Vol. 197, No. 1, 1993
November 30, 1993
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 116-122

ATRIAL NATRIURETIC PEPTIDE ATTRACTS HUMAN SPERMATOZOA *IN VITRO*

Nadav Zamir*, Rivka Riven-Kreitman*, Mira Manor†, Amnon Makler‡, Shmaryahu Blumberg¶, Dina Ralt† and Michael Eisenbach†

*Department of Physiology and Pharmacology, Sackler Faculty of Medicine, Tel Aviv University, 69978 Tel-Aviv, Israel

†Department of Membrane Research and Biophysics, The Weizmann Institute of Science, 76100 Rehovot, Israel

‡Infertility Institute, Rambam Medical Center, Faculty of Medicine, Technion, Haifa, Israel

¶Sackler Institute of Molecular Medicine, Sackler Faculty of Medicine, Tel-Aviv University, 69978 Tel-Aviv, Israel

Received September 21, 1993

SUMMARY: Here we report that atrial natriuretic peptide (ANP), a known activator of particulate guanylate cyclase, induces attraction and swimming speed enhancement of human spermatozoa *in vitro*. Using capillary assays under a variety of experimental conditions (ascending or descending gradients of ANP, or no gradient at all) and microscopic assays in which individual spermatozoa could be followed, we found that spermatozoa followed the gradient of ANP and accumulated in it. Speed enhancement was detected in the presence of ANP without a gradient. These observations suggest either that an ANP-like substance is the physiological attractant for human spermatozoa, or, more likely, that ANP directly affects guanylate cyclase in a manner similar to that caused by the physiological attractant.

Sperm chemotaxis to egg (or follicle) secretions is widespread, from metazoa [(1, 2) for reviews] to humans [(3); (4) for review]. In sea invertebrates (where the fertilization is external), most attractants are proteins or peptides (1, 2). For example, resact, a 14-amino acid long peptide isolated from eggs of the sea urchin *Arbacia punctulata*, causes both receptor-mediated chemotaxis (i.e., movement directed by chemical stimuli) and chemokinesis (i.e., enhancement of swimming speed by a chemical) under appropriate *in vitro* conditions (5, 6). The particulate guanylate cyclase serves as a receptor for resact (2, 7). In humans, the attractant(s) has not yet been identified; however, it is probably one (or more) of the constituents of follicular fluid (3). If, however, conservation of signal transduction mechanisms exists across the phyla in the process of fertilization, then activation of particulate guanylate cyclase in mammalian spermatozoa may provide a clue to the mechanism controlling sperm chemotaxis.

Abbreviations: ANP, atrial natriuretic peptide; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid).

0006-291X/93 $4.00

Atrial natriuretic peptide (ANP), a polypeptide hormone, synthesized, stored and secreted from a variety of cell types in mammals (8-11), exerts many of its actions via activation of particulate guanylate cyclase (12-14). The presence of ANP in human follicular fluids (15, 16) and the existence of specific ANP receptors on human spermatozoa (17) may suggest a role for ANP prior to or during fertilization.

The aim of this study was to test the effects of ANP on the locomotive behavior of human spermatozoa *in vitro*. Our observations suggest that ANP induces chemotaxis and chemokinesis of human spermatozoa.

MATERIALS AND METHODS

Preparation of spermatozoa. Human ejaculates were collected by masturbation from normal healthy donors. Each ejaculate was allowed to liquefy at room temperature and then washed twice (using centrifugation at 120 g for 30 min) with Biggers, Whitten and Whittingham medium (18) supplemented with HEPES (10 mM, pH 7.4) and 0.1% polyvinylpyrrolidone 40 (M_r 40,000, Sigma) to reduce sperm adsorption. This solution is denoted below as "buffer". The spermatozoa were resuspended in this buffer to a concentration of $1\text{-}2\text{x}10^7$ cells/ml (for capillary assays) or $1\text{-}5\text{x}10^8$ cells/ml (for microscopic assays) and incubated for 2 h at 37°C under 5% CO_2.

Microscopic assay. Microscopic assays were carried out in a sealed chamber with a depth of 10 μm, thus confining the ability of the spermatozoa to swim in only two dimensions within the focal plane of a microscope throughout the observation period (19). The behavior of the spermatozoa in the chamber was recorded on video and then analyzed (3). The video recording commenced within 1 min after the chamber had been sealed and continued for 15 min.

