MODIFICATION OF HAMSTER SPERM ADENYL CYCLASE BY CAPACITATION IN VITRO

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SUMMARY: It was found that epididymal hamster sperm contains an adenyl cyclase which at 25 mM $MnCl_2$ synthesized 50 nmoles cAMP/10^8 sperm/hour. This was 20 times the activity observed at 25 mM $MgCl_2$ in the presence or absence of fluoride. The rate of cAMP formation was nearly linear for at least 90 minutes. The specific activity was essentially independent of sperm concentration over the range tested. When hamster sperm were pre-incubated in a medium causing them to become capacitated, they synthesized more cAMP in the presence of manganese than did the uncapacitated sperm control. The significance of this to sperm capacitation is discussed.

Spermatozoa of mammals including man cannot fertilize ova unless they are first modified by the female through a process called capacitation (1,2,3). It is presently impractical to study capacitation of human sperm because it is difficult to obtain the required human ova. However, in vitro sperm capacitation has been accomplished on a limited scale with some rodents (4,5,6). Recently, we have found a way to capacitate sufficient numbers of hamster sperm to investigate the biochemistry of this important process (B. E. Morton, B. J. Rogers and T. S. K. Chang, unpublished).

Here we report both that hamster sperm contain an active adenyl cyclase and that a significant increase in the ability of manganese to stimulate cAMP synthesis by this enzyme occurs when hamster sperm are incubated in a capacitating medium.

METHODS

Epididymal hamster sperm (0.5 - 2.5 x 10^7) contained in stoppered test tubes were incubated at 37° for 4 hours. They were either suspended in 1 ml of Tyrode's solution (7) as a control, or in 0.5 ml Tyrode's solution and

0.5 ml human serum added to capacitate them. The pH 7.5 serum had previously been heated at 56° for 30 min (8) and passed through a 0.3 μ orifice millipore filter. That the sperm were capacitated after this 4 hour incubation in serum was verified by the presence of multiple, swelling sperm heads within the cytoplasma of ova 1-2 hrs after gamete combination.

The adenyl cyclase assay was based upon the formation and separation of ^{32}P-cAMP from α-^{32}P-ATP. This was accomplished as follows: An intervals during the 4 hour capacitation incubation period, 25 μl samples of the sperm suspension were withdrawn for analysis of adenyl cyclase activity. These were placed in 10 x 75 mm plastic tubes (#2039 Falcon Plastics) and incubated for 45 minutes at 30°C. The 100 μl incubation medium contained, in addition to the components contributed by the sperm sample, 16 mM caffeine, 15 mM KCl, 30 mM Tris, pH 7.5, 5 mM $MgCl_2$, 10 mM phosphoenolpyruvate, 50 μg/ml crystalline pyruvate kinase, 1.2 mM ATP and 1-2 x 10^6 cpm ^{32}P-ATP. (1-5 C/mMole Int. Chem. Nuclear Corp., Waltham, Mass.). The incubation was stopped by the addition of 20 μl of 0.1 M EDTA, 6 mM cAMP and 102 x 10^3 cpm ^{3}H-cAMP (14.3 C/mMole, Schwarz/Mann, Orangeburg, N.Y.), followed by the addition of 1 ml 0.05 M Tris, pH 7.6. After centrifugation for 5 min at 3000 g the resultant supernatant fraction was passed through alumina as described by White and Zenser to separate cAMP from the ATP (9). The resulting samples in Bray's solution were counted for 3H and ^{32}P cAMP and the p moles of cAMP synthesized during the incubation was determined using the following relationship:
p moles cAMP = $\frac{H_1}{H_2}$ $(P_1-P_2)/P_3$ where H_1 = cpm H^3 cAMP added in the stopping reagent. H_2 = cpm H^3cAMP recovered after the sample had been processed. P_1 = total ^{32}P cpm in the processed sample that originally contained sperm. P_2 = total ^{32}P cpm in the processed sample that did not contain sperm. P_3 = specific activity of the ATP^{32} substrate = cpm ^{32}P added/total p moles ATP added from all sources (about 1.2 x 10^5). The use of an Olivetti Programma 101 simplified these calculations. The specific activity of the adenyl cyclase was expressed as n moles cAMP/10^8 sperm/hr.

Table 1. The effect of potential activators upon the activity of hamster sperm adenyl cyclase

Agent	nmoles cAMP/10^8 sperm/hr
$MgCl_2$, 25 mM	2.5
KF, 10 mM	2.4
$MnCl_2$, 25 mM	50.0
Detoxified serum, 25%	2.5
Human albumin 10 mg/ml	2.3
Serum blank (apparent activity)	24.9*
Other blanks	0.0

The assay conditions are described in Methods. The incubation time was 45 minutes. *This apparent activity was caused by a change in the absorptive properties of the alumina column, allowing ^{32}P-ATP to elute in the fraction usually containing only cAMP.

