A STUDY OF THE INTERACTION OF HUMAN SPERMATOZOA MEMBRANE WITH ATP AND CYCLIC-AMP

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Received November 5, 1973

SUMMARY

Human spermatozoa suspensions produce a specific fluorescence spectrum which is significantly quenched when adenine nucleotides (AMP, ATP and cAMP) are added to the medium. These same nucleotides modify the pattern of interaction of the human spermatozoa membrane with a fluorescent probe (ANS). ATP induces a decrease in the polarity of the membrane, increases the number of ANS binding sites, and the quantum yield per site, and induces a blue shift of the membrane tryptophan intrinsic fluorescence. Cyclic-AMP is 2 x as active as ATP, but induces an increase in the polarity of the membrane. AMP is considerably less effective than the other two nucleotides.

Although the fact that mammalian spermatozoa acquire fertilizing capacity through a process called capacitation has been recognized for more than 20 years (1, 2), little is known about the intrinsic mechanisms of this process, and even less about the agent (s) that may participate. However, the recent development of technics for *in vitro* induction of sperm capacitation in several mammalian species, including man, offers a good opportunity to investigate the structural and biochemical characteristics of this important process (3, 4, 5).

Adenyl-cyclase activity has recently been found in the spermatozoa of several mammalian species (6, 7, 8). Furthermore, the possible participation of this enzyme in the process of capacitation has been stressed (8, 9). These data are in accord with our own findings which point toward cyclic-AMP (c-AMP) as one of the participants in human (10, 11) and rabbit spermatozoa capacitation (4).

Since modification by capacitation of the spermatozoa plasma membrane has been repeatedly demonstrated (3) and since the biological activity of adenyl-

cyclase and/or c-AMP are also related to this organelle, we decided to study the interaction of c-AMP with the spermatozoa membrane by observing the quenching of the intrinsic fluorescence and the binding of 1-anilino-8-naphtalene sulfonate (ANS) to the membrane of normal and treated human sperm cells.

METHODS

Freshly prepared suspensions of human spermatozoa -- prepared as previously described (12) -- were adjusted to about 10^8 cells/ml, as determined with a hemocytometer, and the numbers were checked again afterwards by absorbance measurements at 230 nm (13).

Fluorescence determinations were made in a Perkin-Elmer MPF-2A recording spectrophotofluorimeter in the ratio mode at 25°C. Slit widths of excitation and emission were 6 nm, unless stated otherwise. The intrinsic fluorescence spectrum of human spermatozoa was measured between 300 and 450 nm, 288 nm being the wavelength of excitation. The number of spermatozoa was kept as low as possible to minimize quenching by self absorption. All fluorescent titrations were carried out 30s after the addition of the reagents to the cell. The observed fluorescence intensities, when required were corrected for the emission of the spermatozoa and/or of free ANS. In all titration the total dilution was maintained below 10% and was usually 5%. Unless stated otherwise, all experiments were performed in 0.20 M tris-buffer pH 7.38 prepared in 0.14 M NaCl. All reagents used, including ANS, were always prepared in this same buffer.

The magnesium salt of ANS was obtained from Eastman Organic Chemical (Cat. No.10990) and used without further purification. ANS concentration was calculated as previously described (12). Cyclic-AMP, (N_6, O_2'-dibutyryl cyclic-AMP), ATP and AMP were obtained from Sigma Chemical Co. All other reagents used were of the best quality available.

RESULTS

Human spermatozoa suspensions produce a specific fluorescence spectrum, when excited at 288 nm, which is almost identical to that obtained by the excitation of biological membrane (Fig.1a). When the area under the spectrum

