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Role of spermine in mammalian sperm capacitation and acrosome reaction

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The binding properties of seminal polyamines to ram spermatozoa and their possible role in sperm capacitation and the acrosome reaction were studied. Binding and release of [^{14}C]spermine from ram spermatozoa occurred at a rate faster than in somatic cells and were not energy-dependent. Release of bound spermine was further facilitated by heparin, a constituent of the female reproductive tract which was reported to induce capacitation and the acrosome reaction. High- and low-affinity polyamine-binding sites were identified, of which the high-affinity site was specific to polyamines with three or more amino groups. We also found that spermine inhibited the acrosome reaction and propose that it is the major seminal decapacitating factor. Since precise timing of capacitation and the acrosome reaction are critical for successful fertilization, it is suggested that the role of seminal spermine is to prevent premature capacitation and the acrosome reaction.

INTRODUCTION

Spermine is an ubiquitous polyamine which is present in milimolar concentrations in the seminal plasma of many species, including man, rat and ram [1]. Although its occurrence in semen has been known for many years, its role in sperm physiology is largely unknown. When mammalian spermatozoa travel through the male and female reproductive tracts to reach the location of fertilization finally, they are exposed to extensive variations in their immediate environment, which affect their physiology [2]. At the time of ejaculation, epididymal spermatozoa come into contact with secretions of the male accessory glands containing high concentrations of prostatic spermine. In spermatozoa, spermine was found to enhance the glycolytic rate [3], adenylate cyclase [4] and ATPase [5] and to inhibit phosphodiesterase [6]. Furthermore, binding of spermatozoa to oocytes [7,8] was affected by polyamines. The acrosome reaction was stimulated by the polyamines polyarginine and compound 48/80 [8], whereas another polyamine, neomycin, inhibited the acrosome reaction [9]. Spermine and other polyamines serve as natural acceptor amines for seminal transglutaminase action, thus attenuating protein cross-linking and premature clotting of the ejaculate [10]. So far, however, no information on the effect of the seminal constituent spermine on the acrosome reaction has been reported.

The acrosome reaction is a prerequisite for successful fertilization in mammals, defined as a Ca^{2+}-dependent exocytotic event in sperm, in which membrane fusion takes place between the outer acrosomal membrane and the overlying plasma membrane, thereby allowing release of the acrosomal contents [11,12]. Before the acrosome reaction, the mammalian sperm in the female reproductive tract undergoes a capacitation process [13,14] in which several modifications of sperm membranes occur [2,15], including increased lateral mobility of membrane proteins and alterations in membrane fluidity. It was reported that spermine inhibits lateral mobility of membrane proteins in other cells [16] and affects membrane fluidity [17]; therefore its influence on the acrosome reaction was determined here. We also characterize for the first time the binding properties of polyamines to sperm and demonstrate their involvement in the mechanism of the acrosome reaction.

EXPERIMENTAL

Materials

[^{14}C]Spermine (111 mCi/mmol), [^{14}C]spermidine (117 mCi/mmol) and [^{14}C]putrescine (112 mCi/mmol) were obtained from Amersham. Silicone oil DC 500 was purchased from Serva. Dinonyl phthalate was obtained from BDH. All other reagents were from Sigma.

Sperm preparation

Semen was collected from rams by electric induction [16] and was immediately diluted (1:1) with buffer A (110 mM-NaCl/5 mM-KCl/10 mM-Mops, pH 7.4). The cells were washed by three centrifugations at 780 *g* for 10 min at 25 °C and resuspended in buffer A to a final concentration of (1–3) $\times$ 10^9 cells/ml.

Binding of ^{14}C-labelled polyamines to spermatozoa

Sperm cells (5×10^8/ml) were incubated in buffer A containing various ^{14}C-labelled polyamines at 37 °C. At the end of the incubation time, a 0.1 ml portion was layered on top of 0.3 ml of phthalate ester mixture (dibutyl phthalate/dinonyl phthalate, 2:1) in polypropylene tubes. When cells were suspended in buffer B (0.2 M-sucrose/10 mM-Mops, pH 7.4), the samples were layered on top of a mixture of dinonyl phthalate and silicone oil DC 500 (3:7, v/v). The tubes were centrifuged for 1 min at 13000 *g*. The supernatants were aspirated, the pellets were suspended in 0.1 ml of buffer A and the radioactivity was counted.

