

Atrial natriuretic peptide inhibits the actions of FSH and forskolin in meiotic maturation of pig oocytes via different signalling pathways

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Abstract

Atrial natriuretic peptide (ANP) as well as its receptors is found in mammalian ovary and follicular cells and its function in oocyte meiotic maturation has also been reported in *Xenopus*, hamster and rat. But the results are controversial and the physiological mechanism of ANP on oocyte maturation is not clear, especially the relationship between gonadotrophin and ANP as well as the signal transduction, and these need further study. The present study conducted experiments to examine these questions by using drug treatment and Western blot analysis and focused on pig oocyte meiotic maturation and cumulus expansion *in vitro*. The results revealed that ANP could inhibited FSH-induced pig oocyte maturation and cumulus expansion and prevent the full phosphorylation of mitogen-activated protein kinase in both oocytes and cumulus cells, and that these inhibitory effects could be mimicked by 8-Br-cyclic guanosine 5'-monophosphate (8-Br-cGMP), but blocked by a protein kinase G (PKG) inhibitor KT5823. Zaprinast, a cGMP-specific phosphodiesterase inhibitor, could enhance the inhibitory effect of ANP on oocyte maturation. A specific analogue of ANP, C-ANP-(4–23), which binds to the natriuretic peptide receptor-C (NPRC), had no effect in either FSH-induced or spontaneous oocyte maturation. Treatment with forskolin, a stimulator of adenylate cyclase, had a biphasic effect; 44 h treatment induced cumulus expansion but inhibited oocyte maturation while 2 h treatment induced maturation of cumulus-enclosed oocytes (CEOs). Both ANP and C-ANP-(4–23) could inhibit the effect of forskolin on CEO maturation, and these inhibitory effects of ANP/C-ANP-(4–23) could be blocked by preincubation with pertussis toxin (PT), consistent with mediation by a Gi protein(s) in the cumulus cells. All these results suggest that ANP is a multifunctional regulator of FSH and forskolin on pig CEO maturation by two signalling mechanisms: one is via a cGMP/PKG pathway, the other is via NPRC receptors in cumulus cells and the activation of the PT-sensitive Gi protein(s).

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Introduction

Atrial natriuretic peptide (ANP) or atrial natriuretic factor (ANF) is one of a family of hormones all sharing a common 17-amino acid ring closed by a disulphide bound between two cysteine residues and varying only in the length of their N- and C-terminal extensions (Flynn *et al.* 1983). ANP is involved in the regulation of blood pressure, salt and water excretion, cell proliferation, body fluid homeostasis (for review see De Bold *et al.* 1991) and the release of aldosterone, vasopressin, androgen, progesterone and growth hormone (for review see Rosenzweig & Seidman 1991). The diverse physiological responses of ANP are manifested by binding to its specific cell-surface receptors. There are four types of natriuretic peptide receptors that have been identified by molecular cloning techniques: natriuretic peptide receptor-A (NPRA), natriuretic peptide receptor-B (NPRB), natriuretic

peptide receptor-C (NPRC) and natriuretic peptide receptor-D (for review see Takei 2001). NPRA receptors exhibit high affinity for ANP and brain natriuretic peptide, and NPRB is specific to C-type natriuretic peptide (for review see Takei 2000). Both NPRA and NPRB receptors can activate particulate guanylate cyclase and stimulate cyclic guanosine 5'-monophosphate (cGMP) to produce physiological responses in many tissues and cells (Pandey *et al.* 2000). ANP also binds with high affinity to NPRC, which has been suggested to clear ANP from the circulation (for review see Maack 1992) and to play a mediatory role in the ANP-dependent inhibitory action on adenylyl cyclase, but has no intrinsic ability to generate cGMP (Drewett *et al.* 1992). The 5-amino acid, ring-deleted ANP analogue, (des(Gln¹⁸Ser¹⁹Gly²⁰Leu²¹Gly²²)rANF-(4–23)-NH₂), which is designated as C-ANP-(4–23), has been reported to be specific for the NPRC receptor (Maack *et al.* 1987, Bovy *et al.* 1989), even though it

interacts with the NPRA and NPRB receptors at high (>1 mM) concentrations (Konrad *et al.* 1991).

It is found that ANP localizes in mammalian oviduct (Russinova *et al.* 2001), ovary (Steegers *et al.* 1990), follicular fluid (Anderson *et al.* 1994), granulosa cells (Ivanova *et al.* 2003), oocytes (Kim *et al.* 1993) and the corpus luteum (Vollmar *et al.* 1988). Because of a local production, ANP may also act as an autocrine and/or paracrine hormone that influences the function of many tissues including ovarian growth or steroidogenesis (Gutkowska *et al.* 1993), regulation of follicular fluid dynamics (Kim *et al.* 1992, Gutkowska *et al.* 1999), oviductal motility (Kim *et al.* 1997), and communication within the female reproductive tract (Usuki *et al.* 1993, Gerbes *et al.* 1994). NPRA receptor has been found in human and bovine ovary (Kim *et al.* 1987, Saheki *et al.* 1989) and the receptor numbers increase with follicular cell growth (Kim *et al.* 1987). This finding, together with the findings that the ovary appears to be a site of ANP synthesis (Vollmar *et al.* 1988, Johnson *et al.* 1994), suggests that in the ovaries the locally synthesized ANP may act as a functional natriuretic peptide to produce physiological responses.

The actions of gonadotrophins on ovarian functions are believed to be mediated in large part through increased production of the second messenger adenosine 3',5'-cyclic monophosphate (cAMP), and subsequent activation of specific signalling pathways involving phosphorylation of mitogen-activated protein kinase (MAPK) (Eppig 1993, Fan *et al.* 2003). Recent studies indicate that the second messenger cGMP also mediates a wide range of influences on the function of the ovary (for review see La Polt *et al.* 2002). ANP can affect oocyte maturation by cGMP, i.e. ANP participates in ovum development by stimulation of cGMP accumulation and activation of cAMP-phosphodiesterase, and thereby promotes *Xenopus* ovum maturation (Sandberg *et al.* 1993) and resumption of meiosis in hamster oocytes (Hubbard & Price 1988). On the other hand, ANP dose-dependently inhibits spontaneous maturation of rat oocytes via cGMP accumulation (Törnell *et al.* 1990). We have also shown that ANP inhibits follicle-stimulating hormone (FSH)-induced pig cumulus-enclosed oocyte (CEO) maturation in a dose-dependent manner (M Zhang, Y Tao, G Xia, H Xie, H Hong, F Wang & L Lei, unpublished observations). These varied results suggest that there may be different signalling pathways participating in the ANP-mediated action on mammalian oocyte meiotic maturation.

Forskolin, a stimulator of adenylate cyclase (Homa 1988, Sirard 1990) which increases cAMP levels in both CEOs and denuded oocytes (DOs) (Olsiewski & Beer 1983, Dekel *et al.* 1984), has been used in several studies to investigate the role of cAMP in the regulation of meiotic maturation in mammalian oocytes (Racowsky 1985, Xia *et al.* 1994, 2000). Forskolin mimics the effects

of FSH by the stimulation of cAMP synthesis and cAMP-dependent protein kinase A (PKA) activation (Cameron *et al.* 1996), induces cumulus mass expansion (Racowsky 1985), and inhibits or promotes oocyte maturation after treatment for different times (Xia *et al.* 2000, Lu *et al.* 2001). It has been reported that ANP significantly reduces forskolin-stimulated cAMP accumulation via NPRC receptors and reduction of ligand-stimulated adenylyl cyclase activity in rat alveolar epithelial type 2 cells (Panchenko *et al.* 1998) and in guinea pig airway smooth muscle (Devlillier *et al.* 2001). All these reported mechanisms of ANP participation in other tissues or cells are not clarified in oocyte development and maturation.