Capillary assay. Capillary assays were carried out in a system consisting of a series of Teflon wells and polyethylene tubes (Intramedic, Parsippany, N.J., PE-50, I.D. 0.058 mm, denoted below as "capillaries") (3). The wells were filled with 100 μl of spermatozoa at a concentration of $1\text{-}2\text{x}10^7$ cells/ml suspended in buffer or in ANP, as indicated. The capillaries were filled with either the buffer or ANP and sealed at one end with a clamp. The open side of each capillary was inserted into the well and the capillaries were incubated for a period of 20-30 min in a 37°C incubator under 5% CO_2. At the end of the incubation period the total content of the capillary was transferred into an Eppendorf tube containing 3 μl of 8% glutaraldehyde in water and the average concentration of the spermatozoa in each capillary was determined by direct counting under the microscope by a haemocytometer.

Endopeptidase assay. Neutral endopeptidase (EC 3.4.24.11) activity was measured in microplates, using the substrate (3-carboxypropanoyl-alanyl-alanyl-leucine 4-nitroanilide) as described elsewhere (20).

Data analysis. The statistical significance of the results in the capillary assays, expressed as the mean ± S.E.M., was calculated by one way analysis of variance (ANOVA) followed by Fisher test.

RESULTS

To evaluate the effects exerted by ANP on the locomotive behavior of the spermatozoa we monitored, under the microscope, their response to a spatial gradient of ANP in a sealed chamber that had been developed for this purpose (19). The circular chamber (see inserts in Fig. 1 for a diagram) contained four wells, two of which (top and bottom) were filled with

