The expression of atrial natriuretic peptide in the oviduct and its functions in pig spermatozoa

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Abstract

Locally synthesized atrial natriuretic peptide (ANP) and its receptors have been found in reproductive tissues of various mammals, and play an important role in the acrosome reaction of human sperm. The objective of the present study was to examine the expression of ANP and its receptors in pig spermatozoa and oviduct, and the effect of ANP on pig spermatozoa function. The expression of ANP and its receptors was analyzed by RT-PCR. Only natriuretic peptide receptors-A (NPRA) mRNA was detected in fresh sperm. While the levels of natriuretic peptide receptors-C (NPRC) mRNA were low with no obvious change among different oviductal phases, the levels of ANP mRNA were high in oviduct(OT)1, OT3 and OT5, but were very low in OT2. On the other hand, the levels of NPRA mRNA were low in OT1 and OT2, increased in OT3 and reached a maximum in OT4 and OT5. Western blot analysis revealed that the level of ANP was high in OT1, decreased in OT2 and OT3, and arrived at the nadir in OT4 and OT5. The effect of ANP on spermatozoa function was studied by the acrosome reaction and IVF. Incubation with ANP for 1 h

significantly induced acrosome reaction of preincubated spermatozoa, and maximal response of acrosome reaction ($34.1 \pm 2.3\%$) was achieved at 1 nM ANP treatment. Both C-ANP-(4–23), a selective ligand of NPRC, and caffeine had no effect on the acrosome reaction. The stimulatory effect of ANP on acrosome reaction could be mimicked by the permeable cGMP analog, 8-Br-cGMP. ANP and caffeine had a similar effect on improving the oocytes penetration rate, polyspermy rate and the average number of sperm per penetrated oocyte. Also, ANP treatment had a similar effect on cleavage rate, blastocyst formation rate and the number of cells per blastocyst as that of caffeine treatment. The effects of ANP on the acrosome reaction and the parameters of oocyte penetration could be blocked by cGMP-dependent protein kinase (PKG) inhibitors KT5823 and/or Rp-8-pCPT-cGMPS. These results suggest that the expression of ANP in the oviduct may be involved in the regulation of the acrosome reaction and the fertilising ability of pig spermatozoa, and the PKG pathway possibly participates in the process.

Journal of Endocrinology (2006) **189**, 493–507

Introduction

Atrial natriuretic peptide (ANP) or atrial natriuretic factor (ANF) is a member of a family of peptides that share a common 17-amino acid ring closed by a disulfide bond between two cysteine residues and varying only in length of their N- and C-terminal extensions (Flynn *et al.* 1983). ANP is mainly involved in the regulation of blood pressure, salt and water excretion, cell proliferation and body fluid homeostasis (Ruskoaho 1992). In addition, extra-atrial sites that synthesize ANP have been found in discrete areas, including the hypothalamus, pituitary gland, adrenal medulla, gastrointestinal tract and thymus (Gutkowska & Nemer 1989). ANP and its receptors have been found in the corpus luteum (Vollmar *et al.* 1988), ovary and follicular fluid (Anderson *et al.* 1994), granulosa cells (Kim *et al.* 1992, Ivanova *et al.* 2003), oocytes

(Kim *et al.* 1993), oviduct (Kim *et al.* 1997), testis (Pelletier 1988), and spermatozoa (Silvestroni *et al.* 1992). Such findings suggest that the locally synthesized ANP may act in an autocrine or paracrine fashion in certain tissues to produce physiological responses (Tornell *et al.* 1990, Anderson *et al.* 1994, Johnson *et al.* 1994, Zamir *et al.* 1995, Evrard *et al.* 1999). The diverse physiological actions of ANP are manifested by binding to its specific cell-surface receptors (for review, see Takei 2001). ANP exhibits high affinity to natriuretic peptide receptors-A (NPRA) and natriuretic peptide receptors-C (NPRC; for review, see Takei 2000). NPRA receptors can activate particulate guanylate cyclase and stimulate cGMP to produce physiological response in many tissues and cells (Pandey *et al.* 2000), while NPRC receptors have no intrinsic ability to generate cGMP (Drewett *et al.* 1992).

In mammals, sperm are not immediately capable of fertilising oocytes. They must undergo modifications that normally occur in the female reproductive tract (Austin 1951, Chang 1951). The changes that occur in sperm involve at least two components: capacitation (Yanagimachi 1981) and acrosome reaction (Yanagimachi & Usui 1974). Yet, the *in vivo* factors that capacitate sperm and induce the acrosome reaction are unknown in most species. Caffeine and heparin have been widely used as suitable alternatives to improve the success rate of IVF (Parrish *et al.* 1986, Niwa & Ohgoda 1988, Yoshida *et al.* 1990). It has been reported that ANP is synthesized in the mucosal layer of rat oviduct, but its local role is not very clear (Kim *et al.* 1997). ANP induces spermatozoa chemokinesis and chemotaxis (Zamir *et al.* 1993, Anderson *et al.* 1995), which may be beneficial for the spermatozoa to resume high motility and travel the distance between the storage site and the fertilisation site at the oviductal ampulla (Hunter 1993). In addition, some studies provide evidence that ANP induces an acrosome reaction in both human (Anderson *et al.* 1994, Rotem *et al.* 1998) and bovine spermatozoa (Zamir *et al.* 1995), and concentrations of ANP in human follicular fluids are correlated with successful IVF (Anderson *et al.* 1994). Therefore, locally produced ANP may play an important role in mammalian sperm function. Although some agonists (such as caffeine and heparin) are widely used for spermatozoa capacitation and IVF in domestic animals (Parrish *et al.* 1986, Niwa & Ohgoda 1988, Abeydeera & Day 1997b, Zhao *et al.* 2002), the mechanisms of fertilisation *in vivo* are unclear. In the present study, we analyzed ANP and natriuretic peptide receptors gene expression in pig sperm and oviduct. In addition, the effects of ANP on the acrosome reaction and the fertilising ability of pig spermatozoa were also examined.

