\%$ for ovaries perfused with calcium/calmodulin-dependent protein kinase II substrate ($P < 0.01$). When KN 62 was added together with calcium/calmodulin-dependent protein kinase II substrate, it significantly reduced ovulatory efficiency to $6.3 \pm 6.3\%$ ($P < 0.01$). However, progesterone and PGF_{2α} concentrations in perfusates from ovaries treated with either calcium/calmodulin-dependent protein kinase II substrate alone or calcium/calmodulin-dependent protein kinase II substrate together with KN 62 displayed no significant change from control ovaries.

Effects of calcium/calmodulin-dependent protein kinase II inhibitors on hCG-induced ovulation and concentrations of progesterone and PGF_{2α}

Ovulatory efficiency was significantly reduced in ovaries treated with hCG and KN 62 at doses of 10^{-8} , 10^{-7} and 10^{-6} mol l⁻¹ (Table 1). Another calcium/calmodulin-dependent protein kinase II inhibitor, 281-302, also significantly inhibited hCG-induced ovulation from $79.6 \pm 8.3\%$

Table 1. Effect of KN 62, an inhibitor of calcium/calmodulin-dependent protein kinase II, on hCG-induced ovulation in the *in vitro* perfused rabbit ovary

Parameter	KN 62 (mol l ⁻¹)				
	0	10 ⁻⁹	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶
Number of perfused ovaries	8	6	4	4	6
Number of ovaries ovulated	8	6	4	4	4
Number of mature follicles	51	38	22	29	43
Number of ovulations	41	21	9	9	17
Ovulatory efficiency (%) ^a	80.9 ± 5.5	60.3 ± 16.3	40.0 ± 7.1*	32.9 ± 8.7**	37.8 ± 14.9*

^aOvulatory efficiency is expressed as mean ± SEM representing 4–8 ovaries per group.

*P < 0.05, **P < 0.01 compared with control (0 mol l⁻¹).

in ovaries perfused with hCG alone to 31.3 ± 11.9% for hCG plus this inhibitor (10⁻⁶ mol l⁻¹; P < 0.01). Although hCG significantly increased progesterone concentration, neither KN 62 nor calcium/calmodulin-dependent protein kinase II inhibitor 281-302 affected hCG-induced progesterone production (control: 1.3 ± 0.4 ng ml⁻¹; hCG: 41.2 ± 8.2 ng ml⁻¹; hCG + KN 62 [10⁻⁷ mol l⁻¹]: 32.8 ± 14.4 ng ml⁻¹; hCG + inhibitor 281-302: 32.7 ± 13.2 ng ml⁻¹). No significant change was observed in PGF_{2α} concentrations in the perfusate of any treated group when compared with the control group.

Effect of calcium/calmodulin-dependent protein kinase II inhibitor (KN 62) on PGF_{2α}-induced ovulation

PGF_{2α}-induced ovulation was inhibited by KN 62 at a dose of 10⁻⁶ mol l⁻¹. Ovulatory efficiency was significantly reduced in these experiments from 59.9 ± 12.6% for ovaries perfused with PGF_{2α} alone to 21.5 ± 8.9% for PGF_{2α} plus KN 62 (P < 0.05).

Effect of L-NAME on ovulation induced by calcium/calmodulin-dependent protein kinase II substrate and hCG

Both calcium/calmodulin-dependent protein kinase II substrate-induced ovulation and hCG-induced ovulation were significantly inhibited by 10⁻⁵ mol L-NAME l⁻¹ (Table 2).

