The Interaction of cAMP With Modeled Bull Sperm

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Demembranated and membrane disrupted bull sperm models exhibit an increase in motility when exposed to cAMP. Tritium-labeled cAMP was used to locate the initial site of action of cAMP in the modeled sperm preparations. cAMP did not bind selectively to the modeled cells, and the presence or absence of plasma membrane fragments on the models did not significantly alter this result. When suspension medium taken from modeled sperm preparations was subjected to gel filtration on Sephadex G25-150 columns, cAMP bound to a high molecular weight component that eluted with the void volume. The responsible binding factor is a soluble component that is released when the plasma membranes of the sperm are disrupted during the modeling procedure. To test the importance of the cAMP binding factor, modeled sperm were centrifuged, the supernatant solution was decanted, and the cells were resuspended in fresh medium. After this treatment the cells could be restored to motility with Mg-ATP but no longer exhibited a response to cAMP. Furthermore, addition of cAMP binding factor isolated by gel filtration partially restored the response of these sperm to cAMP. Investigation of the properties of the cAMP-binding factor have confirmed that it is specific for cAMP, with a much lower affinity for AMP and cGMP. In the presence of a large excess of unlabeled cAMP the labeled complex has a half-life of approximately 1 hour. Our results indicate that the action of cAMP on the motility of modeled sperm is mediated by its attachment to a high molecular weight, soluble component of the cell cytoplasm.

Key words: sperm, flagellum, motility, cAMP, freeze-thawing

INTRODUCTION

The motility of mammalian sperm, like a very wide spectrum of cellular phenomena, appears to be under the control of a mechanism that involves 3'-5' cyclic adenosine monophosphate (cAMP) as an element in the regulatory pathway. The
motility of bull sperm is increased in the presence of phosphodiesterase inhibitors [Drevius, 1971; Garbers et al, 1971a,b]. Since this early observation, evidence has steadily accumulated that supports a role for cAMP in initiating and stimulating mammalian sperm motility [for reviews see Mann and Lutwak-Mann, 1981; Garbers and Kopf, 1980; Sanborn, Heindel, and Robison, 1980; Blum and Hines, 1979].

It is now well established that cAMP does have primary effects on the motility of sperm. Modeled sperm produced by detergent extraction lack cell membranes and cytoplasm and consequently cannot produce adenosine 5' triphosphate (ATP). Direct addition of cAMP to the modeled sperm causes an increase in level of motility at a constant concentration of ATP [Lindemann, 1978; Mohri and Yanagimachi, 1980; Tash and Means, 1981]. Since cAMP has an effect on the modeled sperm under conditions of constant ATP concentration, cAMP must directly alter the functioning of the motile apparatus of the cells.

In biological systems that are controlled by cAMP, specific cAMP binding proteins are usually present in the cells. The cAMP-protein complex activates one or more protein kinases that in turn phosphorylate specific cellular proteins. Garbers, First, and Lardy [1973] found that cAMP binding proteins and protein kinases are present in mammalian sperm. Most recently Tash and Means [1981, 1982] have made progress in identifying the sites in the mammalian sperm contractile apparatus that are phosphorylated when modeled sperm are incubated with cAMP. Ishiguro, Murofushi, and Sakai [1982] have successfully demonstrated the presence of a cAMP binding protein in invertebrate sperm that can make sperm models cAMP dependent. Preliminary findings from our laboratory have shown that a soluble factor is also present in bull sperm that is needed for the cAMP response of bull sperm models [Lindemann, Lipton, and Shlafer, 1981].

In this paper we report the localization of cAMP binding in modeled mammalian sperm produced by a new freezing and thawing technique [Lindemann, Fisher, and Lipton, 1982]. By using a modeled sperm preparation, it has been possible to localize the target of cAMP under conditions that also allow the motile response of the sperm to be monitored. We have examined the attachment of cAMP to the modeled sperm cells and to components of the modeled sperm mixture. In addition, we have verified the importance of the cAMP binding interactions we located to the motility response of the sperm.