Materials and Methods

Chemicals and media

The synthetic human ANP-(1–28), {des (Gln¹⁸Ser¹⁹Gly²⁰Leu²¹Gly²²) rANF-(4–23)-NH₂} {C-ANP-(4–23)}, anti-ANP, anti-β-actin, 8-Br-cGMP, caffeine, KT5823, Rp-8-pCPT-cGMPS, H89, lysophosphatidylcholine, bovine serum albumin (BSA), insulin, epidermal growth factor (EGF), human chorionic gonadotrophin (hCG), porcine follicle-stimulating hormone (FSH), bismark brown, rose bengal and trypan blue were purchased from Sigma Chemical Company. The ANP, C-ANP-(4–23), 8-Br-cGMP, Rp-8-pCPT-cGMPS, H89 and lysophosphatidylcholine were prepared as 0.1 mM, 0.1 mM, 0.5 M, 50 μM, 5 μM and 10 mg/ml stock solutions in distilled deionized H₂O (dd H₂O), respectively, and KT5823 as 1 mM stock solution in ethyl acetate. These stock solutions were kept at –20 °C and added at the beginning of culture. Human ANP was used in our study, since the

whole hormone sequences of ANP are conserved within mammals (for review, see Takei 2001).

The basic medium used for *in vitro* oocyte maturation (IVM) was 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 (Sigma), 50 μg/ml streptomycin sulfate (Sigma), 5 μg/ml insulin, 10 ng/ml EGF, 10 IU/ml hCG and 0.05 U/ml FSH. Basic IVF medium, designated modified Tris-buffered medium (mTBM), was as described by Abeydeera & Day (1997a). The complete IVF medium contained 0.5% BSA, 2 mM caffeine, 1 nM ANP and/or 1 μM KT5823. Culture medium for embryo development was North Carolina State University (NCSU) 23 (Petters & Wells 1993) containing 0.4% BSA, 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulfate.

RNA isolation and RT-PCR

Fresh semen was collected from fertile boars by the gloved hand method (King & Macpherson 1973), and then washed with the Percoll (Sigma) technique (Parrish *et al.* 1995). Briefly, isotonic Percoll solution was used for preparation of 90% and 45% gradients with phosphate-buffered saline (PBS) medium. The Percoll density gradient was made by layering 2.5 ml of 45% Percoll solution on the 2.5 ml of 90% solution in 15 ml Falcon tubes. On the top of the gradient 1 ml of fresh semen was layered and then tubes were centrifuged for 10 min at 700 g, and the pellets were then washed with PBS for three times (500 g for 10 min). Pig oviducts connected to the ovary were collected from a local slaughterhouse and transported to the laboratory within 2 h at 4 °C. Total RNA was extracted from Percoll-washed spermatozoa and oviduct using TRI reagent (Sigma) according to the instructions of the manufacturer, and dissolved in 20 μl nuclease-free water. According to the follicular characterization of the ovary, five oviductal phases throughout the estrous cycle were defined as follows (Fig. 1A): (1) Oviduct at the small follicle stage (the diameter of all follicles in the ovary was less than 3 mm, OT1); (2) oviduct at the middle follicle stage (a few of 3–6 mm follicles existed in the ovary, OT2); (3) oviduct at the large follicle stage (a few of more than 6 mm follicles existed in the ovary, OT3); (4) oviduct at the corpus rubrum stage (OT4); (5) oviduct at the corpus luteum stage (OT5). Semi-quantitative RT-PCR was performed as described by Piao *et al.* (2004). Briefly, 1 μg total RNA was reverse transcribed at 42 °C for 50 min, denatured at 95 °C for 2 min, and amplified for 32 (β-actin) or 35 (ANP, NPRA and NPRC) cycles of denaturation at 94 °C of 30 s, primer annealing at 54 °C for 30 s, and extension at 72 °C for 30 s, with a final extension step of 5 min at 72 °C. The amplified products were analyzed by electrophoresis on 1% agarose gels. Oligonucleotide primer used for amplification of the ANP was from known cDNA sequence of porcine ANP

Table 1 Primers used for determinations of pig ANP mRNA, NPRA mRNA and NPRC mRNA by RT-PCR

	Primer sequence	Predicted product size (bp)	Amplification
mRNA			
ANP	F: 5'-ACGACGCCAGCATGAGCTCCTTC-3' (1–23) R: 5'-GCTGTATCTTCAGTACCGGAA-3' (453–474)	474	35
NPRA	F: 5'-GAGCCCACTAGCCTTGAGA-3' (2016–2034) R: 5'-CTACCACGCAGTTGGATGAC-3' (2456–2475)	460	35
NPRC	F: 5'-GGGCAGTGAGCGAGTGGT-3' (757–774) R: 5'-CCCCATCCTTCTTGCTGT-3' (1140–1157)	401	35
beta-actin	F: 5'-TCCAGCCTTCCTTCTGGGTAT-3' (865–886) R: 5'-CGCCTTCACCGTTCAGTTT-3' (1402–1421)	557	32

F, forward; R, reverse.

(GenBank accession no. X54669). This primer pair predicts 474-basepair (bp) DNA fragments (Mä gert *et al.* 1990). The primer used for amplification of the NPRA was from known cDNA sequences of human NPRA (GenBank accession no. NM000906). The upstream primer has identical nucleotides 2016–2034 of human cDNA, and the downstream primer represents the reverse complement of nucleotides 2456–2475 (Table 1). This primer pair predicts 460-bp DNA fragments. The primer used for amplification of the NPRC was from known cDNA sequence of rat NPRC (GenBank accession no. NM012868). This primer pair predicts 401-bp DNA fragments (Piao *et al.* 2004). The beta-actin primers were based on the mouse sequence (GenBank accession no. NM007393).

Western blot analysis

Partially purified proteins (15 µg) from pig sperm and oviduct were separated on 15% SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad) by the methods of Fan *et al.* (2003). Non-specific binding was blocked by overnight incubation of membrane in 5% nonfat dry milk in PBS containing 0.1% tween-20 (PBST, pH 7.5). Membranes were incubated with 1:200 goat anti-ANP antibody (Sigma) for 2 h and then washed three times (10 min each wash) with PBST. The membrane was incubated for 1 h at 37 °C with horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G (IgG) diluted 1:1000 in PBST. Proteins on the membrane were visualized using the enhanced chemiluminescence detection system (Amersham). The levels of β-actin were detected at the same time.