Discussion

In the study reported here, a rabbit ovarian *in vitro* perfusion model was used to investigate the role of the calcium/calmodulin-dependent protein kinase II pathway in the process of follicular rupture. A specific substrate for calcium/calmodulin-dependent protein kinase II activates calcium/calmodulin signal transduction by increasing calcium, which, in turn, results in binding of calcium to intracellular acceptors; calmodulin is a major acceptor (Colbran and Soderling, 1990; Hanson and Schulman, 1992). Calcium-bound calmodulin activates various enzymes including calcium/calmodulin-dependent protein kinase II (Colbran and Soderling, 1990; Hanson and Schulman, 1992). In the study reported here, calcium/calmodulin-dependent protein kinase II substrate induced

Table 2. Effect of N-nitro-L-arginine methyl ester (L-NAME) on the ovulatory efficiency of calcium/calmodulin-dependent protein kinase II substrate-induced ovulation and hCG-induced ovulation in the *in vitro* perfused rabbit ovary

	L-NAME (10 ⁻⁵ mol l ⁻¹)	
	–	+
CaMK-II substrate-induced ovulation		
Ovulatory efficiency (%)	72.0 ± 8.5	32.0 ± 13.2*
hCG-induced ovulation		
Ovulatory efficiency (%)	79.8 ± 10.4	17.2 ± 10.6**

Values are expressed as means ± SEM.

*P < 0.05, **P < 0.01 compared with control (L-NAME (–)).

ovulation in the extracorporeal perfused rabbit ovary in the absence of hCG. The mechanism(s) by which calcium/calmodulin-dependent protein kinase II substrate brings about follicular rupture is not clear. It is possible that calcium/calmodulin-dependent protein kinase II substrate, which is a peptide, acts on the outer surface of the cell membrane. If this substrate does affect calcium/calmodulin-dependent protein kinase II, the catalytic domain of the enzyme must be directed towards the outside of the cell or be present in the circulation. In this study, exogenous substrate does not appear to compete with the endogenous substrate(s) and inhibit steps in the calcium/calmodulin-dependent protein kinase II signal transduction pathway. It is feasible that ovulation may be mediated through plasma membrane receptors for calcium/calmodulin-dependent protein kinase II substrate.

Perfusate concentrations of PGF_{2α} and progesterone did not change following exposure to calcium/calmodulin-dependent protein kinase II substrate, suggesting that calcium/calmodulin-dependent protein kinase II substrate-induced ovulation occurs independently of prostaglandin production, although it is possible that only threshold concentrations of prostaglandins are required for ovulation to occur. The mechanism(s) by which prostaglandins leads to follicular rupture requires further investigation.

Arachidonic acid metabolites, in particular PGF_{2α} and prostacyclin (PGI₂), are considered as intermediaries, acting

between preovulatory gonadotrophin secretion and ovulation in rabbits as well as in other species (Wallach *et al.*, 1992). Clearly, many factors are involved in the process of ovulation. Some of these factors may express themselves through an influence on ovarian prostaglandins. Indomethacin, a potent inhibitor of prostaglandin synthesis, inhibits ovulation in response to either gonadotrophin or PGF_{2α} and PGI₂, but does not inhibit ovulation induced by the vasoactive agents, histamine and bradykinin, in the *in vitro* perfused rabbit ovary (Kitai *et al.*, 1985; Yoshimura *et al.*, 1988). The results of the experiments reported here do not refute the concept that prostaglandins mediate follicle wall disruption, but suggest that there are other mechanism(s) that are independent of, or act in conjunction with, prostaglandins.

Although prostaglandins may be involved in the release or activation of plasminogen activator within the follicle, blockade of prostaglandin synthesis does not inhibit tissue plasminogen activator secretion (Espey *et al.*, 1985; Reich *et al.*, 1985). The precise relationship between ovulation, tissue plasminogen activator and prostaglandins remains to be clarified. The results of the experiments reported here suggest that, although prostaglandins may be intermediaries in the process of ovulation, they need not be essential for calcium/calmodulin-dependent protein kinase II substrate-induced follicle rupture.