METHODS
Sperm Collection and Storage

Bull sperm were obtained through cooperation of NOBA, Inc. (Tiffin, OH). Ejaculated semen was collected in an artificial vagina. This bull semen was diluted to approximately 75 × 10^6 live cells per ml (usually a dilution of about 1:5 semen to diluent) with a Tris-base extender containing 2% egg yolk [Davis, Bratton, and Foote, 1963]. The diluted semen was shipped on ice and arrived at our lab within 24–36 hours after collection. We stored the diluted semen at 0–5°C until it was used. The extended semen remained suitable for experimentation for 3 to 4 days.

Preparation of Modeled Sperm

A sample (usually 2 ml) of stored sperm in Tris-egg yolk extender was diluted with two parts of citrate buffer containing 0.097 M sodium citrate, 5 mM MgSO₄,
and 1 mM CaCl$_2$ (pH 7.4). The mixture was centrifuged at 540g for 10 minutes; the supernatant solution was discarded. The resulting pellet was resuspended with citrate buffer and the process was repeated. The final pellet was resuspended in a volume of citrate buffer equal to the initial volume of sperm suspension. The resulting preparation of sperm in citrate buffer was stored on ice.

To produce modeled cells the sperm were suspended in a solution containing 0.200 M sucrose, 0.036 M potassium glutamate, 0.030 M Tris-HCl, 1 mM dithiothreitol, and 1 mM MgCl$_2$ at pH 7.9 (glutamate-Tris buffer), frozen in liquid nitrogen, and thawed. In sperm subjected to this treatment the membranes and mitochondria are severely disrupted but for the most part remain attached to the cell [Lindemann et al, 1982]. Aliquots of 50 µl, 100 µl, or 150 µl of sperm suspended in citrate buffer were added to 1.5 ml of the glutamate-Tris buffer in small polypropylene capsules with lids, which could be sealed and frozen after the sperm were added. The samples were precooled for 10 minutes in cold nitrogen vapor and then plunged into liquid nitrogen for a minimum of 10 minutes. Each sample was thawed in air at room temperature after which it was diluted with 1.5 ml of additional glutamate-Tris buffer. After thawing the samples containing 50 µl or 100 µl of sperm stock suspension were placed into culture dishes with optically flat bottoms for motility observations. ATP was added to a concentration of 1 mM to initiate motility. Thawed samples which had received 150 µl of sperm stock were treated with 1 mM ATP and were then placed into a centrifuge tube for treatment with labeled cAMP.

A modification of the modeling procedure was used to increase the concentration of sperm to assay cAMP binding to soluble cell products. Five milliliters of sperm in egg yolk-Tris were diluted with 5 ml of citrate buffer and centrifuged at 540g for 15 minutes. The supernatant solution was decanted and the pellet resuspended in 10 ml of citrate buffer. The process was repeated and after the second decanting the sperm were suspended in 3 ml of glutamate-Tris buffer and frozen in liquid nitrogen.

When complete demembranation of the sperm was desired in order to look for cAMP binding in the absence of the plasma membrane we used the Triton X-100 method reported by Lindemann [1978] in an earlier study. The only modification of the earlier procedure was that 150 µl of the sperm stock suspension in citrate buffer was added to the Triton X-100 modeling solution.

**Motility Experiments**

Frozen-thawed sperm models were used for the motility observations reported here. Freezing and thawing sperm in a simple medium without egg yolk or cryoprotectants effectively ruptures the plasma membranes and destroys the internal structures of the mitochondria [Lindemann et al, 1982]. After freezing and thawing, the sperm can be reactivated with external ATP and Mg$^{2+}$ to produce motile models. The motility of the frozen-thawed models lasts longer with greater stability than in models produced by Triton X-100 extraction. The frozen-thawed models are also less susceptible to damage from centrifugation and resuspension than detergent extracted cell models. To quantitate the frequency and percent motility of the sperm cells in a sample we used the method reported by Lindemann [1978]. All motility experiments were carried out at ambient temperature (21-23°C).