Preparation of spermatozoa and acrosomal reaction evaluation

Fresh sperm cells were washed twice (500 g for 10 min) with mTBM medium containing 0.5% of bovine serum albumin (BSA) and incubated at a temperature of 37 °C in a humidified 5% CO₂ atmosphere for 2.5 h. All centrifugations were performed in covered tubes. Sperm

cells (1×10^8 cells/aliquot with the volume of 1 ml) were then incubated with the various hormones and/or drugs for further 60 min at 37 °C. Acrosomal sperm status was analyzed by the triple-stain technique, as described by Talbot & Chacon (1981) and Rotem *et al.* (1998). Briefly, sperm were resuspended in PBS without BSA, stained with trypan blue for 15 min, fixed in paraformaldehyde (4% in PBS) for 30 min at room temperature and then washed twice (500 g for 10 min) with double distilled (dd) H₂O. Samples were resuspended in 100 µl of dd H₂O, and pipetted onto a microscope glass slide. The air-dried slides were incubated in bismark brown (0.8%) for 8 min at 40 °C, and then in rose bengal (0.8%) for 25 min at room temperature. Slides were washed to remove excess stain, passed twice through absolute ethanol for dehydration, and cleared twice in xylene (100%). Four hundred spermatozoa were examined by light microscopy under oil immersion (1000 ×) to record the acrosome status as described before (Rotem *et al.* 1992). This method assays the acrosome reaction in viable sperm. For the capacitation, pig sperm were washed twice and resuspended in mTBM medium containing 5 mg/ml BSA, and/or 1 nM ANP for 60 min at 37 °C. At the end of incubation, sperm were exposed to lysophosphatidylcholine (LC; Parrish *et al.* 1988) for further 15 min. The acrosome reaction was determined by triple-stain method.

Oocyte collection and IVM

Immature oocytes were aspirated from 3- to 6-mm follicles of abattoir-derived ovaries. Oocytes with uniform ooplasm and compact cumulus were collected and washed in IVM medium. Groups of 50 oocytes were transferred into an individual well of a four-well Nunclon dish with 0.5 ml maturation medium. The culture was carried out at 39 °C in an atmosphere with 5% CO₂ in air for 44 h.

In vitro fertilisation and embryo culture

Oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase at the end of culture.

Thereafter, oocytes were rinsed and 25–30 oocytes were transferred into 50 µl droplets of the mTBM medium with 0.5% BSA plus 2 mM caffeine, or 1 nM ANP and/or 1 µM KT5823. These droplets were covered with mineral oil (Sigma) and equilibrated for 40 h at 39 °C in 5% CO₂ in air. The dishes were kept in a CO₂ incubator until sperm were added for insemination. Spermatozoa were incubated with mTBM medium containing different drugs to a concentration of 6×10^5 cells/ml, and 50 µl of the sperm sample was added to the same fertilisation droplets containing the oocytes. Six h postinsemination (p.i.), oocytes were washed three times and cultured in 0.1 ml droplets in embryo culture medium. All gamete incubations were performed in microdrops under mineral oil equilibrated at 39 °C in an atmosphere of 5% CO₂ in air. Some oocytes were fixed at 12 h p.i. in acetic ethanol (25%, v/v) to assess meiotic stages, spermatozoa penetration rate, and the incidence of polyspermy, as described previously by Hunter & Polge (1966). Oocytes were considered penetrated when one or more decondensed spermatozoa heads and/or male pronuclei and corresponding spermatozoa tails were present. The rate of polyspermy was determined from the number of oocytes penetrated. Cleavage was examined at 48 h p.i., and blastocyst formation was examined at 144 h p.i. (Wu *et al.* 2002). Only embryos with blastomeres of equal diameter were counted as cleavage rate. Embryos or oocytes found to be degenerated or fragmented were not included (Kikuchi *et al.* 2002). Apparent blastocysts were stained with Hoechst 33342 and mounted on slides for counting of cell nuclei. Blastocyst formation rate was calculated as a percentage of the total number of oocytes inseminated.

Experimental design

Experiment 1: The expressions of ANP gene, NPRA gene and NPRC gene in pig spermatozoa and oviduct In order to determine whether ANP mRNA, NPRA mRNA or NPRC mRNA was expressed in pig spermatozoa and oviduct, total RNA was extracted from cells of 1 ml fresh semen (the average density was $145 \pm 36 \times 10^6$ /ml, $n=3$) and oviducts at different stages, and RT-PCR was performed with ANP, NPRA and NPRC primers, respectively.

Experiment 2: Immunodetection of ANP in pig spermatozoa and oviduct To determine whether there is a potential physiological role for ANP in the oviduct during fertilisation, we detected the level of ANP in pig spermatozoa and oviduct. Proteins were extracted from cells of fresh pig semen and oviducts at different stages, and immunodetection of ANP was performed by SDS-PAGE.

Experiment 3: Effects of ANP, C-ANP-(4–23) and caffeine on the acrosome reaction of pig spermatozoa Sperm was incubated in mTBM containing 5 mg/ml BSA

for 2.5 h at a temperature of 37 °C and sperm viability was examined with 0.4% trypan blue. Pig sperm were then incubated in mTBM containing 5 mg/ml BSA supplemented with various doses of ANP, C-ANP-(4–23) (an analogue of ANP that specifically binds to the NPRC receptors), or 2 mM caffeine for further 60 min at 37 °C. Same dose of vehicle was added to the control group at the same time. Acrosomal sperm status was analyzed by the triple-stain technique as described by Talbot & Chacon (1981) and Rotem *et al.* (1998). The motility of sperm was also evaluated.

