Atrial natriuretic peptide induces acrosomal exocytosis of human spermatozoa

RONIT ROTEM, NADAV ZAMIR, NURIT KEYNAN, DALIT BARKAN, HAIM BREITBART, AND ZVI NAOR. Atrial natriuretic peptide induces acrosomal exocytosis of human spermatozoa. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E218–E223, 1998.—Acrosomal exocytosis in mammalian spermatozoa is a process essential for fertilization. We report here that atrial natriuretic peptide (ANP) markedly stimulates acrosomal exocytosis of capacitated human spermatozoa. Typically, ANP exerts some of its actions via activation of the ANP receptor (ANPR-A), a particulate guanylyl cyclase-linked receptor, and subsequent formation of guanosine 3′,5′- cyclic monophosphate (cGMP). We found that ANP-stimulated acrosome reaction was inhibited by the competitive ANPR-A antagonist anantin, indicating a receptor-mediated process. A linear fragment of ANP, ANP-(13—28), and another ANP-like compound, brain natriuretic peptide, were inactive. The stimulatory effect of ANP on acrosome reaction was mimicked by the permeable GMP analog, 8-bromo-cGMP (8-Br-cGMP). Addition of the protein kinase C (PKC) inhibitors, staurosporine and GF-109203X, resulted in a dose-related inhibition of ANP-induced acrosome reaction. Also, downregulation of endogenous PKC activity resulted in inhibition of ANP- but not 8-Br-cGMP-induced acrosome reaction. Removal of extracellular Ca2+ abolished ANP-induced acrosome reaction. Thus ANP via Ca2+ influx, PKC activation, and stimulation of particulate guanylyl cyclase may play a role in the induction of acrosomal reaction of human spermatozoa.

Acrosomal reaction; guanosine 3′,5′- cyclic monophosphate; calcium; protein kinase C

AT THE TIME OF FERTILIZATION, capacitated mammalian spermatozoa undergo an exocytotic process termed acrosome reaction (28, 30). The acrosome reaction involves fusion between the outer acrosomal membrane and the underlying plasma membrane, leading to the exposure and release of acrosomal hydrolytic enzymes (28, 30). The acrosome reaction enables the sperm cell to penetrate the zona pellucida and to fuse with the egg's plasma membrane (28, 30). The acrosome reaction is therefore a prerequisite for successful fertilization. Thus elucidation of the mechanism regulating acrosome reaction is important for understanding human fertilization.

Several lines of evidence suggest that agonists derived from the egg's extracellular coat or zona pellucida or constituents of the female reproductive tract may trigger the acrosome reaction of capacitated spermatozoa in a receptor-mediated mechanism (30). Atrial natriuretic peptide (ANP) may be a candidate for induction of the acrosome reaction. ANP was detected in rat ovaries (8, 13, 14), oocytes (15), and follicular fluids (2, 24, 25). In addition, high-affinity binding sites for ANP were localized in human spermatozoa (23). We have previously demonstrated that ANP-induced chemotaxis and chemotaxis of capacitated human spermatozoa (32). More recently, it was reported that ANP induces an acrosome reaction of human (2, 5) and bovine spermatozoa (31), but only guanosine 3′,5′- cyclic monophosphate (cGMP) was implicated as a mediator in ANP action (2, 31). The present study was undertaken to further elucidate the mechanism underlying ANP-induced acrosome reaction of capacitated human spermatozoa in vitro. We have recently demonstrated the presence of protein kinase C (PKC) in mammalian sperm and its possible involvement in sperm motility and acrosome reaction (6, 11, 16, 20–22). We therefore examined here the possible involvement of PKC in ANP-induced acrosome reaction. We propose that ANP-induced acrosome reaction is mediated by Ca2+, PKC, and cGMP.

MATERIALS AND METHODS

ANP-(1—28) (human), ANP-(5—28) (human, rat), ANP-(13—28) (rat), and porcine brain natriuretic peptide (BNP) were purchased from Peninsula Laboratories (Belmont, CA). A-23187, 8-bromo-cGMP (8-Br-cGMP), 12-O-tetradecanoylphorbol 13-acetate (TPA), and rose bengal were purchased from Sigma Chemical (St. Louis, MO). Bismark brown was from Searle Diagnostics. Staurosporine was purchased from Kyowa Medix (Tokyo, Japan). Trypan blue was purchased from Fluka (Switzerland) and ananatin from Bachem (Bubendorf, Switzerland). GF-109203X was purchased from Calbiochem (La Jolla, CA).