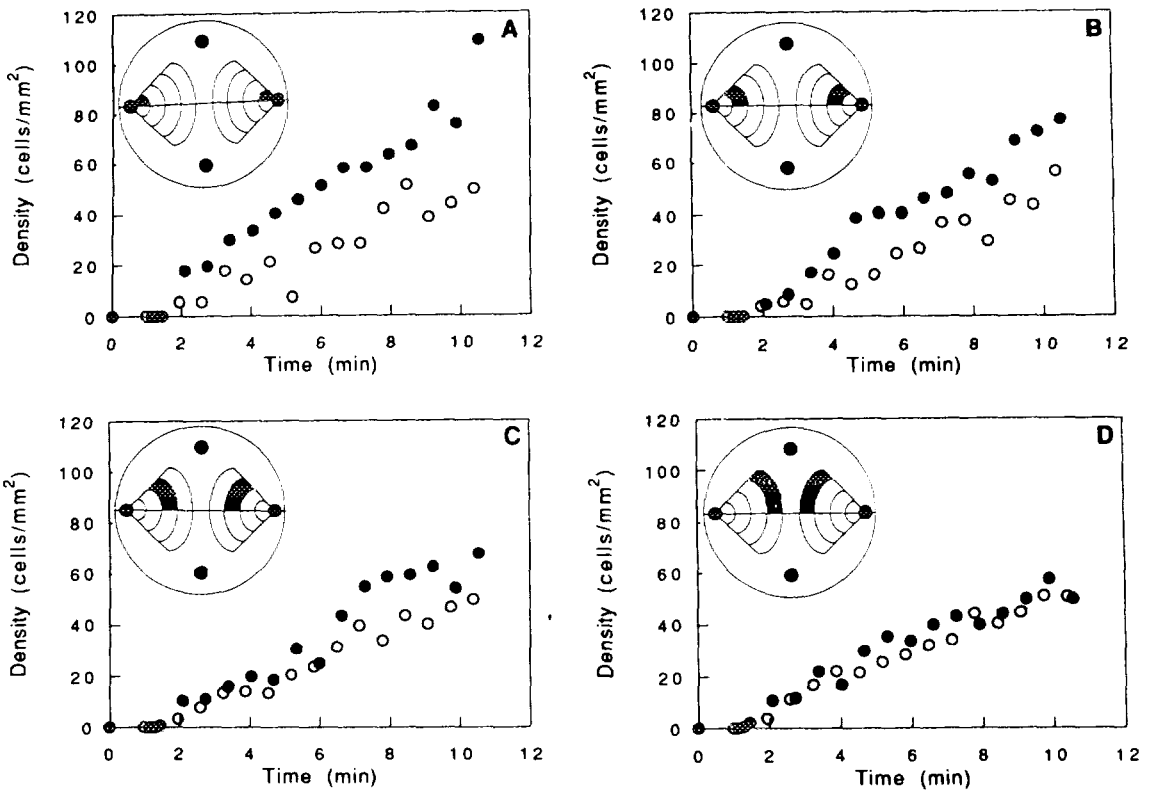

Fig. 1. Distribution of spermatozoa near wells containing ANP and buffer in a microscopic assay. The experiment was carried out as described in the text. The entire chamber was video-recorded. Since the observation field under the microscope was smaller than the field defined by the area between the four wells, we covered most of this area by recording two smaller areas in sequence. Each of these areas was recorded for 10 s every 30 s. Analysis of cell density was carried out separately for the various zones adjacent to the wells as illustrated by the shaded zones in the insert to each panel. •, Sperm density near the ANP (10^{-5} M)-containing well; ○, sperm density near the buffer-containing well. Inserts, a top view diagram of the chamber; the shaded areas indicate the zones studied.

spermatozoa, and the other two (left and right) were filled with buffer and ANP (diluted in buffer), respectively. A gradient of ANP was established by diffusion and the behavior of the spermatozoa in it was recorded on video and then analyzed. Initially (after 2 min) sperm accumulation was observed only in the zone adjacent to the ANP-containing well (Fig. 1A). As the ANP diffused away from the well, accumulation was also observed in more distal zones (e.g. after 4 min in Fig. 1B and after 6 min in Fig. 1C). No difference between the density of the spermatozoa was detected in the most distal zones (Fig. 1D). It should be noted that only a fraction of the sperm population, at any given moment, are chemotactic (21). This is probably the reason for the observation that the difference between the sperm densities near the wells was moderate.

To determine the nature of the sperm attraction to ANP, we measured sperm accumulation in capillary assays in a variety of combinations, i.e., ascending or descending gradients of ANP as well as no gradient at all. The four combinations employed are:

1 ANP in well, buffer in capillary (Fig. 2A-gray column)
2 ANP in well, ANP in capillary (Fig. 2A-black column)
3 Buffer in well, buffer in capillary (Fig. 2B-gray column)
4 Buffer in well, ANP in capillary (Fig. 2B-black column).

In the presence of a descending gradient of ANP (combination no. 1), the number of spermatozoa that migrated down the gradient into the capillary was significantly ($P<0.01$) lower than in the absence of a gradient (combination no. 2). However, in the presence of an ascending gradient of ANP (combination no. 4), the number of spermatozoa that migrated up the gradient into the capillary was significantly ($P<0.01$) higher than in the absence of a gradient (combination no 3). This dependence of the sperm migration on the ANP gradient is in line with sperm chemotaxis induced by ANP. Furthermore, in the absence of a gradient, when both the well and the capillary contained equally-diluted ANP (combination no. 2), the number of spermatozoa that migrated into the capillary was significantly ($P<0.01$) higher than the number that migrated when both the well and the capillary contained just buffer (combination no. 3), indicative of enhancement of swimming speed by ANP, i.e., sperm chemokinesis.

Neutral endopeptidase is a cell surface peptidase, widely distributed in mammalian tissues, known to inactivate a variety of biologically active peptides, including ANP (22). The enzyme is very abundant in semen and although most of its activity is found in the seminal fluid, some activity can also be detected in 2x-washed spermatozoa. We found that the neutral endopeptidase activity of the washed cell suspensions used in the above experiments was in the range of 1-10 nmol/min/ml of hydrolyzed 3-carboxypropanoyl-alanyl-alanyl-leucine 4-nitroanilide. In view of this residual peptidase activity, we repeated the above experiments in the presence of 1.7 μM phosphoramidon, an inhibitor of the endopeptidase activity [K_i = 1.1 nM for

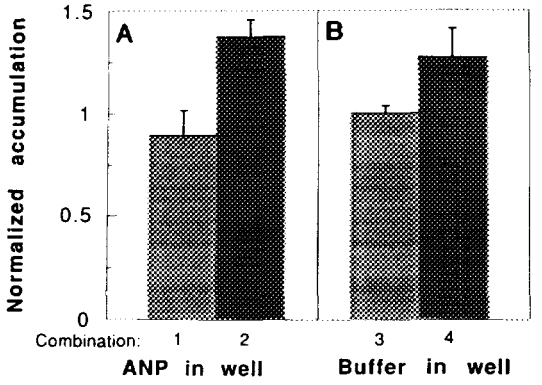

Fig. 2. Effects of ANP on sperm accumulation in capillary assays. Capillary assays were carried out as described in Materials and Methods. Each column represents the mean of 18 determinations (3 experiments, 6 determinations in each) ± S.E.M. Gray columns, buffer in capillary ; black columns, ANP in capillary . In all cases, the spermatozoa were only in the wells at the beginning of the assay. **A**, ANP in the well; **B**, buffer in the well. The data were normalized according to the mean accumulation in combination no. 3.