Experiment 4: The possible pathway of ANP on the acrosome reaction of pig sperm As ANP could bind to its specific cell-surface receptors (natriuretic peptide receptors-A; NPRA) and activate particulate guanylate cyclase and stimulate cGMP to produce a physiological response, we used permeable cGMP analog 8-Br-cGMP to mimic the effect of ANP on acrosome reaction. After incubation in mTBM medium containing 5 mg/ml BSA for 2.5 h at a temperature of 37 °C, pig sperm were then incubated with 0.1, 0.5, 1 and 5 mM cGMP for further 60 min at 37 °C (Rotem *et al.* 1998) and acrosomal sperm status was analyzed by the triple-stain technique. The involvement of cGMP-dependent protein kinase (PKG) pathway in ANP-induced acrosome reaction was also tested by incubation of 1 nM ANP, 1 µM KT5823 (Pandey *et al.* 2000), and/or 0.5 µM Rp-8-pCPT-cGMPS (for review, see Smolenski *et al.* 1998) for further 60 min at 37 °C. Same dose of vehicle was added to the control group at the same time. The possible effect of cAMP-dependent protein kinase (PKA) pathway in the high concentration of 8-Br-cGMP (1 mM) was also studied by PKA inhibitor H89 (0.05 µM; for review, see Smolenski *et al.* 1998).

Experiment 5: Effect of ANP on the capacitation The possible effect of ANP on the capacitation was studied by lysophosphatidylcholine. Pig sperm were washed twice and resuspended in mTBM medium containing 5 mg/ml BSA, and/or 1 nM ANP for 60 min at 37 °C. At the end of incubation, sperm were exposed to 0 or 100 µg/ml lysophosphatidylcholine for further 15 min. The acrosome reaction was determined by triple-stain method.

Experiment 6: Effect of ANP on *in vitro* fertilisation Spermatozoa were incubated in mTBM medium containing 5 mg/ml BSA supplemented with 2 mM caffeine, 1 nM ANP and/or 1 µM KT5823 for 90 min at 37 °C, and then 50 µl of the sperm sample (6×10^5 cells/ml) was added to the same fertilisation droplets containing the oocytes. Same dose of vehicle was added to the control group. As the total incubation time is 7.5 h, sperm was not preincubated before drug treatments. The proportions of

Figure 1 The expressions of ANP gene, NPRA gene and NPRC gene in the oviduct. (A) According to the follicular characterization in the ovary, the five oviductal phases throughout the estrous cycle were defined as OT1, OT2, OT3, OT4 and OT5. (B) RT-PCR analysis of ANP in the oviduct. The intensity of the amplified bands was quantified by densitometric scanning. The respective value of either ANP was normalized according to those of β -actin to evaluate arbitrary units of the relative abundance of the targets. The means $\pm$ S.E.M. have been calculated in three independent experiments. Values with different superscripts differ significantly ($P < 0.05$). (C) RT-PCR analysis of NPRA in the oviduct. The intensity of the amplified bands was quantified by densitometric scanning. Values with different superscripts differ significantly ($P < 0.05$). (D) RT-PCR analysis of NPRC in the oviduct. The intensity of the amplified bands was quantified by densitometric scanning. No obvious change was observed among the five oviductal phases.

oocyte penetration, cleavage and blastocyst formation were examined at 12 h, 48 h and 144 h, respectively.

Statistical analysis

Experiments were performed with different semen and oocyte samples. Results are given as the mean $\pm$ S.E.M. of at least three experiments, with each assessment done in triplicate. All proportional data were subjected to an arcsine transformation and analyzed by ANOVA using StatView software (SAS Institute, Inc, Cary, NC, USA). When there was a significant F ratio, groups were compared using Fisher's protected least significant difference *post hoc* test. Statistical significance was defined as $P < 0.05$.

Results

The expressions of ANP gene, NPRA gene and NPRC gene in pig spermatozoa and oviduct

After washed with Percoll technique, little seminal plasma particulate material (such as spermatogenic cells, neutrophils and/or cytoplasmic droplets) was observed in the semen. Partial cDNAs were amplified by RT-PCR from pig spermatozoa and oviduct using each primer set corresponding to the sequences of porcine ANP, human NPRA and rat NPRC, respectively (Table 1, Fig. 1, Fig. 2). The partial porcine ANP sequence was the same as that reported by Mägert *et al.* (1990). The partial porcine NPRA sequence showed a high degree of similarity to human (94.1%), mouse (89.6%), and rat (90.0%) sequences (Fig. 2B). The partial porcine NPRC sequence also showed a high degree of similarity to human (92.3%), mouse (91.3%), and rat (90.8%) sequences (Fig. 2C). Sequence comparisons indicate that the partial porcine cDNAs amplified with either NPRA primer or NPRC primer conformed to the porcine NPRA region or the NPRC region, respectively. Fig. 2A shows that the ANP gene, NPRA gene and NPRC gene were expressed at all stages of the oviducts, but only NPRA mRNA was detected in the spermatozoa. The levels of the ANP gene expression were high in OT1, dramatically decreased in OT2, and then increased in OT3 (Fig. 1B). The levels of the NPRA gene expression were low in OT1 and OT2, gradually increased in OT3, and then reached a maximum in OT4 and OT5 (Fig. 1C). The levels of the NPRC gene expression were low without obvious change among all phases of the oviduct (Fig. 1D).

Immunodetection of ANP in pig spermatozoa and oviduct

Western blot analysis revealed that ANP protein was not detected in spermatozoa but existed in all stages of the oviduct (Fig. 3). The highest level of ANP was detected at

Figure 2A The expressions of ANP gene, NPRA gene and NPRC gene in pig spermatozoa. (A) RT-PCR analysis of ANP mRNA, NPRA mRNA and NPRC mRNA in pig spermatozoa.

OT1 stage. The level of ANP at OT4 and OT5 stages was lower than that at OT1 ($P < 0.01$), OT2 ($P < 0.05$) and OT3 ($P < 0.05$) stages.

ANP induced acrosome reaction of pig spermatozoa

The motility of fresh sperm was $92.5 \pm 2.9\%$. After incubated, the motilities of sperm in control, ANP (1 nM), C-ANP-(4–23) (1 nM) and caffeine treatment were $82.5 \pm 2.9\%$, $78.8 \pm 4.8\%$, $80.0 \pm 4.1\%$ and $83.8 \pm 4.8\%$, respectively. Preincubated sperm were incubated with various concentrations of ANP for 1 h, and the proportion of spermatozoa that underwent the acrosome reaction was increased ($P < 0.01$) by incubation with 0.1 nM ANP ($26.2 \pm 2.2\%$) or 1 nM ANP ($34.1 \pm 2.3\%$) compared with untreated cells ($16.5 \pm 1.8\%$; Fig. 4). The maximal proportion of spermatozoa that underwent the acrosome reaction (~ 2.0 -fold relative to controls) was detected at 1 nM ANP, while C-ANP-(4–23) (in concentrations that ranged from 10 pM to 100 nM) and caffeine (2 mM) had no effect on the acrosome reaction (Fig. 4, Fig. 5 and data not shown).