The aim of this study was to examine whether ANP could influence the actions of FSH and forskolin on meiotic maturation of pig oocytes *in vitro* and, in that case, by which mechanism, since ANP has been found in granulosa cells of the pig ovarian follicle (Kim *et al.* 1992, Ivanova *et al.* 2003).

Materials and methods

Chemicals

The synthetic human ANP-(1–28) and des(Gln¹⁸Ser¹⁹Gly²⁰Leu²¹Gly²²)rANF-(4–23)-NH₂ (C-ANP-(4–23)) were prepared as 0.1 mM stock solutions. 8-Br-cGMP was prepared as a 0.1 M stock solution. 2-(2-propyloxyphenyl)-8-azapurin-6-one (zaprinast) and forskolin were prepared as 20 mM stock solutions in dimethylsulphoxide and KT5823 as a 1 mM stock solution in ethyl acetate. All chemicals were purchased from Sigma unless otherwise indicated. Human ANP was used in our study, since the whole hormone sequences of ANP are conserved within mammals (Takei 2001).

Collection of pig CEOs and DOs

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory within 3 h. CEOs were aspirated from follicles of 3–6 mm in diameter in Tyrode's lactate-Hepes medium with polyvinyl alcohol (Tatemoto *et al.* 2001). Pig follicular fluid was harvested at the same time, and was centrifuged at 1000 *g* at 4 °C for 15 min. The supernatant fluid was collected, supplemented with 100 IU/ml penicillin G potassium (Sigma) and 25 µg/ml streptomycin sulphate (Sigma), sterilized by a 0.22 µm filter and stored at –20 °C until use. Only oocytes with a uniform ooplasm and a compact cumulus cell mass were selected for maturation culture. DOs were obtained by removing the cumulus cells with 0.1% (w/v) hyaluronidase.

In vitro maturation of pig oocytes

Collected CEOs or DOs were cultured in modified NCSU-37 solution (Funahashi *et al.* 1994) containing 10% porcine follicular fluid (v/v), 1.0 mM glutamine, 0.6 mM L-cysteine, 100 IU/ml penicillin G potassium and 50 µg/ml streptomycin sulphate. This medium is termed maturation medium. Groups of 50 oocytes were transferred into individual wells of a four-well Nunclon dish with 0.5 ml maturation medium containing different drugs. The maturation medium was equilibrated overnight before culture. The culture was carried out at 39 °C in an atmosphere with 5% CO₂ in air for 44 h.

Evaluation of oocyte maturation and cumulus expansion

Oocytes were denuded mechanically by repeated pipetting to remove cumulus cells at the end of the culture period. The oocytes were mounted on glass slides after rinsing with saline, fixed in acetic alcohol (acetic acid:alcohol, 1:3, v/v) for 48 h, stained with 1% acetic-orcein for 5–10 min and examined by phase-contrast microscopy (Leica, Germany) to reveal the stage of meiosis. Oocytes were assessed for maturation scoring for germinal vesicles (GVs) (meiotic arrestment), GV breakdown (GVBD) (meiotic resumption) and for the first polar body (PB1) (the first meiotic maturation).

The cumulus expansion was observed under a phase-contrast microscope. The degree of expansion was scored according to Downs (1989) and Vanderhyden (1993) from 0 to 4 grades. Briefly, no response was scored as 0, minimum observable response was scored as 1, expansion of outer layers was scored as 2, expansion of all layers except the corona radiata was scored as 3 and expansion of all layers was scored as 4. A cumulus expansion index (CEI) was calculated (range, 0–4.00) as described in detail previously (Fagbohun & Downs 1990).

SDS-PAGE and Western blot analysis

For detection of active ERK1/2, proteins from 50 oocytes or cumulus cells of 50 CEOs were extracted using 2 × SDS sample buffer during the different culture times and heated to 100 °C for 4 min. After cooling on ice and centrifuging at 12 000 *g* for 4 min, samples were frozen at –20 °C until use. The total proteins were separated by SDS-PAGE according to the method indicated by Fan *et al.* (2003); briefly, 4% stacking gel and 10% separating gel for 20 min at 56 V and 4.5 h at 110 V respectively, and then electrophoretically transferred onto nitrocellulose membranes for 2 h at 200 mA at 4 °C. The membrane was blocked overnight at 4 °C in TBST (20mM Tris-HCl, 150mM NaCl and 0.1%

Tween 20 pH 7.5) buffer containing 5% low-fat milk, and then incubated for 1 h in TBST with 1:500 mouse anti-p-ERK1/2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes for 10 min each in TBST, the membrane was incubated for 1 h at 37 °C with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin G (IgG) diluted 1:1000 in TBST. Proteins on the membrane were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham).

For reprobing total ERK2, the membrane was washed in stripping buffer (100 mM β-mercaptoethanol, 20% SDS and 62.5 mM Tris pH 6.7) to strip off bound antibody after ECL detection at 50 °C for 30 min. The membrane was reprobed with polyclonal rabbit anti-ERK2 antibody (Santa Cruz Biotechnology) diluted 1:300, incubated with HRP-labelled goat anti-rabbit IgG diluted 1:1000, and finally processed as described above. All experiments were repeated at least three times.

Experimental design

Experiment 1

To study the possible effect of ANP on pig oocyte maturation, CEOs isolated from follicles were cultured in maturation medium with or without FSH (0.05 U/ml), supplemented with 100 nM ANP (an appropriate concentration based on our previous study) or C-ANP-(4–23) for 44 h. Then the nuclear status of the oocytes and cumulus expansion were determined.

Experiment 2

The possible signalling pathway of ANP on the effect of FSH was studied by using zaprinast (a cGMP-specific phosphodiesterase inhibitor), KT5823 (a protein kinase G (PKG) inhibitor) and a MAPK phosphorylation assay. 8-Br-cGMP was also used to mimic the effect of ANP on oocyte maturation and MAPK phosphorylation. Pig CEOs were cultured in maturation medium containing 0.05 U/ml FSH supplemented with 100 nM ANP, 10 µM zaprinast, 1 µM KT5823 and/or different dose of 8-Br-cGMP for 44 h. Then the cumulus expansion and nuclear status of the oocytes were determined. Oocytes and cumulus cells were collected at various time intervals for Western blot analysis to investigate the effects of ANP, 8-Br-cGMP and KT5823 on MAPK phosphorylation.