To assess the effects of cAMP, an initial count of frequency and percent motility was taken and a second count was taken after the addition of cAMP. Ten microliters
of 0.001 M cAMP solution was added to a 3-ml sample of sperm for a final concentration of 3.3 μM cAMP in the sperm suspension. To separate modeled sperm cells from their suspension medium the samples of modeled sperm were centrifuged for 3 minutes at 300g. The supernatant solution was then removed and the pellet was either resuspended in 3 ml of fresh glutamate-Tris buffer or resuspended in the original supernatant solution (control). In a variation of this procedure samples were pelleted and after decanting were resuspended in the supernatant solution removed from another sample of modeled sperm. All samples were allowed to stand without agitation for 1 minute in order to allow the pellet to soften before resuspension. Test and control samples were always identically treated and were produced in pairs from the same citrate buffered sperm suspension.

Pretreatment

In some experiments the intact sperm cells were treated with agents that alter the cAMP response of the models subsequently produced. Two pretreatments were used in this study: 1) intact sperm in glutamate-Tris buffer were subjected to 2 mM KCN and 4 mM 2-deoxy-D-glucose (DOG) for 20 or 40 minutes past the cessation of all motility and 2) intact sperm in glutamate-Tris buffer were incubated for 20 minutes with 1 mM theophylline. After treatment the sperm were frozen to produce models.

cAMP Binding to Modeled Sperm Cells

The amount of cAMP bound to sperm was measured in centrifuged pellets of both frozen-thawed and Triton X-100-treated sperm models. A 3-ml suspension of modeled sperm was incubated with 10 μl of a solution of tritium-labeled cAMP made up to 0.1 μCi/μl (total addition of 1.0 μCi) to yield an effective cAMP concentration of $9.2 \times 10^{-9}$M. Following centrifugation for 30 minutes at 10,000g the supernatant solution was carefully collected and measured and the pellet was resuspended in 3 ml of fresh glutamate-Tris buffer. The 0.5 ml samples of the resuspended pellet and of the decanted supernatant were added to 5 ml of Aquasol 2 universal cocktail (New England Nuclear, Boston, MA) in scintillation vials. Radioactivity was assayed on a Packard model 3320 liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL). In one series of experiments the centrifuge tube was weighed dry before the experiment and weighed again after the supernatant was collected to estimate the weight of the pellet. The specific gravity of tightly packed pelleted sperm was also estimated by determining the buoyancy of sperm pellets in sucrose solutions of known specific gravity.

cAMP Binding to Soluble Cell Products

After thawing the sperm suspension was centrifuged for 30 minutes at 10,000g. A 100 μl sample of the supernatant solution was incubated for 5 minutes with 5 μl of tritiated cAMP (0.5 μCi) yielding a final cAMP concentration of $1.4 \times 10^{-7}$ M. The entire sample was incubated for 5 minutes and then layered onto a Sephadex G25-150 column (0.8 cm × 15 cm) that was equilibrated with glutamate-Tris buffer. The column was eluted with glutamate-Tris buffer. Six drops were collected in each of 30 scintillation vials. Five milliliters of Aquasol 2 universal cocktail was added to each vial and the radioactivity was assayed in the scintillation spectrometer.
The cAMP binding capacity of the sperm suspension solution was also tested in the presence of adenosine 5' monophosphate (AMP) and 3'-5' cyclic guanosine monophosphate (cGMP). AMP, cGMP, or unlabeled cAMP was added to the supernatant solution from the modeled sperm 5 minutes prior to adding tritium-labeled cAMP. Each test compound was added to a final concentration of 0.1 mM, which was 700 times the labeled cAMP concentration. The mixture was incubated for 5 minutes with labeled cAMP and then layered on a gel filtration column.

**Soluble cAMP Binding Component**

The elutant from Sephadex columns layered with 100 µl of sperm supernatant was tested for its ability to sensitize motile modeled sperm to cAMP. Frozen-thawed sperm models were used to test for motility response to cAMP after they were centrifuged and resuspended in fresh glutamate-Tris buffer. The sperm were activated with ATP, their motility was measured, and the sample was then treated with 0.5 ml of filtrate eluted from a Sephadex G25-150 column. After addition of the filtrate to the motile sperm sample 10 µl of 1 mM cAMP was also added and the motility was again measured. For these experiments material eluting from the columns was pooled into three samples: 1) drops 1–30, 2) drops 31–72, and 3) drops 84–135. The activity of the filtrate in each pooled sample was tested on motile sperm models.