Role of cGMP in ANP-induced acrosome reaction

Permeable cGMP analog 8-Br-cGMP was used to mimic the effect of ANP on acrosome reaction of

(B)

Porcine:	GAGCCCAGTA	GCCTTGAGAG	GCACCTCCGG	AATGCGGGCA	GCCGGCTGAC	CCTGAGTGGG	60
Human:	-----	-----	---G---	-G-A---	-----	---C---	
Mouse:	C-----C-	---G----	---T---	-GC-T---	-T-----	-----	
Rat:	C-----C-	---G----	---T-T---	-GC-T---	-----	-----	
Porcine:	AGAGGCTCCA	ATTACGGCTC	CCTGCTGACC	ACAGAGGGCC	AGTTCCAAAT	CTTGCCAAG	120
Human:	-----	-----	---A---	-----	---G-	-----	
Mouse:	C-----	---T----	---A---	-G-----	---G-	-----	
Rat:	C-----	---T----	---A---	-C-----	---G-	-----	
Porcine:	ACAGCGTATT	ATAAGGGCAA	CCTCGTGGCT	GTGAAACGTG	TCAACCGGAA	ACGCATTGAG	180
Human:	---A---	-----	-----	-----	-G---T-	-----	
Mouse:	---A-C-	-----	-----	-----	-G-----	-----	
Rat:	---A-C-	-----	---T-----	-----	-G-----	-----	
Porcine:	CTGACGCGGG	AAGTCCTGTT	TGAACTGAAG	CATATGCGGG	ATGTGCAGAA	TGAACACCTG	240
Human:	---A-AA	-----	-----	-----	-----	-----	
Mouse:	T---A-AA	-----	---T-A	-----	-----	---G-AT-	
Rat:	T---A-AA	-----	---T-A	-----	-----	---G-T-	
Porcine:	ACCAGGTTTG	TGGGGGCCCTG	CACTGATCCC	CCCAACATCT	GTATCCTCAC	GGAGTACTGT	300
Human:	-----	---A-----	---C-C---	---T---	-C-----	A-----	
Mouse:	---A---	---A-T-	T-C-C-T	-----	-----	A-----	
Rat:	--A-A---	---T-T-	T-C-C---	-----	-----	A-----	
Porcine:	CCCCGTGGGA	GCCTACAGGA	CATTCTGGAG	AATGAGAGCA	TCACGCTGGA	CTGGATGTTT	360
Human:	-----	---G-----	-----	-----	---C-----	-----	
Mouse:	-----A-	-----	---A---	-----T-	-T-C-----	-----T	
Rat:	-----A-	-----	---A---	-----T-	-C-----	-----T	
Porcine:	CGGTACTCGC	TCACCAACGA	CATCGTCAAG	GGCATGCTGT	TCCTGCACAA	TGGGGCCATT	420
Human:	-----A-	-----T-	-----	-----	-T-A-----	---T-C	
Mouse:	-----A-	-----T-	---T-----	---A---C-	-T-A-----	C-----	
Rat:	-----	-----T-	---T-----	---A---C-	-T-A-----	-----	
Porcine:	TGCTCCCATG	GGAACCTCAA	GTCATCCAAC	TGCGTGGTAG			
Human:	--T-----	-----	-----	-----			
Mouse:	--T-----	-----	-----	-----			
Rat:	--T-----	-----	-----	--T-----			

Figure 2B The expressions of ANP gene, NPRA gene and NPRC gene in pig spermatozoa. (B) Comparison of partial cDNA sequences of NPRA primers-RT-PCR with human NPRA (accession no. NM000906), mouse NPRA (accession no. NM008727), or rat NPRA (accession no. NM012613).

pig spermatozoa. As shown in Fig. 6, incubation with 8-Br-cGMP (0.1–5 mM) caused a similar effect on spermatozoa acrosome reaction as that incubated with ANP, and the maximal proportion of spermatozoa that underwent the acrosome reaction was detected at 1 mM 8-Br-cGMP ($25.7 \pm 2.1\%$).

The effect of PKG inhibitors on ANP-induced acrosome reaction

ANP interacts with the ANP receptor, with subsequent generation of cGMP in target cells. The PKG inhibitors KT5823 and Rp-8-pCPT-cGMPS were used to test the

(C)

Porcine:	GGGCAGTGAG	CGAGTGGTGA	TCATGTGTGC	GAGCAGCGAC	ACCATCCGCA	GCATCATGCT	60
Human:	—C—	—A—	—	—T—	—G—	—	
Mouse:	A—C—	—	—	C—TG—T—	—T—G—	A—T—	
Rat:	—	—	—	C—TG—T—	—T—G—	A—T—	
Porcine:	GGCGGCCAC	AGGCACGGGA	TGACCAGCGG	AGACTACGCC	TTCTTCAACA	TTGAGCTCTT	120
Human:	—T—G—	—T—C—	—T—	—	—	—	
Mouse:	—TG—	A—C—	—T—	—T—	—	A—	
Rat:	—TG—	A—C—	—T—	—T—T—	—	A—	
Porcine:	CAACAGCTCT	TCTTACGGAG	ATGGCTCATG	GAGGAGAGGA	GACAAGCACG	ACTTTGAAGC	180
Human:	—	—C—T—	—	—A—	—A—	—	
Mouse:	—T—	—C—	—G—	—	—A—	—C—	
Rat:	—T—	—	—G—	—A—G—	—A—	—	
Porcine:	TAAGCAAGCG	TACTCATCCC	TCCAAACAAT	CACTCTACTG	AGGACAGTGA	AACCTGAGTT	240
Human:	—A—	—G—	—G—G—	—	—	—	
Mouse:	A—A—	—G—	—G—	—C—	—C—	—	
Rat:	—T—	—	—G—	—C—G—	—C—C—	—G—	
Porcine:	TGAGAAGTTT	TCCATGGAGG	TGAAAAATTC	TGTTGAGAAG	CAAGGGCTCA	GTGAGGAGGA	300
Human:	—	—	—G—	A—A—	—	A—AT—	
Mouse:	—	—	—G—	—A—	—	A—	
Rat:	—	—	—G—	—A—	—	A—	
Porcine:	TTACGTGAAC	ATGTTTGTG	AAGGATTCCA	CGACGCCATC	CTCCTCTACG	TCCTGGCTTT	360
Human:	—T—	—	—	—T—	—	—T—C—	
Mouse:	—	—	—G—	T—	—	—T—	
Rat:	—	—C—	—C—	T—T—	—	—T—	
Porcine:	ACATGAAGTG	CTCAGAGCTG	GCTACAGCAA	GAAGGATGGG	G		
Human:	—A—	—	—T—	A—A—	—		
Mouse:	—A—	—	—	—	—		
Rat:	G—	—	—	—	—		