Experiment 3

To investigate the relationship between ANP and cAMP on oocyte maturation, pig CEOs and DOs were cultured in FSH-free maturation medium supplemented with

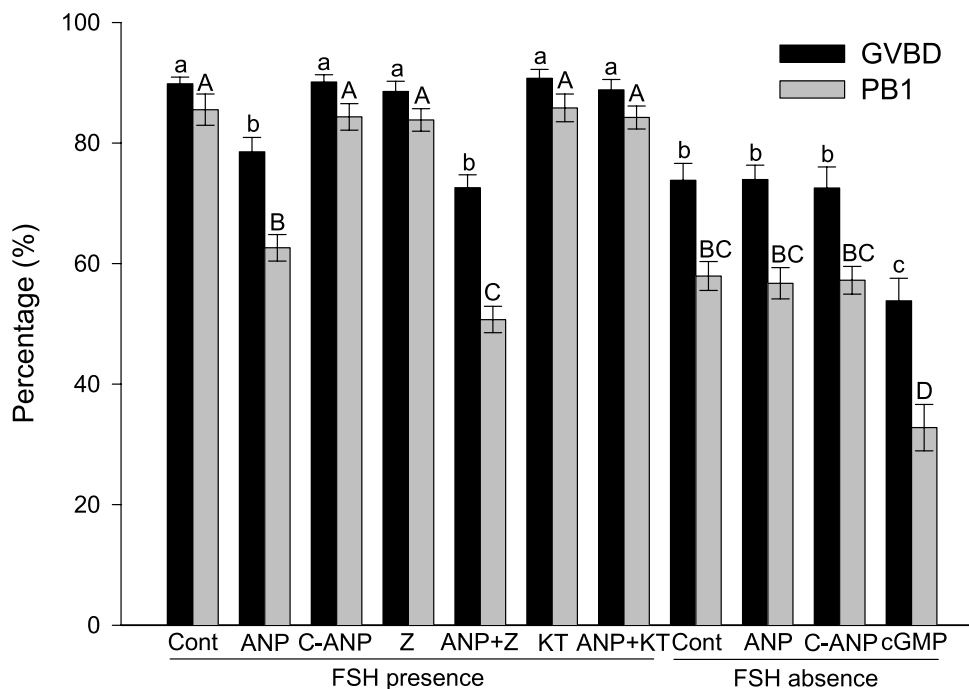


Figure 1 Effects of ANP, C-ANP-(4–23) and 8-Br-cGMP on pig oocyte maturation. Pig CEOs were cultured in NCSU-37 medium with or without FSH (0.05 U/ml), containing 100 nM ANP, 100 nM C-ANP-(4–23), 0.5 mM 8-Br-cGMP, 10 μ M zaprinast and/or 1 μ M KT5823 for 44 h. The median number of oocytes in each group was 330. The means \pm S.E.M. have been calculated from four independent experiments. Different superscripts denote a statistical difference at a $P < 0.05$ level of significance in the GVBD or PB1 respectively. In this and subsequent figures, GVBD is the total number of oocytes which showed GVBD or PB1 stage at the end of culture, and PB1 percentages were calculated as a % of the total oocytes examined. C-ANP, C-ANP-(4–23); Z, zaprinast; KT, KT5823; cGMP, 8-Br-cGMP.

10 μ M forskolin (a stimulator of adenylate cyclase) and different doses of ANP or C-ANP-(4–23) for 44 h, or for 2 h and then cultured in drug-free maturation medium until 44 h. Then the nuclear status of the oocytes and cumulus expansion were determined.

Experiment 4

The possible pathway of ANP in the effect of forskolin during pig oocyte maturation was studied by using pertussis toxin (PT) (Sigma). After pretreated with 0.5 μ g/ml PT for 2 h to block Gi protein(s) activity, pig CEOs and DOs were cultured in maturation medium with 10 μ M forskolin, 100 nM ANP and/or C-ANP-(4–23) for another 42 h, or for another 2 h followed by culture in drug-free medium until 44 h. The nuclear status of the oocytes and cumulus expansion were determined.

Statistical analysis

Experiments were performed at least three times and the values are given as means \pm S.E.M. In the presented data

on oocyte maturation n refers to number of oocytes. Maturation frequencies were subjected to arcsine transformation and analysed by ANOVA followed by Duncan's multiple range tests. $P < 0.05$ was considered statistically significant.

Results

ANP inhibited FSH-induced pig CEO meiotic resumption and the first meiotic maturation

ANP and C-ANP-(4–23) were used to explore the possible participation of ANP in regulating meiotic maturation of pig oocytes. As shown in Fig. 1, FSH had a significant effect on meiotic resumption (89.8% GVBD) and the first meiotic maturation (85.5% PB1, $n = 327$) compared with the FSH-absent treatment (73.8% GVBD and 57.9% PB1, $n = 341$; $P < 0.01$). While C-ANP-(4–23) had no effect on FSH-induced oocyte maturation (90.1% GVBD and 84.3% PB1, $n = 320$), ANP significantly inhibited FSH-induced pig oocyte maturation (78.5% GVBD and 62.6% PB1, $n = 339$).

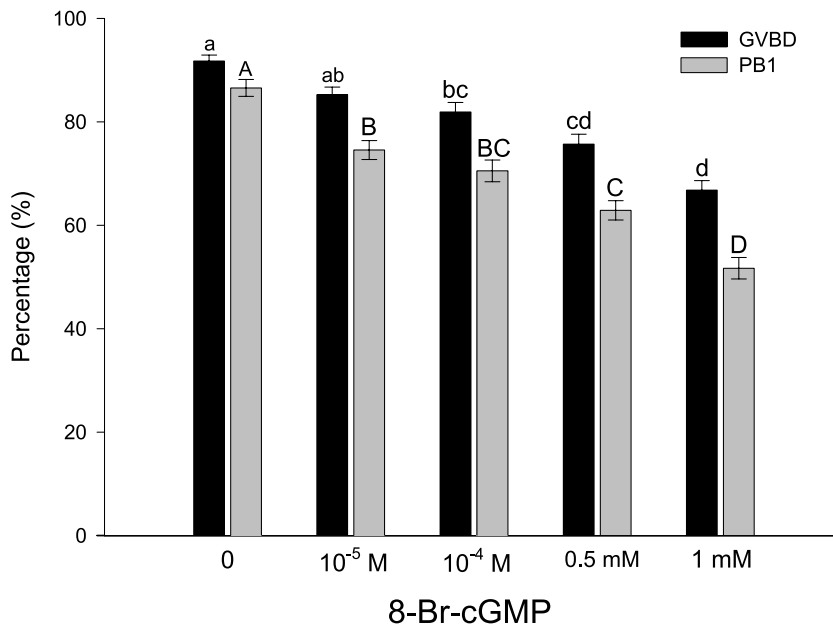


Figure 2 The effect of 8-Br-cGMP on FSH-induced pig CEO maturation. Pig CEOs were cultured in NCSU-37 maturation medium containing 0.05 U/ml FSH and different doses of permeable cGMP analogue 8-Br-cGMP for 44 h. The median number of oocytes in each group was 340. The means \pm S.E.M. have been calculated from four experiments. Different superscripts denote statistical difference at a $P < 0.05$ level of significance in the GVBD or PB1 respectively.

Both ANP and C-ANP-(4–23) were ineffective in pig CEO spontaneous maturation.