RESULTS

The effect of several potential activators upon hamster sperm adenyl cyclase is shown in Table 1. Although fluoride, an agent that stimulates the adenyl cyclase of other tissues, was completely without effect, manganese ion stimulated adenyl cyclase about twenty fold over that stimulated by magnesium at the 25 mM concentrations tested. Serum appeared to change the adsorptive properties of the alumina columns and allow ^{32}P-ATP to elute with the cAMP fraction.

The relative effect of magnesium and manganese concentration upon the activity of adenyl cyclase is shown in Figure 1.

Using 10 mM manganese, the time course data of Figure 2 was obtained. The adenyl cyclase was highly active over a period of at least 90 minutes. Forty five minutes was chosen as the time of incubation for cAMP synthesis in subsequent experiments.

The effect of sperm concentration upon the kinetics of sperm adenyl

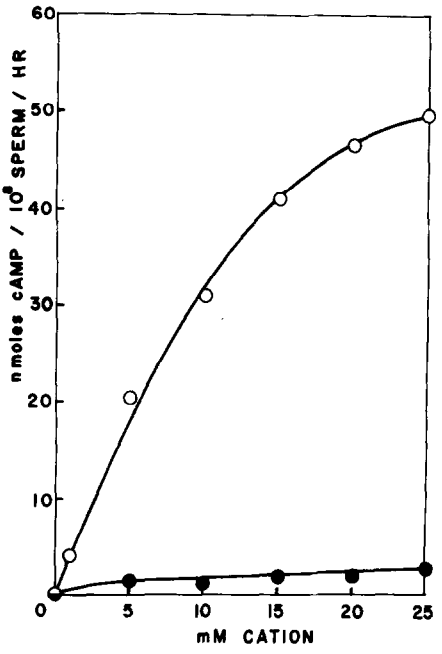

Fig. 1.

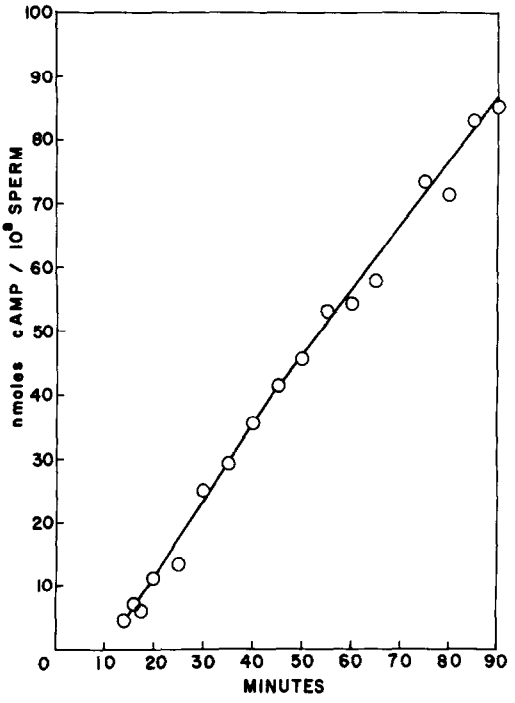

Fig. 2.

Figure 1. The effect of manganese and magnesium ions upon the activity of hamster sperm adenyl cyclase. Sperm from epididymis were dispersed in 5 ml Tyrode's solution and centrifuged for 5 minutes at 2000 g and the pellet resuspended in 30 ml of 0°, 10 mM Trizma buffer, pH 7.6. These were centrifuged at 4000 g for 10 min and the pellet suspended in 0.75 ml of the same buffer. Dark circles = magnesium, light circles = manganese.

Figure 2. THe time course of hamster sperm adenyl cyclase activity. Assay conditions were as described in Methods and Figure 1. 10 mM $MnCl_2$ was present. There were 1.5 x 10^6 sperm/tube.

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cyclase activity is shown in Figure 3. Although the rate of cAMP synthesis/cell is quite similar over the 1.5 - 8.8 x 10^6 sperm/assay tested, the lag period before the appearance of linear kinetics is reduced at high cell concentrations. At 45 minutes the effect of this difference is minimal.

We next investigated the effect of preincubation of the hamster sperm from 0 to 4 hours in a medium known to capacitate them. Because this medium contained heat detoxified serum, shown in Table I to elevate counts in the absence of sperm, extensive sperm-free blanks were first run to evaluate the magnitude of this interference. It was found that the serum blanks did

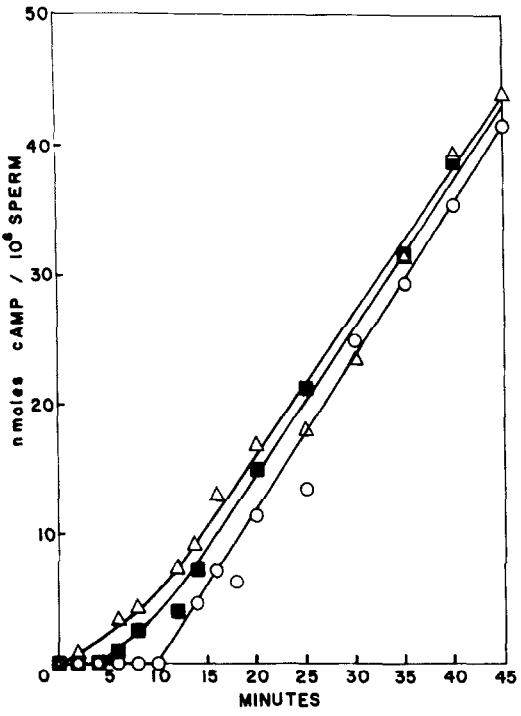

Fig. 3.