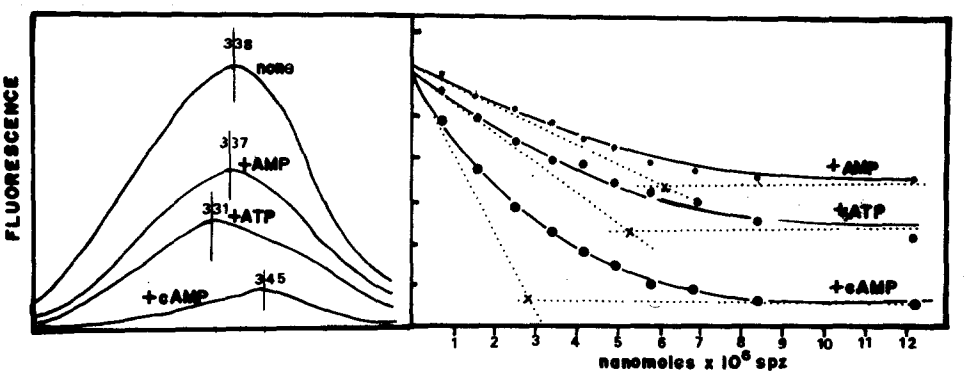

Fig.1 (a) Fluorescence emission spectra of human spermatozoa. The numbers indicate the wavelengths of the maximal fluorescence in the absence and presence of the indicated nucleotides at saturation concentrations (12 nmoles per 10^6 spermatozoa) (b) fluorescence quenching and stoichiometric points obtained by the addition of increasing amounts of the indicated nucleotides in the manner proposed by Velick (14). The wavelength of excitation was always 288 nm, all the values presented have been corrected by non-specific quenching due to absorbance of the nucleotides at this wavelength.

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is integrated by planimetry the relation between fluorescence and number of spermatozoa is linear between 0.5 and 20 x 10^6 cells/ml. The fluorescence emission spectrum of the suspensions of human spermatozoa is altered when adenine nucleotides are added to the medium (Fig.1 and Table I). The fluorescence intensity drops as the nucleotide concentration is raised. All the traces tend to show an asymptotic decrease to a minimum that was lower for c-AMP than for ATP and for non cyclic AMP. The end point is clearly discernible by extrapolation of the linear initial portion of the curve to the terminal base line. From several titrations of this type the equivalent combining weight of each nucleotide can be calculated in the manner suggested by Velick et al (14), (Table I). In addition a small but reproducible blue shift in the wavelength of maximum fluorescence was produced by ATP, while the addition of c-AMP induces, on the contrary, a red shift (Table I).

The binding of ANS to intact human spermatozoa results in a considerable

TABLE I
EFFECT OF NUCLEOTIDES ON ANS FLUORESCENCE IN THE PRESENCE OF HUMAN SPERMATOZOA
The data represent the mean ± S.D. obtained from 5 determinations

Additions	% maximal fluorescence per 10^7 spermatozoa	n (nmoles per 10^6 spermatozoa)	$\bar{K} \times 10^5$ (M)
None	100	2.67 ± 0.09	2.16 ± 0.12
AMP (0.33×10^{-3} M)	128*	3.18 ± 0.12*	2.01 ± 0.08
ATP (0.015×10^{-3} M)	163*	3.37 ± 0.14*	1.76 ± 0.11*
c-AMP (0.015×10^{-3} M)	75*†	2.78 ± 0.08†	2.54 ± 0.09*†

* $P < 0.01$ when compared with the group with no additions.

† $P < 0.001$ when compared with the group with ATP.

P values obtained by the use of the Student's non paired t test.

enhancement of fluorescence, 48 times greater than the fluorescence of ANS alone, and in a blue shift of the fluorescence maximum from 520 to 470 nm (12). When increasing concentrations of dye are added to a fixed concentration of spermatozoa, fluorescence increases to a limiting value. That is, it appears to be possible to saturate all the binding sites on the spermatozoal surface with excess dye (Fig.3a). Significant increases and decreases in fluorescence were observed with the addition of ATP and c-AMP respectively to a mixture containing spermatozoa almost saturated with dye (Fig.2). These modifications in fluorescence were not accompanied by any significant change in the blue shift. When fixed amounts of spermatozoa were titrated with increasing amounts of ANS in the presence of 0.015 mM ATP or c-AMP or 0.33 mM AMP, the results obtained (Fig.3a), plotted as the reciprocals of the obtained values (Fig.3b) and then analyzed by Scatchard method (15), indicate that the action of ATP upon the spermatozoal membrane produces new binding sites and an increase in the quantum yield per site and in the apparent dissociation constant (Table II). On the contrary, c-AMP, without changing the number of binding sites, induces a decrease in the quantum yield and in the apparent dissociation constant (Table II).

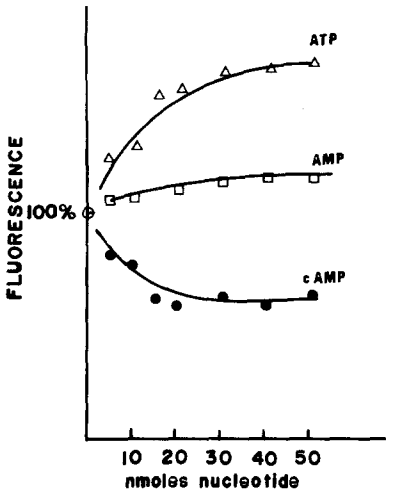

Fig.2 Effect of the addition of increasing amounts of adenine nucleotides upon the fluorescence of ANS-saturated human spermatozoa. In all the cases 10^7 human spermatozoa were saturated with ANS until no further increase in fluorescence was observed and then the indicated concentrations of adenine nucleotides were added. The points indicate the means of 4 to 5 determinations. All the differences are statistically significant.