Measurement of acrosome reaction

This was done by measuring released acrosin activity with benzoylarginine ethyl ester as substrate, and recording the increase in A_{259} as described in detail in [18]. Occurrence of the acrosome reaction was confirmed by observing thin sections of spermatozoa by transmission electron microscopy [18].

Mathematical analysis

K_d values and the number of binding sites were estimated by using the LIGAND data-analysis computer program [19].

RESULTS

Binding characteristics of polyamines to spermatozoa

Binding of [^{14}C]spermine as well as other seminal polyamines to spermatozoa was studied. A rapid rate of [^{14}C]spermine binding was observed, with saturation reached by 5 min at 37 °C (Fig. 1). No further change in the level of bound spermine was

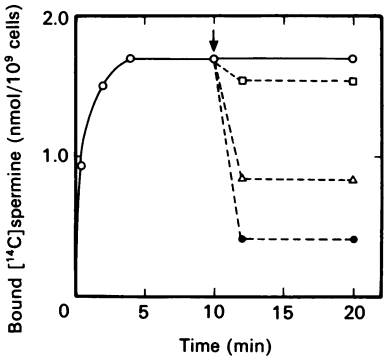

Fig. 1. Reversible binding of spermine to spermatozoa

Washed cells (5×10^8 cells/ml) were incubated with [^{14}C]spermine (0.3 μCi/ml) at 37 °C in buffer A. At the indicated times, cells (0.1 ml) were separated by centrifugation on phthalate esters and bound [^{14}C]spermine was counted (○). To determine the dissociation of [^{14}C]spermine, binding was carried out as described above for 10 min. The 13000 *g* pellets were resuspended in 0.4 ml of buffer B (□), 0.1 ml of buffer A (△) or 0.4 ml of buffer A (●). At the indicated time, the suspensions were centrifuged through phthalate esters and the pellets were counted for radioactivity as described.

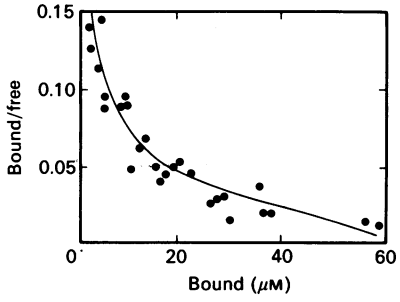

Fig. 2. Scatchard plot of spermine binding to spermatozoa

Washed cells were incubated for 5 min at 37 °C with [^{14}C]spermine (0.3 μCi/ml) in buffer A and with increasing concentrations (0.002–2 mM) of unlabelled spermine. Cells were separated and bound [^{14}C]spermine was counted. Binding data were analysed by the LIGAND program. Values were taken from four separate experiments. Non-specific binding was about 1%.

noticed within 20 min of incubation. Release of bound spermine upon resuspension of the washed cell pellet in fresh buffer was determined. When spermatozoa loaded with [^{14}C]spermine were resuspended in a non-ionic medium (buffer B), almost no release of the bound spermine was observed. However, resuspension in an ionic medium (buffer A) resulted in a rapid release of bound spermine at a rate which was comparable with the initial binding rate, and the extent of release was volume-dependent (Fig. 1). Similar results (not shown) were obtained with spermidine and putrescine.

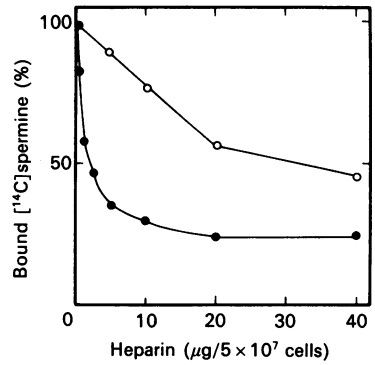

Fig. 3. Heparin-aided dissociation of spermine from sperm cells

Sperm cells were incubated with [^{14}C]spermine in buffer A (5 min, 37 °C). The cells were washed and the pellet was resuspended together with increasing concentrations of heparin in either buffer A (0.1 ml, ○) or buffer B (0.1 ml, ●). After 2 min the cells were centrifuged and their radioactivity was counted.