The stability of the cAMP interaction was tested by adding unlabeled cAMP to 0.1 mM concentration to a 1.0 ml test sample of sperm suspension medium 5 minutes after tritiated cAMP had been added. The sample was then incubated at ambient temperature and 0.1-ml aliquots were removed at intervals and subjected to gel filtration. The amount of label eluting as a bound complex was analyzed as a function of incubation time.

The temperature sensitivity of the cAMP binding factor was measured by immersing test tubes with 0.1-ml samples of the supernatant solution from the modeled sperm into a water bath at the desired temperature and incubating the sample for 5 minutes. This procedure was performed with the labeled cAMP added prior to incubation and with the labeled cAMP added after incubation of the sample at temperature.

The concentration of sperm in our preparations was determined using a microscope stage counting chamber (improved Neubauer type, Scientific Products, McGraw Park, IL).

**Reagents**

Tritium-labeled cAMP [2,8-3H]- was obtained from New England Nuclear (Boston, MA) in the form of the ammonium salt in 1:1 ethanol:water, with an initial specific activity of 36 Ci/m mole. The 250 µl initial volume was diluted to 2.5 ml by addition of 2.25 ml of 1:1 ethanol:water solution and was stored at -20°C. Aquasol 2 scintillation cocktail was also obtained from New England Nuclear (Boston, MA). cAMP, cGMP, AMP, dithiothreitol, Triton X-100, Sephadex G25-150, and Trizma base were all obtained from Sigma Chemical Company (St. Louis, MO). ATP was obtained from Boehringer Mannheim Corporation (New York, NY). All solutions were prepared with distilled and deionized water. The tritiated cAMP was cochromatographed with AMP and cold cAMP on PEI cellulose 300 thin layer chromatog-
raphy plates to rule out the possibility that an appreciable amount of tritiated AMP could be present in the sample. A 0.85 M KH₂PO₄ solution at pH 3.9 was used as the running buffer for this chromatography.

RESULTS

CAMP Binding Studies

Very little tritium labeled cAMP is bound to sperm models when these models are recovered in the pellet after centrifugation. An average of 1.2% of the total radioactivity was observed in the sperm pellets in a total of 37 experiments, Table I. Moreover, no significant difference in cAMP binding was observed when Triton X-100 extracted models were compared to frozen-thawed models. This suggests that the plasma membrane is not the site of binding because much of the membrane is still present in frozen-thawed sperm, whereas the membranes are removed from the Triton -extracted sperm. The sensitivity of modeled sperm to cAMP is increased by inhibiting the sperm with KCN and DOG prior to modeling the cells [Lindemann, 1978]. Table I shows that no difference in bound cAMP can be attributed to the pretreatment of the sperm samples with KCN and DOG before modeling the cells. In 11 experiments sperm motility (frequency and percent of moving cells) was measured in conjunction with measurements of labeled cAMP binding to the cells. We confirmed that KCN-DOG pretreated frozen-thawed models exhibit a greater motility response to cAMP than untreated sperm models; however, the sperm in the treated and untreated samples did not show a significant difference in cAMP binding.

The molar quantity of cAMP bound to the sperm cells was estimated. In 11 experiments the volumes of the pellets were determined as well as the amount of label present in the pellets. The pellet volumes were found from the pellet weights and a determination of the specific gravity (1.06) of pelleted sperm. The fraction of the total label associated with the pellets was 0.0133±0.0027, and the fraction of the sample volume that was present in the pellet was 0.0077±0.0013. If a uniform distribution of label is assumed, then the relative labeling should approximate the division of volume between pellet and supernatant. Subtracting this contribution to the label in the pellets we find an excess label present in the pellets equal to 0.0056 of the total label in the sample. The total dosage of cAMP in each experiment was

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Number of experiments</th>
<th>Fraction° of label in pellets (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100® extracted sperm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with KCN-DOG® pretreatment</td>
<td>3</td>
<td>0.0123 ± .003</td>
</tr>
<tr>
<td>without KCN-DOG pretreatment</td>
<td>6</td>
<td>0.0111 ± .003</td>
</tr>
<tr>
<td>Frozen-thawed sperm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>With KCN-DOG® pretreatment</td>
<td>12</td>
<td>0.0116 ± .004</td>
</tr>
<tr>
<td>Without KCN-DOG pretreatment</td>
<td>16</td>
<td>0.0126 ± .005</td>
</tr>
</tbody>
</table>