Preparation of spermatozoa. Human sperm were obtained from fresh ejaculates of healthy donors (22–30 yr old) after 72 h of abstinence. Samples were allowed to liquefy at room temperature for 30–60 min. Sperm cells were then washed twice (750 g for 10 min) with Ham's F-10 medium containing 0.5% of human serum albumin (HSA) and incubated for 2.5 h at a temperature of 35°C for capacitation.

Acrosomal reaction evaluation. Capacitated sperm cells (5 × 106 cells/aliquot) were incubated for 60 min with the various hormones and/or drugs at 35°C. Acrosomal sperm status was analyzed by the triple-stain technique, as described by Talbot and Chacon (26). Sperm were resuspended in Ham's F-10 medium without HSA, containing trypan blue (1% in phosphate-buffered saline), for 15 min. Samples were centrifuged (750 g for 5 min) and washed with saline until the stain disappeared. Sperm were then fixed in glutaraldehyde (3%, in cacodylate buffer) for 30 min at room temperature and later washed twice with distilled deionized H2O (ddH2O), resuspended in 50 µl of ddH2O, and pipetted onto a microscope glass slide. The air-dried slides were incubated in bismark brown (0.8%) for 8 min at 40°C. Slides were then washed twice with ddH2O and mounted with 50% glycerol in a drop of Eukitt (La Jolla, CA).
washed in ddH₂O to remove excess stain and incubated in rose bengal (0.8%) for 25 min at room temperature. Slides were washed to remove excess stain, passed twice through absolute ethanol alcohol dehydration, and cleared twice in xylene (100%). Sperm were then examined by light microscopy under oil immersion to follow the acrosome status (22).

RESULTS

Induction of acrosome reaction by ANP. The acrosome reaction was detected at the light-microscopic level using the triple-stain technique. Addition of human ANP-(5—28) for 60 min caused significant enhancements of acrosome reaction of capacitated human spermatozoa compared with untreated cells (Fig. 1). Maximal response of acrosome reaction (~2.5-fold) was detected at 1 nM ANP. Human ANP-(1—28), human ANP-(5—28), and rat ANP-(5—28) showed similar activities. In further experiments, we utilized human ANP-(5—28).

The percentage of the acrosome-reacted cells induced by 1 nM ANP was 60–70% of that induced by 2 µM of the Ca²⁺ ionophore A-23187 (data not shown).

A linear fragment of ANP, rat ANP-(13—28), had no effect on acrosome reaction, indicating that the 17-member disulfide ring is essential for its biological activity (Fig. 2). Porcine BNP, another ANP-like compound derived from a different gene (7), had no effect on the human acrosome reaction at concentrations up to 100 nM (Fig. 2 and data not shown).

Role of ANP receptor activation in ANP-induced acrosome reaction. ANP exerts many of its actions through interaction with the ANP receptor (ANPR-A), a particulate guanyl cyclase-linked receptor, and subsequent generation of cGMP in target cells. The involvement of ANPR-A in ANP-induced acrosome reaction was tested by a selective ANPR-A antagonist, anantin (17, 29). We found that anantin at 100 nM completely abolished ANP-induced acrosomal exocytosis of capacitated human spermatozoon (Fig. 3). The dose of anantin used here is based on previous observations using bovine sperm cells (31). Anantin by itself had no effect on acrosomal exocytosis or sperm motility.

Role of cGMP in ANP-induced acrosome reaction. Because ANPR-A is a guanylyl cyclase-linked receptor, it was of interest to analyze the role of cGMP in ANP action. Indeed, Anderson et al. (2) have recently demonstrated elevation of cGMP by ANP in human sperm, and we found similar results in bovine sperm (31). Furthermore, we were able to mimic the stimulatory effect of ANP on the acrosome reaction in capacitated human spermatozoa by using a membrane-permeable analog of cGMP, 8-Br-cGMP. Indeed, the cGMP analog caused a similar elevation in acrosome reaction to that

\[ \text{Fig. 1. Dose response for effect of atrial natriuretic peptide (ANP) on human sperm acrosome reaction. Human semen was washed twice with Ham's F-10 medium containing human serum albumin (HSA, 0.5%) and further incubated in above medium for capacitation for 2.5 h. Sperm (5} \times 10^8/\text{tube}) \text{were then washed again and incubated for 60 min with increasing concentrations of human ANP-(5—28) in above medium at 35°C. Acrosome reaction was determined by triple-stain method. In this and subsequent figures, results are means ± SE of 3 experiments, each done in triplicate, and statistical symbols are as follows: *P < 0.05; **P < 0.01; ***P < 0.001 vs. control in paired Student's t-test.} \]