Figure 2C The expressions of ANP gene, NPRA gene and NPRC gene in pig spermatozoa. (C) Comparison of partial cDNA sequences of NPRC primers-RT-PCR with human NPRC (accession no. NM000908), mouse NPRC (accession no. AF131864), or rat NPRC (accession no. NM012868).

involvement of PKG pathway in ANP-induced acrosome reaction. The possible effect of PKA pathway in the high concentration of 8-Br-cGMP (1 mM) was also studied by a PKA inhibitor H89. As shown in Fig. 7, both KT5823 (1 μ M) and Rp-8-pCPT-cGMPs (0.5 μ M) could completely abolish ANP-induced acrosomal exocytosis of pig spermatozoa, but each alone had no effect on the acrosome reaction. On the other hand, H89 (0.05 μ M) could not block the effect of 8-Br-cGMP on the acrosome reaction.

The effect of ANP on the capacitation

The possible effect of ANP on the capacitation was studied by lysophosphatidylcholine. Compared with control, incubation of lysophosphatidylcholine for 15 min had a significant effect on the percentage of sperm that were acrosome-reacted ($P < 0.05$, Fig. 8). However, lysophosphatidylcholine had no obvious effect on the acrosome reaction when sperm were preincubated with ANP. Addition of 100 μ g/ml lysophosphatidylcholine for

Figure 3 Immunodetection of ANP in pig spermatozoa and oviduct. The intensity of the amplified bands was quantified by densitometric scanning. Autoradiogram reflects a representative experiment and bar graphs represent the mean of three independent experiments. The respective value of either ANP was normalized according to those of beta-actin to evaluate arbitrary units of the relative abundance of the targets. Values in different oviduct stages with different superscripts differ significantly ($P < 0.05$). The level of ANP was not detected in spermatozoa.

15 min alone had no effect on the percentage of sperm that were motile or acrosome-reacted (data not shown).

ANP induces fertilising ability of pig spermatozoa

As shown in Table 2, the presence of ANP or caffeine in insemination medium resulted in a higher proportion ($P < 0.05$) of oocytes penetrated and polyspermy, and also resulted in a higher ($P < 0.05$) average number of spermatozoa per penetrated oocyte. The effect of ANP was completely inhibited by KT5823, but KT5823 alone had no effect on these fertilisation parameters. There were no differences between ANP and caffeine treatments in the proportion of oocytes penetrated and polyspermy, the average number of sperm per penetrated oocytes, cleavage rate, blastocyst formation rate or the number of cells per blastocyst (Table 2, Table 3, Fig. 9).

Discussion

It has been reported that the sperm not only contribute the haploid male genome, but also deliver messenger RNAs to the oocyte at fertilisation (Ostermeier *et al.* 2004), suggesting that the messenger RNAs of the sperm may play an important role in fertilisation (Ostermeier *et al.* 2002). In the present study, we examined the

Figure 4 Effects of ANP, caffeine and C-ANP-(4-23) on the acrosome reaction. Pig semen were washed twice with mTBM medium containing 5 mg/ml BSA and then incubated in this medium for 2.5 h. Sperm (1×10^8 /tube) were then washed again and incubated at 37 °C for 60 min with increasing concentrations of ANP, caffeine or C-ANP-(4-23). The acrosome reaction was determined by triple-stain method. Results are means $\pm$ S.E.M. of three experiments, with at least 400 spermatozoa counted per replicate ($n = 3$). ** $P < 0.01$; *** $P < 0.001$ versus control.

Figure 5 Image of ANP-induced acrosomal sperm status. Preincubated sperm were further incubated with 1 nM ANP 60 min, and the acrosomal sperm status was determined by triple-stain method. Arrow points to the acrosome-reacted sperm with white acrosomal region. Bar = 10 μ m.

expressions of the ANP gene, the NPRA gene and the NPRC gene, and only NPRA mRNA was detected in pig spermatozoa. Western blot analysis further indicated that ANP protein did not exist in the spermatozoa. Our results are consistent with previous reports that a single class of ANP receptors (NPR A) is detected in human spermatozoa (Silvestroni *et al.* 1992, Willipinski-Stapelfeldt *et al.* 2004), and that ANP-induced acrosome

Figure 6 Effect of 8-Br-cGMP on the acrosome reaction. Pig spermatozoa were preincubated and then further incubated with increasing doses of permeable cGMP analog 8-Br-cGMP for 60 min. The acrosome reaction was determined by triple-stain method. Results are means $\pm$ S.E.M. of three experiments, with at least 400 spermatozoa counted per replicate ($n=3$). * $P<0.05$; ** $P<0.01$ versus control.