°Counts in the pelleted sperm divided by the total counts in the sample.
®Sperm were treated with 0.1% Triton X-100.
®Sperm were treated with 2 mM KCN and 4 mM 2-deoxy-D-glucose before being modeled. They remained in the presence of inhibitor for 40 minutes after loss of motility and were then used to produce sperm models. The inhibition was conducted at 23°C.
2.8 \times 10^{-11} \text{ moles}, which yields a value for bound cAMP of 1.6 \times 10^{-13} \text{ moles/pellet.}

To test the hypothesis that cAMP interacts with a soluble component of the sperm the supernatant solution from sperm models pelleted by centrifugation was treated with tritiated cAMP and subjected to gel filtration on Sephadex G25-150. The sharp peak of radioactivity that coincides with the void volume indicates the presence of a high molecular weight cAMP complex (Fig. 1). The curves shown are the elution profiles of supernatant solutions from frozen-thawed sperm (B) and from the same preparation of sperm prior to freezing (A). An average of 8.4\% of the label eluted in the bound fraction in ten experiments with frozen-thawed sperm models (Table II). Little or no cAMP complex was present in the supernatant solutions of unfrozen sperm. The amount of cAMP complex that eluted in the void volume was not significantly altered by the presence of AMP in the sample, but was substantially reduced in the presence of cGMP (Table II). The percentage of bound cAMP was only slightly reduced when the supernatant solutions were subjected to 700 times more AMP than labeled cAMP. A stronger interference was noted when the same excess of cGMP was present. The presence of unlabeled cAMP was most effective and completely prevented binding of labeled cAMP. The suspension solutions from intact sperm that were never frozen exhibited little binding activity (Table II). The small amount of binding activity that was present in these samples could have resulted from damage to the cells during centrifugation and resuspension.

Our initial experiments to locate cAMP binding were performed with sperm that received KCN-DOG treatment prior to modeling. A series of three experiments...
were also conducted to test the importance of the KCN-DOG treatment to the cAMP binding results. Pretreatment with KCN-DOG was compared to a 20-minute pretreatment with 1 mM theophylline. Theophylline pretreated cells exhibit stimulated respiration and motility, while KCN-DOG treated cells lose all respiration and motility. In spite of the difference in motility, we found no difference in the amount of free cAMP-binding factor in sperm exposed to the two pretreatment regimens.

The stability of the complex formed by cAMP and the binding agent was examined by monitoring the decay of the complex at various times after the addition of a 700-fold excess of cold cAMP. Three determinations were made with the best determination shown in Figure 2. The decay curve seems to be bimodal with the more stable component accounting for about 14% of the total. The half-life estimated from the first part of the curve is approximately 1.0 hours and from the second part of the curve is approximately 250 hours.

The binding compound is heat sensitive at temperatures greater than 50°C. Rapid decay of the bound complex was observed at 60°C or 75°C. The ability of the soluble factor to bind cAMP also decreased when the supernatant solution was subjected to temperatures of 60°C and 75°C for 5 minutes and then cooled prior to the addition of labeled cAMP. The degree of deactivation was greater with increasing temperature above 50°C. When the supernatant solutions were subjected to 5 minutes incubation at 75°C all binding activity was lost.

**Motility Experiments**

The frozen-thawed sperm responded to cAMP with consistent increases in the flagellar beat frequency and the percentage of motile cells in the sample (Table III). Pretreating the cells before freezing with 2 mM KCN and 4 mM DOG for 40 minutes past the cessation of motility enhanced the cAMP response of models subsequently produced (this data is also in Table III). The removal of the original solution in which the sperm were frozen and thawed decreased the responsiveness of the sperm to cAMP. This finding also held true with KCN-DOG pretreated sperm (Table III).