The affinity of various polyamines to spermatozoa under equilibrium conditions was estimated by Scatchard analysis of saturation or displacement data by using the LIGAND program. Scatchard analysis of [^{14}C]spermine binding yielded a curvilinear plot, which could be resolved into two binding sites differing in their affinity for spermine (Fig. 2). The affinity of spermidine and putrescine was similarly determined (Table 1). The binding characteristics of spermidine were similar to those of spermine, whereas putrescine gave a linear Scatchard plot, corresponding to the lower-affinity site only. The rather low affinity of spermatozoa for polyamines ($K_{d1} = 10^{-5}$–10^{-4} M and $K_{d2} = 10^{-3}$–7×10^{-3} M) was counterbalanced by the high abundance of the sites [site 1, (1–5) $\times 10^6$/cell; site 2, (5–8) $\times 10^7$/cell] and the high concentration of spermine.

Release of bound spermine was found to be facilitated by the polyanion heparin. Resuspending the cells in 0.1 ml of ionic medium resulted in a 50% displacement of bound spermine (Fig. 1), and further displacement of up to 55% of the remaining spermine was obtained by including heparin in the ionic medium

Table 1. Dissociation constant and number of binding sites for polyamines on spermatozoa

(*a*) Spermatozoa (5×10^8 cells/ml) were incubated for 5 min at 37 °C with [^{14}C]spermine (0.3 μCi/ml) and unlabelled spermine (2 μM–2 mM), [^{14}C]spermidine (1 μCi/ml) and unlabelled spermidine (2 μM–2 mM) or [^{14}C]putrescine (2 μCi/ml) and unlabelled putrescine (20 μM–20 mM). Cells were washed, the bound [^{14}C]polyamine was counted for radioactivity and the binding data was analysed by the LIGAND program. (*b*) Spermatozoa were incubated as in (*a*) with [^{14}C]spermine (0.3 μCi/ml) and increasing concentrations (0.2 μM–2 mM) of unlabelled neomycin. Cell-associated radioactivity was counted and binding data were analysed as in (*a*). For the mathematical analysis, K_d values of spermine were taken from line 1 of the Table.

		Site 1		Site 2	
Polyamine	*n*	K_{d1} (M)	No. of sites/cell	K_{d2} (M)	No. of sites/cell
(*a*) Spermine	4	3.58×10^{-5} (±64%)	5.20×10^6 (±55%)	1.21×10^{-3} (±51%)	5.38 ± 10^7 (±24%)
Spermidine	2	7.50×10^{-5} (±90%)	1.48×10^6 (±88%)	3.20×10^{-3} (±38%)	6.69×10^7 (±24%)
Putrescine	2	–	–	6.68×10^{-3} (±25%)	7.90×10^7 (±25%)
(*b*) Neomycin	2	9.50×10^{-7} (±74%)	9.30×10^6 (±8%)	4.90×10^{-4} (±34%)	1.10×10^8 (±13%)

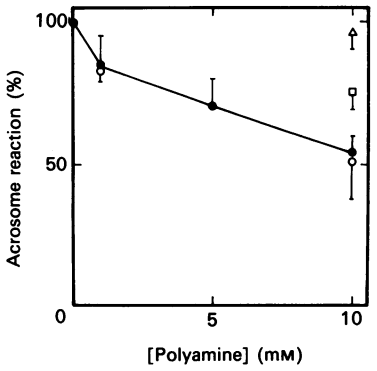

Fig. 4. Inhibition of acrosome-reaction by polyamines

Sperm cells (1×10^8/ml) were incubated in buffer A containing 2 mM-$CaCl_2$ and increasing concentrations of spermine (0–10 mM; ●), neomycin (0–10 mM; ○), spermidine (10 mM; □) or putrescine (10 mM; △). The acrosome reaction was induced by Ca^{2+} ionophore A23187, and after 1 h at 37 °C the extent of the acrosome reaction was determined. The amount of benzoylarginine ethyl ester hydrolysed without added polyamines (about 800 nmol/min per 10^8 cells) was taken as 100% acrosome reaction. Ca^{2+}-independent acrosin activity, obtained in the presence of 0.5 mM-EGTA (instead of Ca^{2+}), was subtracted. Each point represents the mean ± S.E.M. of duplicates from 3 to 9 independent experiments.