The possible mechanism of ANP on oocyte maturation was studied by using zaprinast (a cGMP-specific phosphodiesterase inhibitor), KT5823 (a specific inhibitor of cGMP-dependent protein kinase) and 8-Br-cGMP. The inhibitory effect of ANP was enhanced in the presence of zaprinast (72.6% GVBD and 50.7% PB1, $n=316$; Fig. 1), but completely reversed by KT5823 (88.8% GVBD and 84.2% PB1, $n=331$). Zaprinast or KT5823 alone had no effect on FSH-induced pig CEO maturation (Fig. 1). 8-Br-cGMP had a similar inhibitory effect to ANP on FSH-induced oocyte maturation, and showed a dose-dependent manner in concentrations ranging from 10 μ M (85.3% GVBD and 74.6% PB1, $n=328$) to 1 mM (66.8% GVBD and 51.7% PB1, $n=349$; Fig. 2). 8-Br-cGMP also significantly inhibited spontaneous CEO maturation (53.8% GVBD and 32.7% PB1; Fig. 1). These results revealed that the cGMP/PKG pathway is involved in this process.

ANP inhibited FSH-induced MAPK phosphorylation

The kinetics of MAPK during pig CEO maturation are shown in Fig. 3A. Phosphorylation of ERK1/2 was evaluated by a specific antibody against

phospho-MAPK. ERK1/2 existed in an inactive form in oocytes at the GV stage (Fig. 3A, lane 1). Phosphorylation of kinases was detected at 18 h (lane 2) and 24 h (lane 3) after culture. An abrupt increase was observed at MI stage oocytes (30 h of culture; lane 4), and their full phosphorylation increased in the following hours of culture and remained to the end of culture (44 h; lane 5). However, when the CEOs were cultured in maturation medium containing 100 nM ANP (lanes 11–14) for 18, 24, 30 or 44 h, full phosphorylation of ERK1/2 was obviously inhibited.

Expression and phosphorylation of MAPK was also detected in pig cumulus cells isolated from the same 50 CEOs (Fig. 3A). Active MAPK existed in cumulus cells surrounding GV oocytes freshly isolated from ovaries (lane 6). A significant increase of MAPK activation was detected at 18 h of culture (lane 7), peaking at 24 h (lane 8) and maintained to 44 h (lane 10). Similarly, in cumulus cells isolated from the oocytes treated with ANP (lanes 17–20), full phosphorylation of ERK1/2 was obviously inhibited as observed by Western blotting (Fig. 3A). As shown in Fig. 3B and C, 0.5 mM 8-Br-cGMP had a similar inhibitory effect on the phosphorylation of MAPK, and KT5823 could reverse the inhibitory effect of ANP on the phosphorylation of ERK1/2 of oocytes and cumulus cells, but KT5823 alone had no effect on MAPK phosphorylation.

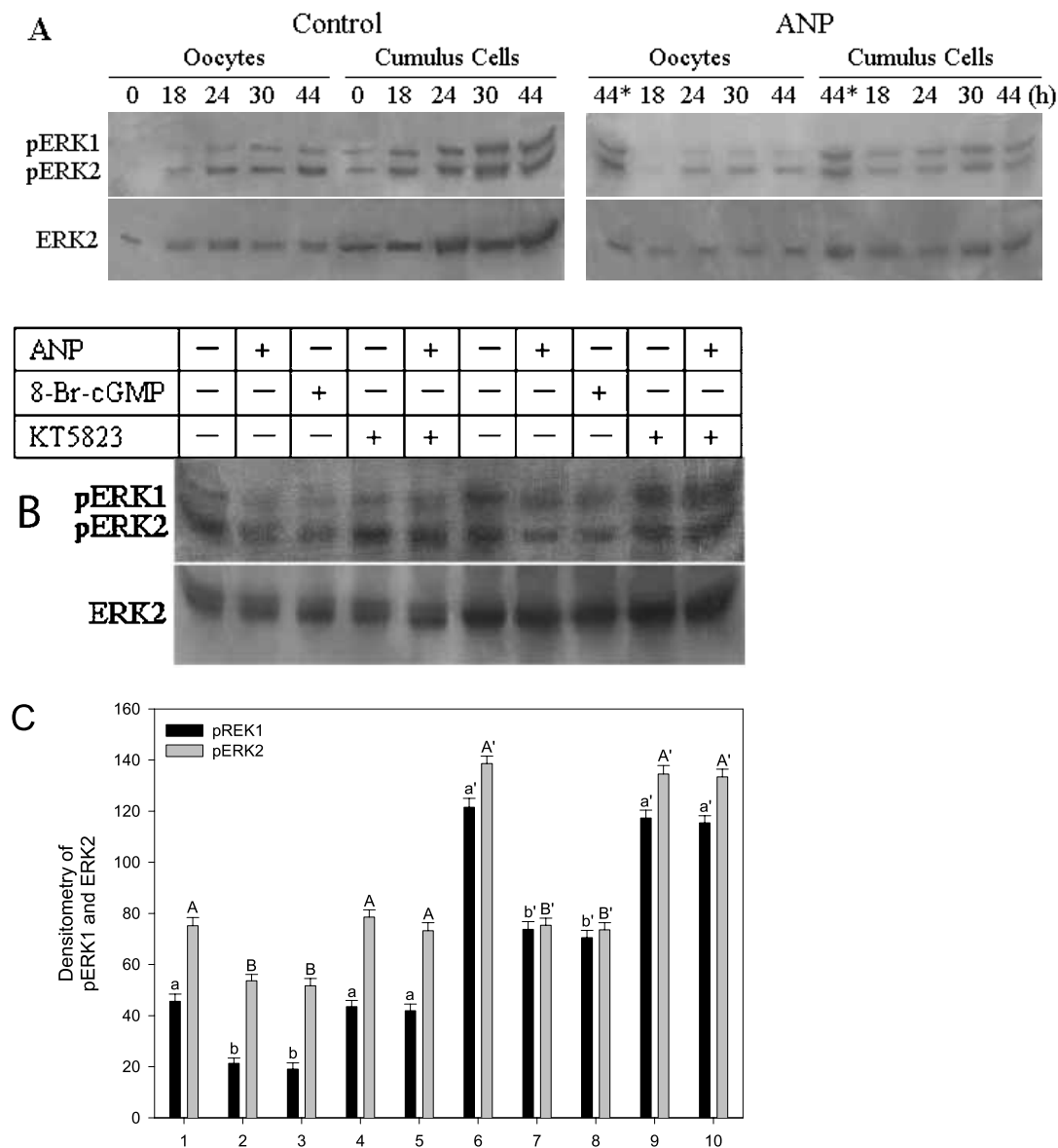


Figure 3 Effects of ANP and 8-Br-cGMP on FSH-induced ERK1/ERK2 phosphorylation in oocytes and cumulus cells. Pig CEOs were cultured for various time intervals in the maturation medium, containing 0.05 U/ml FSH, 100 nM ANP, 0.5 mM 8-Br-cGMP, and/or 10 μ M KT5823. ERK1/ERK2 phosphorylation in oocytes and cumulus cells was assayed as described under Materials and methods. Autoradiogram in (A and B) reflect a representative experiment and bar graphs (C) represent the mean of three independent experiments. (A) Western blot analysis of ERK1/2 phosphorylation during oocyte maturation treated with FSH (lanes 1–10) or treated with FSH and ANP (lanes 11–20). *KT5823 was supplemented in lanes 11 and 16 of the ANP treatment. (B) Western blot analysis of MAPK phosphorylation during oocyte maturation treated with FSH, ANP, 8-Br-cGMP, and/or KT5823 for 44 h. Oocyte group: lanes 1–5; cumulus cell group: lanes 6–10. (C) Quantification of total active ERK1/ERK2 in oocytes and cumulus cells. The means \pm s.e.m. have been calculated from three independent experiments. Different superscripts denote statistical difference at a $P < 0.05$ level of significance in the pERK1 or pERK2 respectively.