When frozen-thawed sperm were centrifuged, decanted, and then resuspended in the supernatant from a second sample of sperm, the sperm showed a high degree of sensitivity to cAMP (Table IV). This test system was used to determine the
Fig. 2. The decay of bound cAMP complex at 23°C. The fraction of bound cAMP in samples of sperm suspension medium was determined by gel filtration on Sephadex G25-150 columns. An initial determination was made 5 minutes after the addition of labeled cAMP. Subsequent determinations were made at various time intervals after the addition of unlabeled cAMP to the mixture (0.1 mM final concentration). The 100 μl aliquots were withdrawn from the incubating mixture for each determination of labeled complex. The fraction of bound complex present at each determination is shown as a function of the incubation time of the sample in the presence of excess unlabeled cAMP. The numbers displayed near the curve are the half-lives estimated from the two parts of the decay curve.

The biological activity of the supernatant solution from frozen-thawed sperm following elution from a Sephadex column (see Table IV). The material eluted from the column before the binding peak had no effect on restoring the cAMP response. Material eluting in drops 84–135 also had no effect. Material eluting between drops 31 and 72, the region of the binding peak, did restore the sensitivity of the sperm to cAMP. An average increase in motility of 8 percentage points and a 20% increase in beat frequency was obtained. Nevertheless, this increase in motility was not as large as was observed with unfractionated supernatant. These findings all support a role for a soluble factor in the cAMP response of modeled sperm.

DISCUSSION

cAMP influences sperm motility by interacting with a soluble constituent released from modeled sperm. The evidence supporting this conclusion can be summa-
TABLE III. Response of Frozen-thawed Bull Sperm to CAMP After Centrifugation and Resuspension*

<table>
<thead>
<tr>
<th>Added supplement</th>
<th>Number of experiments</th>
<th>Percent motile (Average ± SD)</th>
<th>Frequency (Average ± SD)</th>
<th>Percent motile (Average ± SD)</th>
<th>Frequency (Average ± SD)</th>
<th>Motility (Percent points)</th>
<th>Frequency (Hertz)</th>
</tr>
</thead>
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<tr>
<td>No pretreatment</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (same medium)</td>
<td>8</td>
<td>38 ± 10</td>
<td>3.5 ± 1.1</td>
<td>45 ± 11</td>
<td>3.9 ± 1.1</td>
<td>+7</td>
<td>+0.4</td>
</tr>
<tr>
<td>Test (new medium)</td>
<td></td>
<td>40 ± 10</td>
<td>3.5 ± 1.2</td>
<td>32 ± 11</td>
<td>3.4 ± 1.1</td>
<td>−8</td>
<td>−0.1</td>
</tr>
<tr>
<td>KCN-DOG pretreated*</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (same medium)</td>
<td>8</td>
<td>24 ± 9</td>
<td>2.8 ± 1.1</td>
<td>39 ± 13</td>
<td>4.0 ± 1.2</td>
<td>+15</td>
<td>+1.2</td>
</tr>
<tr>
<td>Test (new medium)</td>
<td></td>
<td>34 ± 10</td>
<td>3.2 ± 1.2</td>
<td>35 ± 12</td>
<td>3.3 ± 1.2</td>
<td>+1</td>
<td>+0.1</td>
</tr>
</tbody>
</table>

*All experiments were done in pairs consisting of a control and test sample prepared from the same sperm stock and subjected to the same regime of centrifugation and resuspension. Motility was observed at 23°C.

*Sperm were treated with 2 mM KCN and 4mM 2 deoxy-D-glucose. They were inhibited for 40 minutes after loss of motility before being frozen.

TABLE IV. Restoration of cAMP Sensitivity to Centrifuged and Resuspended Frozen-thawed Sperm*