\[ \text{Fig. 2. Effect of ANP-like compounds on acrosome reaction in human spermatozoa. Capacitated human sperm as above were incubated with ANP [human ANP-(5—28)], porcine brain natriuretic peptide (BNP), or rat ANP-(13—28), each at 1 nM for 60 min. C, control. Other details as above (see Fig. 1).} \]

\[ \text{Fig. 3. Effect of ANP receptor antagonist (ANPR-A) anantin on ANP-induced acrosome reaction in human spermatozoa. Capacitated human sperm as above were incubated with ANP (1 nM), anantin (an, 100 nM), or both for 60 min, and acrosome reaction was determined. For other details see Fig. 1.} \]
obtained with ANP (Fig. 4). These results support the notion that ANP-induced acrosome reaction is mediated via activation of the guanylyl cyclase-linked receptor, ANPR-A, and subsequent formation of cGMP.

Role of PKC in ANP stimulation of acrosome reaction. To evaluate the role of PKC in ANP action, we employed inhibition and depletion of PKC. Incubation of the cells with increasing concentrations of the PKC inhibitors staurosporine and GF-109203X resulted in a dose-related inhibition of ANP-induced acrosome reaction (Figs. 5 and 6). In another approach, we employed PKC-downregulated cells that were obtained by preincubation with TPA (22). Indeed, incubation of noncapacitated human sperm with TPA (500 ng/ml for 3 h) reduced endogenous PKC enzymatic activity from 0.25 pmol 32P·min⁻¹·µg protein⁻¹ to undetectable levels. ANP-induced acrosome reaction was abolished in the downregulated cells, supporting the inhibition experiments (Fig. 7). On the other hand, stimulation of acrosome reaction by 8-BrcGMP was only slightly affected by downregulation of endogenous PKC (Fig. 8). The results suggest that cGMP acts downstream, or in parallel to PKC during ANP action.

Role of Ca²⁺ in ANP-induced acrosome reaction. Acrosomal exocytosis in capacitated human spermatozoa is believed to be a Ca²⁺-dependent process (30). We therefore tested the ANP-induced acrosome reaction to see whether it is Ca²⁺ dependent. We incubated capacitated human spermatozoa in Ca²⁺-free medium with or without ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). Removal of Ca²⁺ alone was not sufficient to block ANP action. On the

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Fig. 4. Effect of 8-bromo-cGMP (8-BrcGMP) on acrosome reaction in human spermatozoa. Capacitated human sperm as above were incubated with increasing doses of permeable cGMP analog 8-BrcGMP for 60 min, and acrosome reaction was determined. For other details see legend to Fig. 1.

Fig. 5. Effect of protein kinase C (PKC) inhibitor staurosporine on ANP-induced acrosome reaction of human spermatozoa. Capacitated human sperm as above were incubated with increasing doses of staurosporine for 5 min followed by ANP (1 nM) for 60 min, and acrosome reaction was determined. For other details see Fig. 1.

Fig. 6. Effect of PKC inhibitor GF-109203X on ANP-induced acrosome reaction of human spermatozoa. Capacitated human sperm as above were incubated with increasing doses of GF-109203X (GF) for 5 min followed by ANP (1 nM) for 60 min, and acrosome reaction was determined. For other details see Fig. 1.

Fig. 7. Effect of downregulation of PKC on ANP-induced acrosome reaction. Noncapacitated human sperm were preincubated with or without 12-O-tetradecanoylphorbol 13-acetate (TPA, 500 ng/ml for 3 h) to achieve downregulation of endogenous PKC (DR). Sperm were then washed and further incubated with or without ANP (1 nM) for 60 min, and acrosome reaction was determined. For other details see legend to Fig. 1.
other hand, removal of Ca\textsuperscript{2+} and addition of EGTA abolished ANP-induced acrosome reaction (Fig 9). The results indicate that ANP-induced acrosomal exocytosis requires extracellular Ca\textsuperscript{2+} influx.

**DISCUSSION**

Multiple physiological agonists probably participate in the regulation of acrosomal exocytosis (2, 3, 10, 19, 28, 30, 31). Such agonists may originate from the egg or its associated cellular and acellular structures or from the female reproductive tract.