(Fig. 3). The inherent ability of heparin to deplete bound spermine was even more apparent in a non-ionic medium (Fig. 3), in which minimal dissociation of spermine upon dilution without heparin was observed (Fig. 1).

Involvement of polyamines in regulation of the acrosome reaction

We found that the acrosome reaction was inhibited by milimolar concentrations of spermine. Significant inhibition was observed at 5 mM-spermine (30%) and at 10 mM-spermine (46%; Fig. 4). Spermidine was less inhibitory than spermine, whereas putrescine did not inhibit the acrosome reaction at all (Fig. 4). At the time of ejaculation, ram sperm cells are exposed to millimolar concentrations of spermine. Some of this spermine is taken up by the cells, and indeed we detected polyamines in the midpiece and the acrosome region of the sperm by cytochemical staining with fluorescamine [20]; results not shown). It has been shown that the polyamine neomycin inhibited the acrosome reaction in ram spermatozoa [10], an observation which was confirmed here (Fig. 4). The binding properties of neomycin to spermatozoa were estimated by its ability to displace [^{14}C]spermine. It was found that neomycin competed for both the high- and low-affinity sites, and its affinity was about 30 times that of the seminal polyamine spermine (Table 1).

DISCUSSION

The binding properties of the seminal polyamines spermine, spermidine and putrescine to ram spermatozoa were investigated. In addition, the possible role of these amines in sperm capacitation and the acrosome reaction was studied. The results indicate that the polyamines bind reversibly to spermatozoa by electrostatic interactions. The rates of both binding and release were significantly faster as compared with other cell types [21]. In addition, metabolic energy is not involved in polyamine binding to spermatozoa, since complete depletion of cellular ATP by pretreatment with 2-deoxyglucose (20 mM) and antimycin A (1 μM) did not affect this binding. In other cell types the binding of spermine was reported to be energy-dependent [21].

When the rather small volume of fluids in the uterus and oviduct [22] is considered, the contribution of heparin as an aid in removal of sperm-associated spermine is probably very significant. Heparin as a constituent of the female reproductive tract [23] can induce the process of sperm capacitation [24]. We suggest that one of the mechanisms by which heparin induces capacitation is by depleting cell-associated spermine. During sperm capacitation several alterations in the plasma membrane occur, one of which is an increase in the lateral diffusion of proteins [2]. Spermine probably inhibits such a lateral diffusion and increases the mechanical stability of plasma membranes, as was reported for other cell types [17]. The seminal plasma was reported to contain decapacitating factors which stabilize the sperm membrane and prevent premature acrosome reaction. Several seminal proteins, including caltrin and acrosome-stabilizing factor (ASF), were identified as decapacitating factors [25,26]. On the basis of our observations and the reported properties of polyamines, we suggest that spermine is the major seminal decapacitating factor.

The observation that putrescine which bound only to the low-affinity sites did not inhibit the acrosome reaction indicates that only the high-affinity sites are associated with control of the acrosome reaction, and that these sites specifically bind polyamines of three or more amino groups. As to the mechanism by which polyamines inhibit the acrosome reaction, it was shown that neomycin acts by blocking the hydrolysis of phosphoinositide [9]. It was also shown elsewhere that physiological concentrations of spermine and spermidine inhibited a phospholipase-C-dependent hydrolysis of phosphoinositide in rat brain [27], platelets [28], ventral prostate and liver [29]. It is likely that the antibiotic neomycin mimics the action of spermine, and we therefore suggest that, similarly to neomycin, spermine inhibits the acrosome reaction by blocking phosphoinositide breakdown.