DISCUSSION

Motility can be increased and prolonged in the spermatozoa of several mammalian species, including man, by cyclic nucleotides (10, 11, 13). The regulation of spermatozoan motility and metabolism (11) by these compounds must be considered as potentially important to understanding the process of sperm capacitation, ovum penetration, and eventual fertilization.

Since the membrane of live human spermatozoa is apparently not permeable to ANS (12), the modification in fluorescence of this probe must reflect changes in the structure of the sperm cell membrane. In the same way, quenching of the intrinsic fluorescence of the spermatozoa must also reflect the interaction and/or the structural changes induced in this organelle by the added nucleotides. That exogenous ATP influences transport phenomena (16) and modifies the membrane structure of human spermatozoa (17) has been pointed out previously. The results

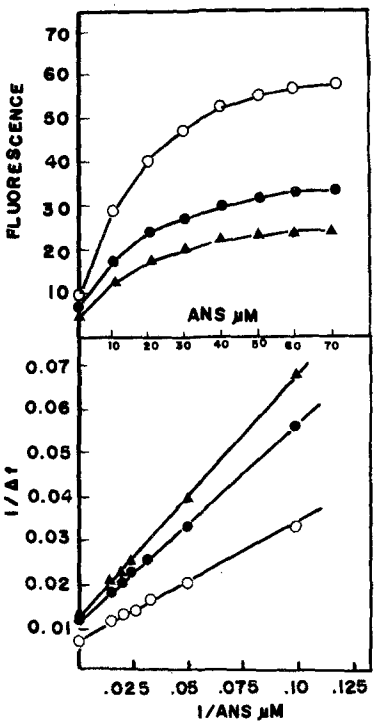

Fig.3 (a) Effect of the presence of 0.015 x 10^{-3} M ATP (o——o) and c-AMP (▲——▲) on the fluorescence produced by adding ANS to a suspension of intact human spermatozoa, as compared with no added nucleotide (●——●) (b) Double-reciprocal plot of the data presented in (a). In both cases the effect produced by AMP has been omitted for the sake of clarity.

presented herein seem to emphasize that exogenous ATP is important in unfolding the membrane (increase in the number of binding sites for ANS) and in decreasing the polarity of these binding sites (increase in fluorescence of bound ANS (18), and blue shift of the tryptophan induced intrinsic fluorescence of human spermatozoa (19)). On the contrary binding of c-AMP to the spermatozoa membrane induces a structural change that seems to expose some of the membrane functional groups to the polar environment (decrease in the fluorescence of bound ANS, red shift of the tryptophan induced intrinsic fluorescence of human sperm cells). This conformational change may be a factor in the increased transport of subs-

TABLE II
CHANGES IN THE INTRINSIC FLUORESCENCE EMISSION SPECTRUM OF HUMAN SPERMATOZOA
The data represent the mean ± S.D. of 5 different observations

Additions	Quenching at stoichiometric point (%)	Maximum Quenching observed (%)	Maximum fluorescence emission at saturation	Equivalent combining weight, nmoles per 10^6 cells
None	——	——	338	——
AMP	38 ± 3.2	47 ± 4.3	336	6.06 ± 0.43
ATP	47 ± 2.9*	66 ± 7.2*	331	5.06 ± 0.23*
c-AMP	64 ± 5.3*†	91 ± 4.0*†	345	2.3 ± 0.21*†

* $P < 0.05$ when compared with the experiments with AMP.
† $P < 0.01$ or less when compared either with the experiments with ATP or with the experiments with AMP.
P Values obtained by the use of the Student's non paired t test.

trates (9) that accompanies the metabolic activation induced by this nucleotide (11).

The observations that partial *in vitro* capacitation of rabbit spermatozoa may be accomplished by c-AMP (4) and that this compound induces changes in the human spermatozoa that may be related to capacitation (10) seem to support the hypothesis that c-AMP acts, in part, by interacting with the spermatozoa plasma membrane. In view of this remarkable interaction of c-AMP with the sperm cell membrane it seems possible to postulate that some of the reported metabolic changes induced by this reagent are due, at least in part, to its action upon the membrane of the sperm cell. It may also be recalled that profound modification of the spermatozoa membrane seems to be necessary in order for capacitation to occur (3).

Acknowledgement

This work was supported in part by a grant from the Ford Foundation.

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