phosphoramidon acting on rat neutral endopeptidase (23)]. Such inhibitor concentrations are commonly used to diminish the enzyme activity (22). As shown in Table 1, in the presence of the inhibitor we could detect ANP-induced chemotaxis and chemokinesis at concentrations as low as 10^{-9}-10^{-8} M. This suggests that the sperm endopeptidase acted on ANP, and that this degradation of the peptide was prevented by phosphoramidon.

In both types of assays there was a concentration range in which ANP was effective. In the microscopic assays, sperm accumulation was observed with ANP concentrations of 10^{-6}-10^{-5} M in the well. (The actual concentration of ANP sensed by the spermatozoa was always lower than the concentration in the well.) In the capillary assays, the range was 10^{-7}-10^{-5} M. This concentration ranges reflect differences in the responsiveness of different sperm donors as well as the stability of the peptide under our experimental conditions.

DISCUSSION

Here we demonstrated that ANP induces chemotaxis and chemokinesis of human spermatozoa *in vitro.* Although the physiological relevance of these findings and their putative role in the process of fertilization are not yet clear, there is growing body of evidence which suggests that ANP may play a role in fertilization. Thus, ANP and ANP transcripts have been detected in rat ovary (10) and oocytes (11), indicating synthesis of ANP in the oocyte; bovine corpus luteum was found to contain ANP-like immunoreactivity (24); and immunoreactive ANP was detected in ovarian follicular fluids of women (15, 16). In line with this notion, specific high-affinity binding sites for ANP have been recently detected and localized in human spermatozoa by using membrane-binding assays and receptor autoradiography (17). The ANP receptors are localized mainly around the sperm midpiece (where mitochondria are abundant) (17). ANP is capable of binding to the mitochondria and may potentially affect respiration and motility of spermatozoa (25). Indeed, in the sea urchin *Arbacia punctulata*, the sperm attractant, resact, stimulates particulate guanylate cyclase and enhances the respiration rate and the motility of spermatozoa under appropriate *in vitro* conditions (6, 26). By analogy, activation of particulate

Table 1. Phosphoramidon effects on sperm attraction to ANP

Combination	ANP in		Sperm concentration ($\times 10^{-4}$) in capillary	
no.	well	capillary	-phosphoramidon	+phosphoramidon
1	+	-	156 ± 22	76 ± 20
2	+	+	129 ± 15	198 ± 6

The capillary assay was carried out as described in Materials and Methods. Each well contained 1.5×10^7 spermatozoa/ml. Each result represents the mean of 6 determinations ± S.E.M. The ANP concentration used was 10^{-9} M, and that of phosphoramidon was 1.7 μM. ANOVA followed by Fisher test showed that, in the presence of phosphoramidon, the differences between combinations no. 1 *vs.* no. 2 were significant ($P < 0.01$).

guanylate cyclase by ANP in human spermatozoa may be involved in changes of their motility behavior and contribute to the precontact sperm-egg communication.

At this stage it is not yet known whether ANP is involved in sperm chemotaxis and chemokinesis *in vivo* or whether the processes discovered in this study occur only *in vitro*. According to the first alternative, it is possible that the physiological attractant for human spermatozoa (3) is an ANP-like substance. However, our experiments demonstrated that, *in vitro*, neutral endopeptidase inhibitor should be present for observing the effect of ANP at physiological concentrations (Table 1). Since this inhibitor is probably absent *in vivo*, the likelihood of this alternative seems to be low, unless the activity of the neutral endopeptidase *in vivo* is much lower than the activity measured *in vitro*. If the second alternative is correct, namely that sperm chemotaxis and chemokinesis to ANP occurs only *in vitro*, it indicates that guanylate cyclase is involved in these processes. Thus, ANP may directly affect guanylate cyclase *in vitro* in a manner similar to that caused by the physiological attractant *in vivo*. Further experiments are required for distinguishing between these alternatives .