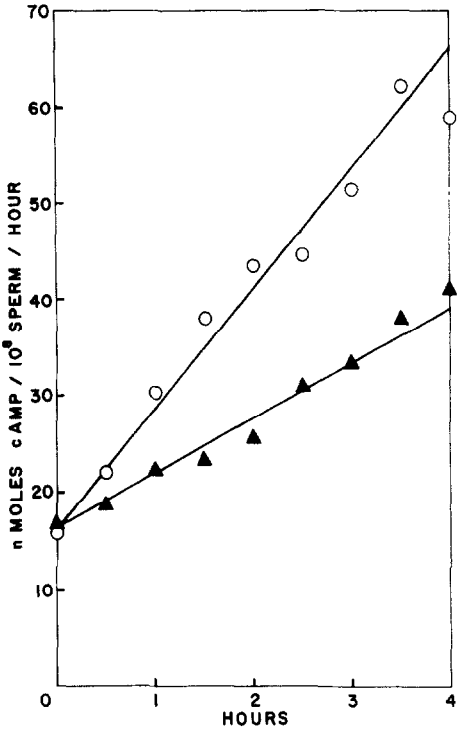

Fig. 4.

Figure 3. The effect of sperm number upon the specific activity of hamster sperm adenyl cyclase. Assay conditions the same as in Figure 2. Triangles = 8.8 x 10^6 sperm/tube, squares = 5.0 x 10^6 sperm/tube and circles - 1.5 x 10^8 sperm/tube. 20 µg/ml rifampicin was present only in the latter tubes.

Figure 4. The effect of capacitation medium upon the activation of hamster adenyl cyclase by manganese. The average of three experiments is plotted. In each the adenyl cyclase activity over 45 minutes at 10 mM $MnCl_2$ was determined in triplicate for each time point. These were corrected by the use of appropriate blanks. 1% Triton X-100 was added at the termination of the assay. Other incubation conditions are given in Methods. Circles = sperm preincubated in heat detoxified human serum. Triangles = sperm preincubated in Tyrode's solution only.

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not vary more than 5 per cent over the 4 hour preincubation and that they were not altered by the combined presence of 25 µg/ml rifampicin, 50 µg/ml streptomycin and 50 IU/ml penicillin. The same antibiotic mixture had no effect upon the activity of sperm adenyl cyclase incubated in Tyrode's solution or serum. Bacterial counts and cultures of the sperm-containing incubation mixture indicated the microorganism levels were very low under these conditions.

A comparison of the adenyl cyclase activity of hamster sperm preincubated

up to 4 hours in Tyrode's solution or in capacitating medium is shown in Figure 4. As may be seen, the manganese-stimulated adenyl cyclase activity of sperm preincubated in capacitating media was significantly greater than that of sperm preincubated in Tyrode's solution alone.

DISCUSSION

Adenyl cyclase of hamster sperm appears to be similar in properties to that in the sperm of other species (10,11,12) in that it was activated, not by fluoride, but by manganese. Although we at present do not understand why the activity of the enzyme increased with time, in a full-length report we will present evidence that this was not due to changes in sperm permeability to nucleotides.

The natural effector of sperm adenyl cyclase is at present unknown. Thyroxine and testosterone at nonphysiological levels have been reported to increase the activity monkey sperm adenyl cyclase (12). Whatever the effector is, our results show that capacitation of hamster sperm brings about an increased rate of cAMP synthesis by adenyl cyclase in the presence of a constant stimulus. This may occur through modification by capacitation of the plasma membrane (13) upon which adenyl cyclase is believed to reside.

An increased ability of capacitated sperm to synthesize cAMP is consistent with the following parallel observations: The cAMP-phosphodiesterase inhibitor, caffeine, or cAMP itself, stimulates sperm motility (14), causes a reduction of ATP levels (15) and activates both oxidative phosphorylation (15) and glycolysis (16). Similarly, capacitation of sperm results in a stimulation of motility (17,18), a reduction in ATP levels (B. J. Rogers and B. Morton, unpublished) and an activation of oxidative phosphorylation (19) and glycolysis (20).

The relationship of the elevation in sperm cAMP levels to the above manifestations appears to have been explained by the discovery of large amounts of cAMP-dependent protein kinase in the cytosol of bull sperm (21,22). Because such a kinase in skeletal muscle phosphorylates troponin, a protein

implicated in the control of muscle contractility (23), it is postulated that the yet unidentified substrate for sperm protein kinase will be a flagellar protein controlling the rate of sperm motility and thus the cellular ATP level and the rate of ATP synthesis. Thus the modification of sperm adenyl cyclase by capacitating components could be sufficient to account for all changes in motility and energy metabolism reported for capacitated sperm.

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