The specific calcium/calmodulin-dependent protein kinase II inhibitor, KN 62, was used in the perfusion model to study the role of the calcium/calmodulin-dependent protein kinase II signal pathway in gonadotrophin-induced ovulation. KN 62, which is specific for calcium/calmodulin-dependent protein kinase II and crossreacts with neither cAMP-dependent protein kinase nor protein kinase C pathways, binds to the calmodulin site of this enzyme (Tokumitsu *et al.*, 1990). In this study, the inhibitors blocked hCG-induced ovulation. A larger dose of KN 62 was required to achieve inhibition of calcium/calmodulin-dependent protein kinase II substrate-induced ovulation than to inhibit hCG-induced ovulation. This supports the concept that calcium/calmodulin-dependent protein kinase II may be involved in gonadotrophin-induced follicular rupture in rabbits. Therefore, it can be postulated that LH uses at least three signal transduction pathways to control separate cellular functions (i.e. cAMP-dependent protein kinase, the protein kinase C, and the calcium/calmodulin-dependent protein kinase II pathways). Little information is available on the effect of LH on calcium/calmodulin-dependent protein kinase II metabolism in rabbits. Other purported intermediaries of ovulation, including PGF_{2α}, have been shown to activate the calcium/calmodulin-dependent protein kinase II pathway in several cell populations (Wegner *et al.*, 1991). Consequently, calcium/calmodulin-dependent protein kinase II substrate-mediated follicular rupture may occur directly via hCG activation, or may result from the role of calcium/calmodulin-dependent protein kinase II as a second messenger for other intermediaries the release of which may be triggered by gonadotrophins.

In the present experiments, KN 62 blocked PGF_{2α}-induced ovulation, implying that PGF_{2α} has a direct effect on activation of the calcium/calmodulin-dependent protein kinase II signal transduction. Prostaglandin F_{2α} has been shown to act on the

proteolytic enzyme cascade, and this action may be either direct or indirect through calcium/calmodulin-dependent protein kinase II (Miyazaki *et al.*, 1991b).

Inhibition of nitric oxide synthesis by L-NAME, an analogue of L-arginine, significantly reduces hCG-induced follicular rupture in rabbits (Hesla *et al.*, 1993). This effect is demonstrated both *in vivo* and *in vitro*, when the ovary was isolated from systemic influences and directly exposed to the compound. Interleukin-1β induces nitric oxide synthase activity in the rat ovary (Ben-Shlomo *et al.*, 1994) and induces ovulation in the rabbit ovary in the absence of gonadotrophin (Takehara *et al.*, 1994). These findings suggest that nitric oxide production by the ovary is a component of the physiological processes that occur during the periovulatory period. There are two isoforms of nitric oxide synthase. The constitutive type (neural and endothelial nitric oxide synthase) is a calcium/calmodulin-dependent enzyme that releases nitric oxide for short periods in response to receptor stimulation (Moncada *et al.*, 1991). The calcium/calmodulin dependence of neural nitric oxide synthase allows for its stimulation through first messengers or agents that increase intracellular calcium concentrations (Moncada *et al.*, 1991). The resultant increase in calcium can then stimulate nitric oxide synthase as well as other calcium-dependent enzymes. The second form of nitric oxide synthase is inducible nitric oxide synthase. This form is calcium-independent and expressed after activation of cytokines (Hibbs *et al.*, 1988). In this study, L-NAME significantly reduced calcium/calmodulin-dependent protein kinase II substrate-induced ovulation, suggesting that calcium/calmodulin-dependent protein kinase II brings about follicle rupture at least in part through stimulating nitric oxide synthesis.

In conclusion, this study demonstrates that the calcium/calmodulin-dependent protein kinase II pathway has a role in gonadotrophin-induced follicular rupture in rabbits. Inhibition of PGF_{2α}-induced ovulation by calcium/calmodulin-dependent protein kinase II inhibitor indicates that PGF_{2α}-mediated follicular rupture may be achieved partially through activation of calcium/calmodulin signal transduction. Furthermore, the data supplement previous observations that suggest a role for nitric oxide in the overall process of follicular rupture.

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