ACKNOWLEDGMENTS

M.E. is an incumbent of Jack and Simon Djanogly Professorial Chair in Biochemistry. This work was supported in part by research grants to M.E. from the Ebner Family Biomedical Research Foundation at the Weizmann Institute of Science in memory of Alfred and Adolph Ebner, and from the Israel Ministry of Labour and Social Affairs.

REFERENCES

1. Miller, R. L. (1985) In Biology of Fertilization (C. B. Metz and A. Monroy, eds.) pp. 275-337, Academic Press, New York.
2. Cosson, M. P. (1990) In Controls of Sperm Motility: Biological and Clinical Aspects (C. Gangnon, ed.) pp. 103-135, CRC Press, Boca Raton.
3. Ralt, D., Manor, M., Cohen-Dayag, A., Tur-Kaspa, I., Makler, A., Yuli, I., Dor, J., Blumberg, S., Mashiach, S. and Eisenbach, M. (1993) Biol. Reprod.,in press.
4. Eisenbach, M. and Ralt, D. (1992) Am. J. Physiol. 262 (Cell Physiol. 31), C1095-C1101.
5. Suzuki, N., Shimomura, H., Radany, E. W., Ramarao, C. S., Ward, G. E., Bentley, J. K. and Garbers, D. L. (1984) J. Biol. Chem. 259, 14874-14879.
6. Ward, G. E., Brokaw, C. J., Garbers, D. L. and Vacquier, V. D. (1985) J. Cell Biol. 101, 2324-2329.
7. Garbers, D. L. (1989) Ann. Rev. Biochem. 58, 719-742.
8. Zamir, N., Skofitsch, G., Eskay, R. and Jacobowitz, D. M. (1986) Brain Res. 365, 105-111.
9. Goetz, K. L. (1990) Hypertension 15, 9-19.
10. Gutkowska, J., Tremblay, J., Antakly, T., Meyer, R., Mukaddam-Daher, S. and Nemer, M. (1993) Endocrinology 132, 693-700.
11. Kim, S. H., Cho, K. W., Oh, S. H., Hwang, Y. H., Lim, S. H., Ryu, H., Seul, K. H., Jeong, G. B. and Yoon, S. (1993) Comp. Biochem. Physiol. 104A, 219-223.
12. Brenner, B. M., Ballerman, B. J., Gunning, M. E. and Zeidel, M. L. (1990) Physiol. Rev. 70, 665-699.
13. Ruskoaho, H. (1992) Pharmacol. Rev. 44, 481-602.
14. Zamir, N., Tuvia, S., Riven-Kreitman, R., Levin, S. and Korenstein, R. (1992) Biochem. Biophys. Res. Commun. 188, 1003-1009.
15. Sundfjord, J. A., Forsdahl, f. and Thibault, G. (1989) Acta Endocrinol. 121, 578-580.
16. Steegers, E. A. P., Hollanders, J. M. G., Jongsma, H. W. and Hein, P. R. (1990) Gynecol. Obstet. Invest. 29, 185-187.

17. Silvestroni, L., Palleschi, S., Guglielmi, R. and Croce, C. T. (1992) Arch. Androl. 28, 75-82.
18. Biggers, J. D., Whitten, W. K. and Whittingham, D. G. (1971) In Methods in Mammalian Embryology (J. D. Daniel, ed.) pp. 86-116, Freeman, San Francisco.
19. Makler, A., Reichler, A., Stoller, J. and Feigin, P. D. (1992) Fertil. Steril. 57, 1066-1074.
20. Indig, F. E., Ben-meir, D., Spungin, A. and Blumberg, S. (1989) FEBS Lett. 255, 237-240.
21. Cohen-Dayag, A., Ralt, D., Tur-Kaspa, I., Manor, M., Makler, A., Dor, J., Mashiach, S. and Eisenbach, M. (1993) Biol. Reprod., in press.
22. Kenny, A. J. and Stephenson, S. L. (1988) FEBS Lett. 232, 1-8.
23. Altstein, M., Bachar, E., Vogel, Z. and Blumberg, S. (1983) Eur. J. Pharmacol. 91, 353-361.
24. Vollmar, A. M., Mytzka, C., Arendt, R. M. and Schulz, R. (1988) Endocrinol. 123, 762-767.
25. Bachar, H., Haver, E., Ilani, A. and Lichtstein, D. (1992) Pflugers Arch 422, 204-206.
26. Garbers, D. L. (1989) J. Androl. 10, 99-107.