ANP and C-ANP-(4–23) inhibited the action of forskolin on pig CEO meiotic resumption and the first meiotic maturation

Pig CEOs were cultured in maturation medium with or without forskolin for 44 h, and the oocyte maturation was evaluated by acetic-orcein staining. As shown in Fig. 4A, both GVBD (73.2%) and PB1 (57.0%, $n=341$) occurred in CEOs cultured in drug-free medium. When CEOs were treated with 10 μM forskolin for 44 h, the GVBD (23.8%) and PB1 (10.2%, $n=363$) were strongly inhibited. Treatment with ANP or C-ANP-(4–23) for 44 h dose-dependently inhibited the function of forskolin in concentrations ranging from 1 nM (30.1% GVBD and 15.1% PB1 with ANP treatment, $n=348$; 33.4% GVBD and 19.7% PB1 with C-ANP-(4–23) treatment, $n=369$) to 1 μM (51.1% GVBD and 36.3% PB1 with ANP treatment, $n=371$; 55.3% GVBD and 37.9% PB1 with C-ANP-(4–23) treatment, $n=355$), although the inhibitory effect of forskolin on the meiotic resumption and the first meiotic maturation of pig CEOs was not completely reversed by even 1 μM ANP or C-ANP-(4–23) (Fig. 4A).

To test the possible mechanism of ANP and C-ANP-(4–23) in regulating the action of forskolin on oocyte maturation, pig CEOs were preincubated with PT for 2 h to block Gi protein(s) activity, and then cultured in maturation medium containing forskolin, ANP and/or C-ANP-(4–23) for another 42 h. As shown in Fig. 4B, the inhibitory effect of ANP and C-ANP-(4–23) on the function of forskolin was completely eliminated after treatment with PT (25.4% GVBD and 9.5% PB1 with ANP treatment, $n=298$; 24.1% GVBD and 11.9% PB1 with C-ANP-(4–23) treatment, $n=312$). PT alone had no effect on the function of forskolin during oocyte meiotic maturation (23.7% GVBD and 11.3% PB1, $n=327$). Short-term priming with forskolin for 2 h could induce pig CEO maturation (89.6% GVBD and 81.9% PB1, $n=374$; Fig. 4C). Similarly, this effect was also inhibited by ANP (78.9% GVBD and 63.7% PB1, $n=346$) and C-ANP-(4–23) (75.8% GVBD and 61.2% PB1, $n=326$), and preincubation with PT could completely eliminate the inhibitory effect of ANP and C-ANP-(4–23) on the function of forskolin short-term priming (Fig. 4C).

ANP and C-ANP-(4–23) were ineffective in pig DO maturation

The role of the cumulus cells was studied by incubating DOs with ANP, C-ANP-(4–23), and/or forskolin for 44 h, or with forskolin for 2 h followed by a 42 h culture in drug-free medium. As shown in Fig. 5, pig oocyte maturation was strongly inhibited when DOs were treated with forskolin for 44 h (25.6% GVBD and

10.5% PB1, $n=278$) or for 2 h (26.4% GVBD and 11.3% PB1, $n=284$). ANP and C-ANP-(4–23) were ineffective in pig DO maturation cultured in drug-free medium or medium with forskolin (Fig. 5).

Effect of ANP on cumulus expansion of pig CEOs

In addition to oocyte maturation, cumulus cells were also evaluated by the CEI. As shown in Table 1 and Fig. 6, FSH induced cumulus expansion of pig CEOs to the maximal degree with a CEI of 3.48. ANP could significantly inhibit FSH-induced cumulus expansion (with a CEI of 2.68). This inhibitory effect of ANP could be mimicked by 8-Br-cGMP (with a CEI of 2.64 with 0.5 mM treatment), and was completely reversed by KT5823 (with a CEI of 3.45). The CEI was 2.19 in pig CEOs cultured in FSH-free medium, but treatment with 10 μM forskolin for 44 h, not for 2 h, could partially induce cumulus expansion (with a CEI of 2.65). ANP was ineffective in cumulus expansion of pig CEOs cultured in drug-free medium or medium with forskolin for 44 h.

Discussion

In the present study, we investigated the role of ANP on the effect of FSH and forskolin in pig oocyte meiotic maturation. The gonadotrophin-induced oocyte maturation, instead of spontaneous meiotic resumption, was inhibited by ANP. ANP also obviously inhibited FSH-induced ERK1/2 phosphorylation. All these inhibitory effects of ANP could be mimicked by 8-Br-cGMP but blocked by KT5823, and zaprinast enhanced the inhibitory effect of ANP on oocyte maturation. In our study, the inhibitory effect of ANP on pig oocyte maturation might be via the PKG/MAPK pathway, since FSH-induced ERK1/2 phosphorylation in both oocytes and cumulus cells is necessary for pig oocyte maturation (Fan *et al.* 2003). On the other hand, both ANP- and C-ANP-(4–23)-inhibited action of forskolin on CEO maturation should be via NPRC receptors and the PT-sensitive Gi protein(s), since PT could eliminate the inhibitory effect of ANP/C-ANP-(4–23) on forskolin-induced oocyte maturation. These results are consistent with previous reports that ANP negatively regulates MAPK phosphorylation and proliferation of rat mesangial cell by the ANP/NPRA system (Pandey *et al.* 2000), and that ANP significantly reduces forskolin-stimulated cAMP accumulation via NPRC receptors and reduction of ligand-stimulated adenylyl cyclase activity in guinea pig airway smooth muscle (Devillier *et al.* 2001). While 8-Br-cGMP significantly inhibited spontaneous oocyte maturation, ANP had no effect on pig oocyte maturation in the