reaction via the elevation of cGMP could be completely inhibited by the selective NPRA antagonist anantin (Anderson *et al.* 1994, Rotem *et al.* 1998). We also examined the changes of the ANP protein, ANP mRNA, NPRA mRNA and NPRC mRNA in the oviduct. The oviduct was divided into five phases by the follicular characterization in the ovary, which may present the different stages of estrous cycle. The data revealed that the levels of both ANP mRNA and ANP protein were obviously high in OT1, consistent with a previous report that the concentration of ANP in rat oviduct is significantly elevated at the diestrus stage of the estrous cycle (Kim *et al.* 1997). The level of ANP protein in OT3 was lower than that in OT1, but was higher than that in OT4 and OT5. Moreover, the level of ANP mRNA was dramatically increased in OT3. The expression of ANP in OT3 may play a physiological role in spermatozoa function, since the spermatozoa displayed NPR A gene expression but not the ANP gene expression. On the other hand, the expression of ANP gene may also be involved in the regulation of oviductal motility (Kim *et al.* 1997). The motility may be regulated by both ANP and NPR A, since we also detected the changes of ANP mRNA and NPR A mRNA among the different oviductal phases. The low levels of NPRC mRNA in the oviduct throughout estrous cycle are consistent with a previous report that ANP bindings in rat oviduct are not reversed by C-ANP-(4–23), that specifically binds to NPRC (Kim *et al.* 1997).

Figure 7 Effect of PKG inhibitors on an ANP-induced acrosome reaction. Pig spermatozoa were preincubated and then further incubated with ANP (1 nM), KT5823 (1 μ M), and/or Rp-8-pCPT-cGMPs (0.5 μ M) for 60 min. The acrosome reaction was determined by triple-stain method. The possible effect of PKA pathway in the high concentration of 8-Br-cGMP (1 mM) treatment was also studied by PKA inhibitor H89 (0.05 μ M). Results are means $\pm$ S.E.M. of three experiments, with at least 400 spermatozoa counted per replicate ($n=3$). ** $P<0.01$; *** $P<0.001$ versus control. KT, KT5823; Rp, Rp-8-pCPT-cGMPs; cGMP, 8-Br-cGMP.

The acrosome reaction enables the sperm cell to penetrate the zona pellucida (ZP) and to fuse with the egg's plasma membrane (Wassarman 1987, Yanagimachi 1994), which is essential for fertilisation. Therefore, elucidation of the mechanisms that control the acrosome reaction is important for understanding fertilisation. Multiple physiological agonists probably participate in the regulation of the acrosome reaction (Joyce *et al.* 1987, Wassarman 1987, Anderson *et al.* 1994, 1995, Roldan *et al.* 1994, Yanagimachi 1994, Zamir *et al.* 1995). ANP could serve as such a physiological agonist. In our study, ANP significantly induced acrosome reaction of preincubated pig spermatozoa; this effect was mimicked by 8-Br-cGMP but abolished by the PKG inhibitors KT5823 and Rp-8-pCPT-cGMPs. However, C-ANP-(4–23), that specifically binds to NPRC (Bovy *et al.* 1989), had no effect on the acrosome reaction, suggesting that the function of ANP on the acrosomal reaction may not be through the NPRC receptors. It is reported that ANP shows high affinity for natriuretic peptide receptors-A (NPRA) and NPRC receptors in various animals (for review, see Takei 2000), and induces the acrosome reaction of capacitated human spermatozoa by NPRA receptors and elevation of cGMP (Anderson *et al.* 1994, Rotem *et al.* 1998). We also showed that only NPRA mRNA was detected in pig spermatozoa. All these results indicate that the ANP-induced acrosome reaction of pig spermatozoa occurs via the NPRA/cGMP/PKG pathway. In contrast, it is shown

Figure 8 Effect of ANP on the capacitation. Pig sperm were washed twice and resuspended in mTBM medium containing 5 mg/ml BSA, and/or 1 nM ANP for 60 min at 37 °C. At the end of incubation, sperm were exposed to 0 or 100 μ g/ml lysophosphatidylcholine (LC) for a further 15 min. The acrosome reaction was determined by triple-stain method. Results are means $\pm$ S.E.M. of three experiments, with at least 400 spermatozoa counted per replicate ($n=3$). * $P<0.05$ versus control.

Table 2 Effect of caffeine, ANP and/or KT5823 on fertilisation parameters of pig oocytes inseminated in mTBM medium

Treatment	n	Oocytes penetrated		Polyspermy† (%)	No. sperm/ penetrated oocyte
		Total (%)	With MPN (%)		
Control‡	124	33.4 ± 2.6 ^b	79.3 ± 2.8	22.4 ± 1.5 ^b	1.13 ± 0.14 ^b
Caffeine§	121	72.2 ± 5.0 ^a	84.2 ± 3.4	59.6 ± 6.8 ^a	2.12 ± 0.35 ^a
ANP§	118	75.2 ± 5.3 ^a	83.6 ± 2.3	62.1 ± 7.8 ^a	2.24 ± 0.31 ^a
KT5823§	106	35.5 ± 3.7 ^b	78.6 ± 3.1	24.5 ± 2.7 ^b	1.06 ± 0.18 ^b
ANP+KT5823§	110	38.6 ± 4.1 ^b	81.4 ± 3.7	26.3 ± 3.7 ^b	1.31 ± 0.22 ^b

Values with different superscripts (a, b) within column differ significantly ($P<0.05$). MPN, male pronucleus. Experiments were repeated four times.
†Percentage of oocytes penetrated.
‡Sperm were preincubated in mTBM containing 5 mg/ml BSA for 90 min, and then 50 µl of the sperm sample was added to the same fertilisation droplets containing the oocytes.
§Sperm were preincubated in mTBM containing 5 mg/ml BSA supplemented with 2 mM caffeine, 1 nM ANP and/or 1 µM KT5823 for 90 min, and then 50 µl of the sperm sample was added to the same fertilisation droplets containing the oocytes.

that PKG I is absent in human sperm, and the effect of high concentration cGMP may be explained by a cross-activation of PKA (Willipinski-Stapelfeldt *et al.* 2004). However, H89, a PKA inhibitor, could not block cGMP-induced acrosome reaction in our study. The absence or presence of PKG I in pig sperm needs further study. It is also possible that other signal transduction pathways (such as cGMP-gated ion channels) might be involved in this process (Willipinski-Stapelfeldt *et al.* 2004). The optimal concentration of ANP for induction of the acrosome reaction in pig spermatozoa in the present study was consistent with dissociation constants obtained for ANP binding to human spermatozoa (Silvestroni *et al.* 1992) and also with concentrations in follicular fluids (Anderson *et al.* 1994), suggesting a physiological role for ANP in the regulation of the acrosome reaction.