ANP could serve as such a physiological agonist. It is synthesized in granulosa cells (14) and oocytes (15) of mammalian ovaries (8, 13, 14) and secreted into the follicular fluids. Indeed, ANP is found in the follicular fluids of human and other species (2, 24, 25). ANP was reported to play a role in the development of ovarian follicles (27), in steroidogenesis in ovarian granulosa cells (14), and in the process of oocyte maturation (27). Cyclic changes in ovarian ANP levels were observed during the estrous cycle of the rat (13). ANP derived from the ovary may therefore act on spermatozoa and affect their function. Indeed, high-affinity ANPR-A (particulate guanylyl cyclase) has been localized in human spermatozoa (23). Thus ANP derived from granulosa cells and/or oocytes may act on spermatozoa before and during fertilization. ANP-stimulatory actions on mammalian spermatozoa have been reported in recent studies (2, 5, 31, 32). We have demonstrated that ANP-induced attraction (chemotaxis) and enhanced swimming speed (chemokinesis) in human spermatozoa in vitro (32). ANP was also reported to induce acrosome reaction both in human (2, 5) and bull spermatozoa (31), apparently by cGMP elevation (2, 31). The results of the present study further elucidate the mechanisms underlying ANP-induced acrosome reaction of capacitated human spermatozoa. Induction of acrosomal exocytosis of capacitated human spermatozoa by ANP is in good agreement with dissociation constants obtained for ANP binding to different somatic cell types (7) and human spermatozoa (23) and also with the levels observed in follicular fluids (2). These observations support a putative physiological role for the hormone in the regulation of acrosomal exocytosis. ANP may also contribute to successful fertilization, since Anderson et al. (2) have found that ANP levels in human follicular fluids are correlated with successful in vitro fertilization. The effect of ANP on human spermatozoa is specific and mediated by plasma membrane ANPR-A as evident by inhibition of ANP action by the selective ANPR-A antagonist, anantin.

ANP induces acrosome reaction of human sperm, apparently by Ca\textsuperscript{2+} influx, PKC activation, and cGMP formation. Removal of extracellular Ca\textsuperscript{2+} had no inhibitory effect on ANP action (2). This has led Anderson et al. (2) to suggest that ANP does not require extracellular Ca\textsuperscript{2+} to exert its stimulatory response on acrosome reaction. We investigated the role of Ca\textsuperscript{2+} further and demonstrate here inhibition of ANP action by removal of extracellular Ca\textsuperscript{2+} and addition of EGTA to chelate residual Ca\textsuperscript{2+}. We therefore suggest that ANP-induced acrosome reaction is dependent on extracellular Ca\textsuperscript{2+} influx by ANP.

We also found that PKC is involved in ANP-induced acrosome reaction. The PKC inhibitors, staurosporine and the more selective drug, GF-109203X, produced a dose-related inhibition of ANP action. Furthermore, downregulation of endogenous PKC by preincubation with TPA also resulted in inhibition of ANP action. Because previous studies have shown that PKC-induced acrosome reaction does not depend on extra- or intracellular Ca\textsuperscript{2+} levels (22), we propose that PKC acts downstream to Ca\textsuperscript{2+} during ANP action.

The interaction of ANP with the ANPR-A results in cGMP elevation in bovine and human sperm (2, 31). Indeed, cGMP seems to be involved in ANP action, since the addition of the permeable analog, 8-Br cGMP, stimulated acrosome reaction (present results). Further-
more, a particulate guanylyl cyclase inhibitor, LY-83583, was found capable of inhibition of ANP action on human sperm acrosome reaction (2). The results are in agreement with our findings that anantin blocked the effect of ANP. To analyze the site of cGMP action in relation to PKC, we used downregulated cells. As mentioned above, downregulation of endogenous PKC abolished the effect of ANP but had no significant effect on 8-BrcGMP. The results suggest that cGMP acts independent to or downstream to PKC during ANP action on human sperm acrosome reaction.

Our proposed signaling for ANP action seems to differ from that observed in renal glomerular cells, in which ANP was reported to antagonize the PKC signal (4). Nevertheless, ANP was reported to stimulate phosphoinositide turnover (1, 9). In this case, Ca²⁺ might be activated via ANPR-C, whereas cGMP is thought to inhibit phosphoinositide turnover (1). In addition, downregulation of PKC did not block 8-BrcGMP-induced acrosome reaction, indicating that cGMP acts downstream or in parallel to PKC.

Our results suggest that ANP may join other sperm ligands, such as zona pellucida glycoproteins (e.g., ZP-3) and progesterone, in eliciting acrosome reaction of human sperm.

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