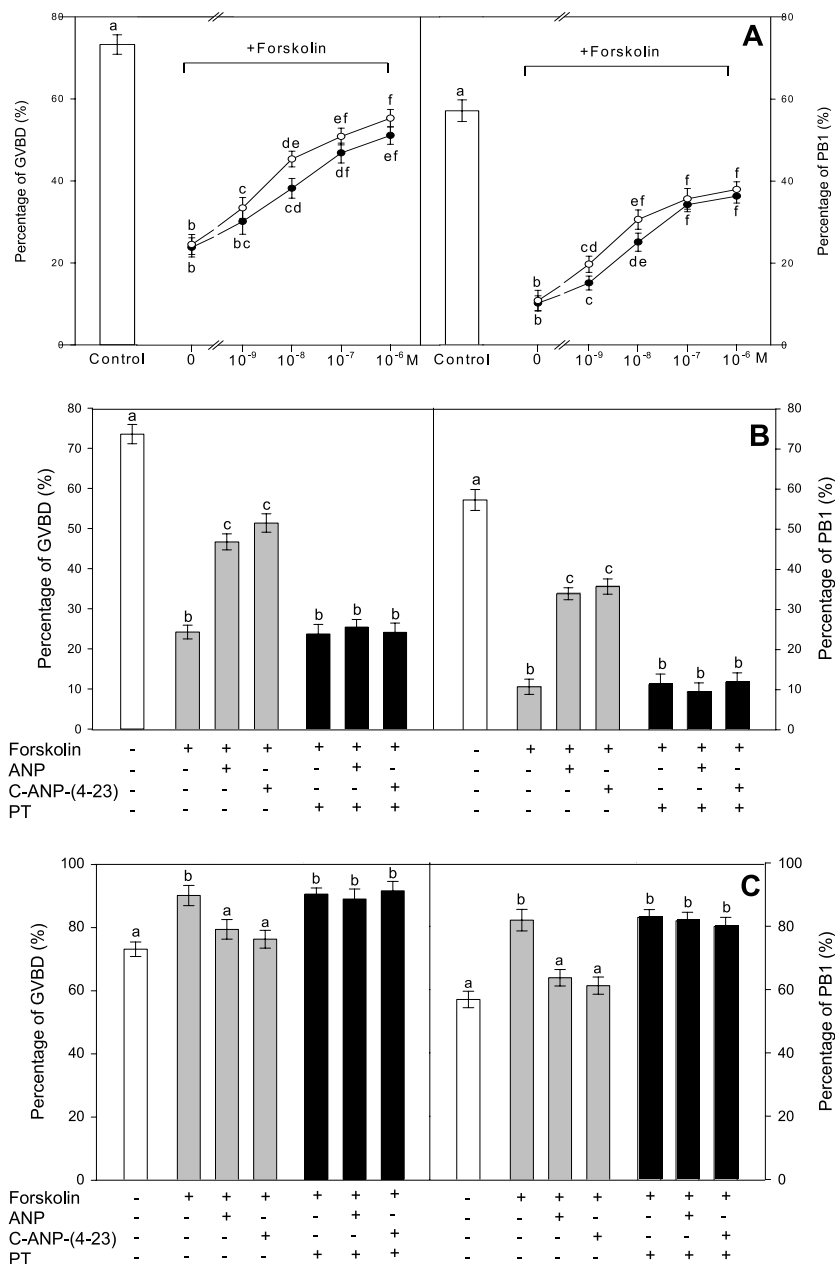


Figure 4 The effects of ANP and C-ANP-(4-23) on the action of forskolin during pig oocyte maturation. (A) Pig CEOEs were culture in FSH-free medium supplemented with forskolin (10 μM) and various doses of ANP (●) and C-ANP-(4-23) (○) for 44 h. The median number of oocytes in each group was 340. The means±S.E.M. have been calculated from four independent experiments. Different superscripts denote statistical difference at a *P*<0.05 level of significance in the GVBD or PB1 respectively. Similar demonstrations of statistical analysis are also used in the following panels. (B and C) After preincubation with 0.5 μg/ml PT for 2 h, pig CEOEs were cultured in 10 μM forskolin, 100 nM ANP and/or C-ANP-(4-23)) for another 42 h, or for another 2 h followed by culture in drug-free medium until 44 h.

absence of FSH. The possible reason is that the receptor expression increases in the presence of the hormone (Gutkowska *et al.* 1999).

Previous results showed that ANP potentiates progesterone-induced *Xenopus* oocyte maturation by stimulation of cGMP accumulation and activation of

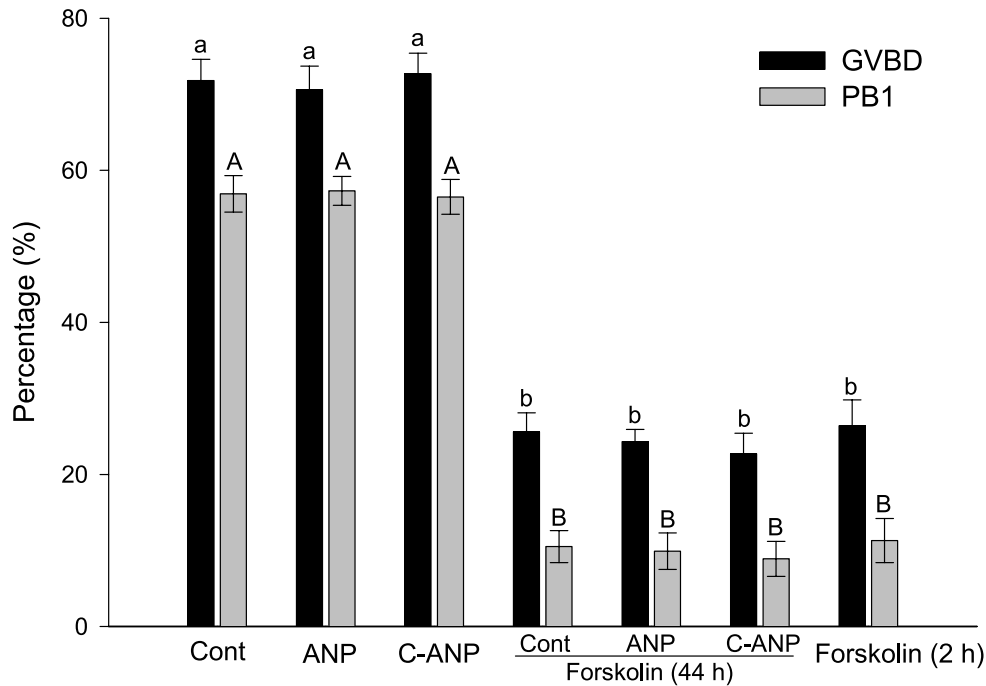


Figure 5 Effect of ANP and C-ANP-(4–23) on pig DO spontaneous maturation. Pig DOs were cultured in maturation medium with 100 nM ANP, 100 nM C-ANP-(4–23), and/or 10 μ M forskolin for 44 h, or with 10 μ M forskolin for 2 h followed by a 42 h culture in drug-free medium. The median number of oocytes in each group was 300. The means \pm S.E.M. have been calculated from four independent experiments. Different superscripts denote statistical difference at a $P < 0.05$ level of significance in the GVBD or PB1 respectively. C-ANP, C-ANP-(4–23).

Table 1 Effects of ANP, C-ANP-(4–23), 8-Br-cGMP and forskolin on cumulus expansion of pig CEOs

Treatment	Degree of cumulus expansion					CEI ^b	n
	0 (%) ^a	+1 (%) ^a	+2 (%) ^a	+3 (%) ^a	+4 (%) ^a		
FSH presence (0.05 U/ml)							
Control	0	2.1 \pm 1.3	11.3 \pm 4.2	22.7 \pm 4.1	63.9 \pm 3.9	3.48 \pm 0.12	327
100 nM ANP	0	16.4 \pm 3.3	27.1 \pm 3.1	28.7 \pm 2.9	27.8 \pm 3.7	2.68 \pm 0.09**	339
100 nM C-ANP-(4–23)	0	1.8 \pm 1.7	15.7 \pm 4.6	22.5 \pm 4.9	60.0 \pm 4.1	3.41 \pm 0.13	320
500 μ M 8-Br-cGMP	0	17.5 \pm 2.4	27.3 \pm 3.0	28.6 \pm 3.3	26.6 \pm 3.4	2.64 \pm 0.15**	318
1 μ M KT5823	0	2.5 \pm 2.3	16.5 \pm 3.8	20.4 \pm 3.7	60.6 \pm 3.2	3.40 \pm 0.10	351
ANP+KT5823	0	1.6 \pm 1.9	12.8 \pm 4.5	24.7 \pm 3.9	60.9 \pm 4.2	3.45 \pm 0.16	331
FSH absence							
Control	5.3 \pm 2.4	23.3 \pm 3.2	31.5 \pm 3.4	26.1 \pm 2.9	13.8 \pm 3.3	2.19 \pm 0.15	341
100 nM ANP	6.3 \pm 3.3	20.1 \pm 2.8	32.4 \pm 2.7	29.7 \pm 3.1	11.5 \pm 3.4	2.20 \pm 0.10	349
100 nM C-ANP-(4–23)	5.4 \pm 2.5	21.5 \pm 3.5	33.8 \pm 2.9	26.4 \pm 2.4	12.9 \pm 3.1	2.20 \pm 0.21	328
10 μ M forskolin (44 h)	0	14.9 \pm 2.7	29.3 \pm 4.1	31.9 \pm 4.2	23.9 \pm 3.8	2.65 \pm 0.13*	363
ANP+forskolin (44 h)	0	17.6 \pm 2.1	28.7 \pm 2.0	32.6 \pm 1.3	21.0 \pm 3.2	2.57 \pm 0.09*	359
C-ANP-(4–23)+forskolin (44 h)	0	15.4 \pm 2.1	25.9 \pm 2.0	35.7 \pm 2.3	23.0 \pm 3.5	2.66 \pm 0.12*	336
10 μ M forskolin (2 h)	4.7 \pm 2.8	21.3 \pm 3.5	30.5 \pm 3.1	28.1 \pm 3.2	15.8 \pm 3.6	2.29 \pm 0.14	374