Fresh pig sperm were used for the fertilisation experiments, as in the acrosome study. It has been reported that the treatment of spermatozoa with caffeine results in

increased *in vitro* penetration rates of bovine (Niwa & Ohgoda 1988) and porcine (Wang *et al.* 1991, Zhao *et al.* 2002) oocytes, and also results in a higher proportion of fertilisation (Parrish *et al.* 1986, Niwa & Ohgoda 1988). However, the incidence of sperm penetration was either low or nil in mTBM medium without caffeine (Abeydeera & Day 1997b, Zhao *et al.* 2002). ANP had a similar effect on the proportion of oocytes penetrated and fertilisation as that of caffeine, suggesting that ANP may play a physiological role in fertilisation. The effect of ANP on IVF might occur via the PKG pathway, since the parameters of ANP-induced fertilisation could be completely reversed by KT5823.

The acrosome reaction is absolutely required for fertilisation. Before sperm can penetrate the egg, they are subjected to physiological alteration (capacitation) in the female reproductive tract. Capacitation prepares the sperm cell for binding to the egg and exocytosis (acrosome reaction). It is becoming increasingly clear that the

Table 3 Development of pig oocytes inseminated with caffeine or ANP preincubated spermatozoa

Treatment	No. oocytes	Cleavage rate (%)	Blastocyst rate (%)	No. cells/ blastocyst†
Control	374	14.8 ± 5.7	0	—
Caffeine‡	325	62.7 ± 4.6	25.8 ± 2.6	35.6 ± 1.9
ANP§	346	60.2 ± 5.1	26.7 ± 3.2	38.4 ± 2.3
KT5823	258	16.4 ± 5.3	0	—
ANP+KT5823	236	19.7 ± 4.2	0	—

Values are mean ± S.E.M. of four replicates and are not significantly different between caffeine and ANP groups ($P>0.05$).
†Number of blastocysts counted: caffeine, 134; ANP, 138.
‡Sperm were preincubated in mTBM containing 5 mg/ml BSA and 2 mM caffeine for 90 min, and then 50 µl of the sperm sample was added to the same fertilisation droplets containing the oocytes.
§Sperm were preincubated in mTBM containing 5 mg/ml BSA and 1 nM ANP for 90 min, and then 50 µl of the sperm sample was added to the same fertilisation droplets containing the oocytes.

Figure 9 Image of blastocyst obtained from pig oocytes inseminated with ANP treated sperm. (A) Overall picture of blastocysts formed in a culture well 6 days after IVF. Note variation in morphology and size of apparent blastocysts. Bar=100 μ m. (B) Enlarged image of a blastocysts. Bar=100 μ m. (C) Fluorescent image of a blastocyst stained with Hoechst 33342. Bar=100 μ m.

acrosome reaction is normally restrained until oocyte ligands bind to sperm receptors. Caffeine has been known to enhance and prolong the motility of bull and boar spermatozoa (Garbers *et al.* 1971, 1973) and accelerate the rate of capacitation by precociously increasing the concentration of cAMP (Fraser 1979), but had no effect on the acrosome reaction in our study. Sperm treated with caffeine may reach and bind to the ZP by hyperactivated motility, and then receive a signal that causes the acrosome react (Yanagimachi 1994). Recent evidence suggests that sperm may initiate the acrosome reaction during capacitation which could lead to exposure of acrosomal protein(s) (e.g., sp56) that permit binding to ZP3 and fertilisation (Wassarman *et al.* 2001, Primakoff & Myles 2002). Although ANP had a similar effect on improving the fertilising ability of pig spermatozoa to that of caffeine, different mechanisms may exist during this process. In the present study, an ANP-induced acrosome reaction (not the capacitation) may have contributed to the higher proportion of oocytes that are penetrated and fertilized, in previous studies, chondroitin sulfate and heparin could facilitate bovine IVF by inducing the acrosome reaction (Lenz *et al.* 1983, Parrish *et al.* 1986). Although the majority of sperm that bind to the ZP are acrosome-intact regardless of the percentage of acrosome-reacted sperm in suspension (Fazeli *et al.* 1997), sperm attachment to the zona does not require capacitation and is not a functional indicator of zona penetration (Lynham & Harrison 1998). On the other hand, ANP can induce attraction (chemotaxis) and enhance swimming speed (chemokinesis) in human spermatozoa *in vitro* (Zamir *et al.* 1993, Anderson *et al.* 1995), which might also be involved in this process.

It is reported that high-affinity NPRA (particulate guanylyl cyclase receptors) has been particularly localized

in human spermatozoa (Silvestroni *et al.* 1992), and that ANP is synthesized in the mucosal layer of the oviduct in rat, and the concentration of oviductal ANP begins increase at the estrus and metestrus stage of estrous cycle (Kim *et al.* 1997). In our study, the high levels of ANP gene were expressed at OT3 stage (the large follicle stage), and the expression of NPRA gene was detected in pig spermatozoa. All these results suggest that the locally synthesized ANP may serve as a physiological agonist in the regulation of the acrosome reaction and fertilisation. Elucidation of the mechanism of ANP-induced acrosome reaction and the fertilising ability would provide further understanding of this fundamental biological process, and generate clinical advances for treatment of infertility. In addition, the prevention of spermatozoa acrosome reaction induced by ANP may become an exciting new approach to contraception.

In conclusion, the ANP mRNA and protein is expressed in the oviduct, but only NPRA gene is detected in pig spermatozoa. ANP induces acrosome reaction in preincubated spermatozoa, apparently via a PKG pathway. Furthermore, ANP also enhances the fertilising ability of spermatozoa. These results imply that ANP, which is locally produced in the oviduct, may serve as a physiological agonist in the regulation of fertilisation in the intact animal.

Acknowledgements

This research was supported by grants for National Outstanding Youth foundation of China (No. 30025032), National Natural Science Foundation of China (No. 30470868) and Beijing Natural Science Foundation (No.

5052017). The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 13 January 2006

Accepted 25 January 2006

Made available online as an Accepted Preprint
15 February 2006