In conclusion, we suggest that, in species that have millimolar concentrations of spermine, it is rapidly incorporated into sperm during ejaculation, temporarily inhibiting capacitation and the acrosome reaction. A progressive depletion of cellular spermine would then follow during the travel of sperm through the female genital tract, enhanced by the presence of heparin, so that sperm capacitation and consequently the acrosome reaction will take place at the appropriate time when the sperm reaches the vicinity of the oocyte.

REFERENCES

1. Mann, T. (1964) The Biochemistry of Semen and the Male Reproductive Tract, Methuen and Co., London
2. Yanagimachi, R. (1988) in The Physiology of Reproduction (Knobil, E. & Neill, J., eds.), pp. 135–185, Raven Press, New York
3. Pulkkinen, P., Sinervirta, R. & Jänne, J. (1975) Biochem. Biophys. Res. Commun. **67**, 714–722
4. Casillas, E. R., Elder, C. M. & Hoskins, D. D. (1980) J. Reprod. Fertil. **59**, 297–302
5. Sheth, A. R., Gunjikar, A. N. & Shah, G. V. (1979) Andrologia **11**, 11–14
6. Shah, G. V. & Sheth, A. R. (1976) Experientia **34**, 980–981
7. Sailing, P. M., Storey, B. T. & Wolf, D. P. (1978) Dev. Biol. **65**, 515–525
8. Fleming, D. A. & Armstrong, D. T. (1985) J. Exp. Zool. **233**, 93–100
9. Roldan, E. R. S. & Harrison, R. A. P. (1989) Biochem. J. **259**, 397–406
10. Williams-Ashman, H. G. (1984) Mol. Cell. Biochem. **58**, 51–56
11. Yanagimachi, R. (1981) in Fertilization and Embryonic Development *in vitro* (Mastorianni, L., Jr. & Biggers, J. D., eds.), pp. 82–182, Plenum Press, New York
12. Russell, L., Peterson, R. & Freund, M. (1979) J. Exp. Zool. **208**, 41–56

13. Chang, M. C. (1951) Nature (London) **168**, 697–699
14. Austin, C. R. (1952) Nature (London) **170**, 326–328
15. Sailing, M. P. (1989) in Oxford Reviews of Reproductive Biology (Milligan, S. R., ed.), pp. 339–388, Oxford University Press, Oxford
16. Schindler, M., Koppel, E. D. & Sheetz, M. P. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 1457–1461
17. Ballas, S. K., Mohandas, N., Marton, L. J. & Shohet, S. B. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 1942–1946
18. Ben-Av, P., Rubinstein, S. & Breitbart, H. (1988) Biochim. Biophys. Acta **939**, 214–222
19. Munson, P. J. & Rodbard, D. (1980) Anal. Biochem. **107**, 220–239
20. Larrson, L. I., Morch-Jorgensen, L. & Hougaard, D. M. (1982) Histochemistry **76**, 159–174
21. Seiler, N. & Dezeure, F. (1990) Int. J. Biochem. **22**, 211–218
22. Casslén, B. & Wilsson, B. (1984) Am. J. Obstet. Gynecol. **150**, 877–881
23. Homburger, F., Brenfeld, P., Tregier, A., Grossman, M. S. & Harpel, P. (1963) Ann. N.Y. Acad. Sci. **106**, 683–688
24. Parrish, J. J., Susko-Parrish, J. L., Winer, M. A. & First, N. L. (1988) Biol. Reprod. **38**, 1171–1180
25. Wilson, W. L. & Oliphant, G. (1987) Biol. Reprod. **37**, 159–169
26. Rufo, G. A., Schoff, P. K. & Lardy, H. A. (1984) J. Biol. Chem. **259**, 2547–2552
27. Fichberg, J., Zetusky, W. J., Bell, M. E. & Cavanagh, E. (1981) J. Neurochem. **36**, 1868–1871
28. Nahas, N. & Graff, G. (1982) Biochem. Biophys. Res. Commun. **109**, 1035–1040
29. Ahmed, K., Goveli, A. S. & Williams-Ashman, H. G. (1988) Biochim. Biophys. Acta **966**, 384–389

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