^aPercentages (means \pm S.E.M. for four replicates) were based on CEOs used for maturation. *Significantly lower ($P < 0.05$) than control value in the FSH absence treatment and **significantly lower ($P < 0.01$) than control value in the FSH presence treatment.

^bCEI, cumulus expansion index. This represents the mean expansion value for the complexes, with 0 equal to no expansion, and 4 equal to maximal expansion.

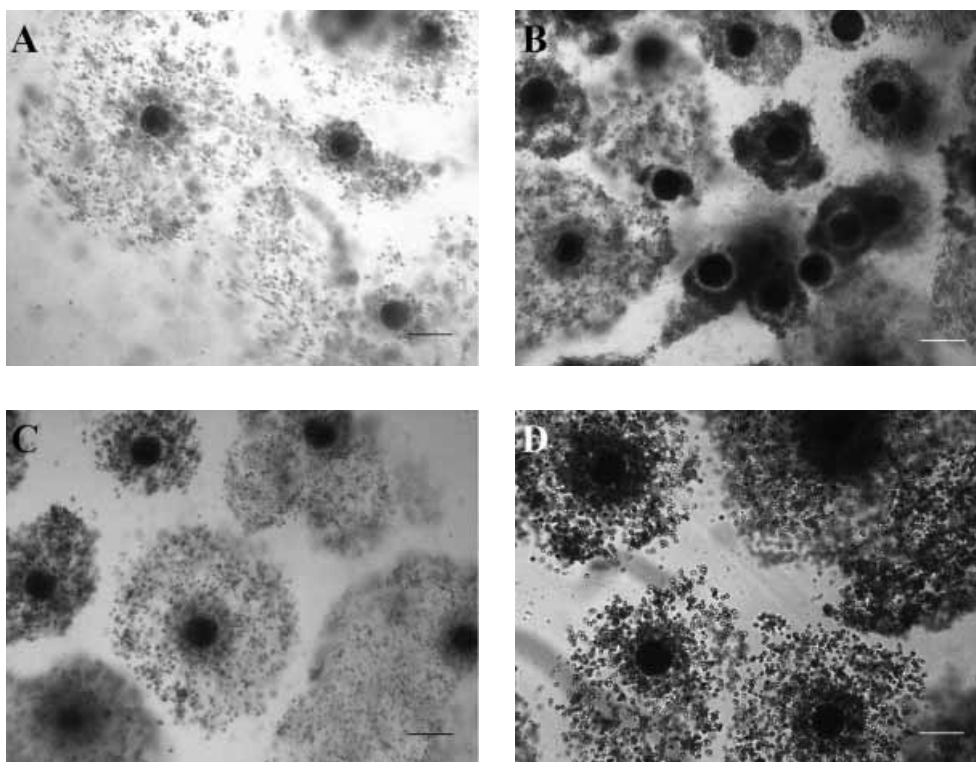


Figure 6 Photomicrographs of pig cumulus expansion. Pig CEOOs were cultured in maturation medium containing different drugs for 44 h. (A) FSH treatment showed a maximal degree of cumulus expansion. (B) Control treatment without any drug showed an obviously lower degree of cumulus expansion. (C) FSH+ANP treatment and (D) forskolin treatment showed a similar degree of cumulus expansion. Bars=150 µm.

cAMP phosphodiesterase (Sandberg *et al.* 1993), but inhibits spontaneous rat oocyte maturation by cGMP accumulation (Törnell *et al.* 1990). However, suppression of MAPK activation in *Xenopus* oocytes does not inhibit progesterone-induced GVBD, suggesting that the activation of MAPK is actually not necessary for the resumption of meiosis, and therefore, an alternative pathway may exist in frog oocytes for triggering the resumption of meiosis (Fisher *et al.* 1999). Similarly, different pathways may also exist in rat oocytes, since FSH has no obvious function in rat oocyte maturation *in vitro* (Zhang & Armstrong 1989).

A drop in intracellular cAMP levels followed by the decrease of the cAMP-dependent protein kinase (PKA) activity is associated with oocyte resumption of meiosis (Sun *et al.* 1999). Forskolin, stimulating adenylyl cyclase by a combination of direct interaction with adenylyl cyclase and activation of G_s protein (for review see Taussig & Gilman 1995), strongly induces the cAMP content of CEOOs and DOs in a dose-dependent manner (Racowsky 1985), and inhibits the meiotic resumption and MAPK activation (Sun *et al.* 1999). In this study, treatment with forskolin for 44 h strongly inhibited the meiotic maturation of both CEOOs and DOs. However,

both ANP and C-ANP-(4–23) did not completely inhibit the action of forskolin (44 h) in the maturation of pig CEOOs, suggesting that the inhibitory effect of ANP/C-ANP-(4–23) is not sufficient to alter the functional responses induced by forskolin. ANP seems to ‘attenuate’ the action of forskolin, consistent with previous results that the cAMP levels stimulated by forskolin in guinea pig tracheal smooth muscle could only be partially blocked by ANP (Devillier *et al.* 2001). The attenuated effect of ANP and C-ANP-(4–23) on forskolin was completely eliminated by pretreatment with PT, suggesting that this effect is therefore probably related to adenylyl cyclase inhibition by the activation of the PT-sensitive Gi protein(s) (Panchenko *et al.* 1998).

It is generally accepted that increased cAMP in oocytes maintains them in the meiotic arrest stage, but increased cAMP in the intra-cumulus cells plays an important role in controlling oocyte maturation. When activated by FSH, MAPK may stimulate the synthesis of specific proteins and then the secretion of a uncharacterized factor(s) of somatic cell origin (Su *et al.* 1999), for example follicular fluid meiosis-activating sterol (FF-MAS) secreted by follicular somatic cells in response to gonadotrophic stimulation (Byskov *et al.*

1997, Thomas *et al.* 2002, Xie *et al.* 2004). The secretion of FF-MAS by granulosa cells is itself a cAMP-dependent process. Forskolin can mimic the effect of gonadotrophin on oocyte maturation by short-term priming (Xia *et al.* 2000, Lu *et al.* 2001). Treatment with forskolin for 2 h induced pig CEOs but inhibited DO spontaneous maturation in our study. These different effects of forskolin on progression of meiosis in CEOs and DOs may be consistent with a role of factor(s) secreted by cumulus cells in response to high levels of cAMP that override the meiosis-inhibiting action of cAMP on completion of meiosis (Byskov *et al.* 1995, 1997). Similarly, ANP and C-ANP-(4–23) could inhibit the effect of forskolin on the maturation of pig CEOs, and pretreatment with PT could eliminate this inhibitory effect. These results further suggest that ANP has a regulatory effect on forskolin-mediated oocyte maturation via the PT-sensitive Gi protein(s).

It has been reported that the cumulus cell, not the oocyte itself, has ANP receptors and guanylate cyclases in the rat (Törnell *et al.* 1990). Here we have no evidence suggesting whether the NPRA or the NPRB receptor mediates the effect on FSH-induced oocyte maturation. ANP shows high affinity for NPRA receptors (for review see Takei 2000), and the concentrations of ANP used in this study were in the same range as those used to regulate MAPK phosphorylation by the ANP/NPRA system (Pandey *et al.* 2000), suggesting that the inhibitory effect of ANP on FSH-induced pig oocyte maturation is more likely involving the specific receptor NPRA.

As both ANP and C-ANP-(4–23) could block the function of forskolin on CEO maturation, the present results provide evidence that NPRC receptors are functional in pig cumulus cells as in other cells (Devillier *et al.* 2001), but the oocyte lacks NPRC receptors mediating the attenuation. In spite of binding to NPRC receptors, ANP may also bind to NPRA receptors to promote the accumulation of cGMP (Panchenko *et al.* 1998). The possible accumulation of cGMP induced by ANP was apparently ineffective in forskolin-mediated oocyte maturation in our study, since PT pretreatment to block the NPRC-Gi protein(s) pathway could completely reverse the effect of ANP on the action of forskolin. However, the forskolin-induced cAMP pathway may block GVBD by inhibiting maturation promoting factor activation rather than inhibiting MAPK phosphorylation, since MAPK activation is not the prerequisite of spontaneous meiotic maturation in mammalian oocytes (Su *et al.* 2002, Fan *et al.* 2003). Alternatively, ANP should not affect the FSH-induced maturation of pig CEOs via binding to NPRC receptors, since the inhibitory effect of ANP was completely reversed by KT5823, and C-ANP-(4–23) was ineffective in FSH-induced oocyte maturation. It seems that ANP could produce physiological actions in the ovary via two different signal pathways: one is to induce cGMP accumulation via NPRA (or NPRB) receptors,

and the other is to inhibit adenylyl cyclase in a PT-sensitive manner via NPRC receptors as in other tissue (Anand-Srivastava *et al.* 1996).

It is puzzling that ANP inhibits FSH-induced oocyte maturation via the PKG pathway, but inhibits the action of forskolin on oocyte maturation via NPRC receptors and PT-sensitive Gi protein(s), in spite of the fact that both FSH and forskolin affect oocyte maturation primarily via the activation of the PKA pathway (Racowsky 1985, Conti & Jin 1999). Gonadotrophin receptors may be coupled to other heterotrimeric G proteins (such as Gq protein), except in the cAMP signalling pathway (for review see Conti 2002), but we showed that anti-Gq α (G₁₁ α) had no effect on FSH-induced pig CEO maturation (data not shown). Studies on endocrine and non-endocrine cells have shown that cAMP-phosphodiesterases 4 (cAMP-PDE4s) expression in granulosa and theca cells but not in oocytes (Tsafiriri *et al.* 1996), are part of a feedback control of cAMP levels stimulated by gonadotrophin (for review see Conti 2000). Stimulation of cultured rat granulosa cells with FSH causes a significant increase in PDE4s activity, which may in turn decrease the cAMP levels (Conti *et al.* 1984). FSH, forskolin or db-cAMP significantly increases activin β A expression while suppressing the expression of activin β B, but no significant desensitization is noticed for forskolin and db-cAMP over the concentration ranges tested (Wang & Ge 2003). Furthermore, a portion of the cAMP generated in response to forskolin is formed in a functional compartment that lacks access to cAMP-dependent protein kinase (Zhou *et al.* 1992). From the discussion above, we postulate that ANP may not be sensitive to the relatively low cAMP levels stimulated by FSH because of the feedback control by PDE4s, but obviously inhibits the increase of MAPK phosphorylation via the PKG pathway. On the contrary, ANP may be sensitive to the large increase in cAMP content stimulated by forskolin and directly induce the inhibitory effect on forskolin via NPRC receptors and PT-sensitive Gi protein(s). Does ANP moderately modulate the actions of FSH and forskolin on pig oocyte maturation via different signalling pathways in order to prevent the intense physiological reaction? Its further regulation mechanism remains to be elucidated. Compared with FSH, treatment with forskolin for 44 h exhibited a relatively low sensitivity to ANP and C-ANP-(4–23), since this diterpene rapidly stimulates the catalytic subunit of adenylyl cyclase (Seamon & Daly 1981) and generates a large enough increase in cAMP content to elicit an optimal activation of cAMP-dependent protein kinase (Devillier *et al.* 2001).

Cumulus expansion induced by gonadotrophin requires the participation of MAPK (Su *et al.* 2002). Inhibition of MAPK activation prevents gonadotrophin-stimulated resumption of meiosis as well as cumulus

expansion (Su *et al.* 2003). In our study, the inhibitory effect of ANP on FSH-induced cumulus expansion might be via the inhibition of MAPK phosphorylation, since FSH-induced MAPK phosphorylation was obviously inhibited by ANP in both oocytes and cumulus cells. However, forskolin-induced cumulus expansion was not inhibited in the presence of ANP or C-ANP-(4–23). The possible explanations are that forskolin generated a large enough increase in cAMP content to induce cumulus expansion in spite of the presence of ANP or C-ANP-(4–23).

The fact that treatment with forskolin for 44 h induced cumulus expansion but inhibited pig CEO maturation, while treatment with forskolin for 2 h only induced CEO maturation, supports the previous conclusions of Eppig (1979) and Racowsky (1985) that oocyte maturation is not a prerequisite for cumulus expansion. In the mouse, cumulus expansion induced by FSH is dependent upon specific cumulus-expansion-enabling factor(s) secreted by the oocyte (Buccione *et al.* 1990, Salustri *et al.* 1990), but in the pig, cumulus expansion induced by FSH or forskolin is not dependent on the oocyte (Procházka *et al.* 1991). However, compared with the almost full expansion induced by FSH, the extent of expansion stimulated by forskolin is limited to the outer layers of the cumulus cells as in our and other studies (Racowsky 1985, Procházka *et al.* 1991), suggesting that the normal interaction between oocytes and cumulus cells in the pig may be needed for the full cumulus expansion.

In conclusion, ANP inhibits the actions of FSH and forskolin on pig oocyte maturation via different receptors and signalling pathways. One is via the cGMP/PKG pathway, and the other is via NPRC receptors in cumulus cells and the activation of PT-sensitive Gi protein(s). ANP alone is ineffective in oocyte maturation cultured in drug-free medium. These results suggest that the diverse responses of ANP could mediate the action of cAMP via different signalling pathways during pig follicular development.

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