<table>
<thead>
<tr>
<th>Added supplement</th>
<th>Number of experiments</th>
<th>Percent motile (Average ± SD)</th>
<th>Frequency (Average ± SD)</th>
<th>Percent motile (Average ± SD)</th>
<th>Frequency (Average ± SD)</th>
<th>Motility (Percent points)</th>
<th>Frequency (Hertz)</th>
</tr>
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<tr>
<td>Whole supernatant from frozen sperm (3 ml)</td>
<td>6</td>
<td>14 ± 12</td>
<td>1.8 ± .5</td>
<td>34 ± 11</td>
<td>2.6 ± .9</td>
<td>+20</td>
<td>+0.8</td>
</tr>
<tr>
<td>Sephadex filtrate drops 1-30 (0.5 ml)</td>
<td>5</td>
<td>17 ± 9</td>
<td>3.7 ± .2</td>
<td>16 ± 7</td>
<td>4.0 ± .9</td>
<td>−1</td>
<td>+0.3</td>
</tr>
<tr>
<td>Sephadex filtrate drops 31-72 (0.5 ml)</td>
<td>10</td>
<td>21 ± 17</td>
<td>3.6 ± 1.1</td>
<td>29 ± 15</td>
<td>4.3 ± .8</td>
<td>+8</td>
<td>+0.7</td>
</tr>
<tr>
<td>Sephadex filtrate drops 84-135 (0.5 ml)</td>
<td>4</td>
<td>22 ± 6</td>
<td>3.8 ± 1.1</td>
<td>16 ± 6</td>
<td>3.4 ± 1.0</td>
<td>−6</td>
<td>−0.4</td>
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</table>

*All sperm used in these tests were KCN-DOG inhibited for 20 minutes and then disrupted by freezing and thawing. After thawing, the samples were centrifuged and the sperm resuspended into fresh reactivation medium before using the sperm to test for cAMP sensitivity.

*After centrifugation the sperm pellet was resuspended in media removed from a second sample of modeled cells that were prepared and centrifuged in the same way.
Action of cAMP in Sperm

Action of cAMP in Sperm was summarized as follows: 1) cAMP interacts weakly with the bodies of modeled sperm and with the membranes of modeled sperm; 2) cAMP reacts strongly with a high molecular weight component found in the suspension medium of disrupted sperm; 3) sperm deprived of the original suspension media in which they were modeled no longer show a motility response to cAMP; and 4) sperm deprived of their original suspension medium will respond to cAMP in the presence of the suspension media obtained from other modeled sperm, or high molecular weight material separated by gel filtration.

The observed binding of cAMP to the structural protein of the sperm was shown to be minimal; even when the concentration of labeled cAMP was 1/1000, the cAMP needed to experimentally saturate the motility response [Lindemann, 1978]. Even so, the percentage of the cAMP that was bound was less than 1% of the total. The amount of cAMP sequestered by the sperm averaged $8 \times 10^3$ molecules per sperm based on the average of the unaccounted for excess cAMP given in Results, and the concentration of sperm in the stock solution. The low level of cAMP binding to the sperm cells or to the sperm membrane appears to be insignificant when the absolute amounts of cAMP involved are considered. The small amount of radioactivity associated with the pelleted sperm could easily be caused by an interaction of complexed cAMP with the sperm structure. This would be consistent with the binding factor forming an association with a protein kinase. Alternately a small amount of nonspecific binding may also be taking place between the structural components of the sperm and the labeled cAMP.

A much larger fraction of the total radioactivity was bound to high molecular weight soluble material in the sperm suspension. An estimate of the number of moles of bound cAMP in the filtered mixture (based on the average figure given in Table II) yields a value of $1.2 \times 10^{-12}$ moles. The average number of sperm contributing binding factor for the same sample was approximately $1.5 \times 10^7$ in these experiments based on counts of sperm concentration. Therefore approximately $8 \times 10^{-20}$ moles of cAMP were bound per sperm ($5 \times 10^4$ molecules per sperm).

Despite the differences in the experimental protocol for measuring cAMP binding to sperm cell bodies and to soluble factors, the ratio of moles of cAMP to numbers of sperm was fairly similar in both experimental regimes. In the pelleted sperm experiments the ratio was approximately $1.0 \times 10^{-6}$ mg cAMP/1 $\times 10^6$ sperm and in the soluble factor experiments $0.33 \times 10^{-6}$ mg cAMP/1 $\times 10^6$ sperm. For this reason the difference in the total amount of cAMP sequestered by the soluble binding factor and by the sperm cell bodies should constitute a fair comparison.

The cAMP binding component we have isolated from modeled sperm is a high molecular weight constituent. It preferentially binds the cyclic form of AMP and is specific for cyclic AMP over cyclic GMP. The stability of the complex that is formed with cAMP suggests a tight binding. The stability data also indicates that two components or two binding sites are present in the mixture with 86% of the binding being in the form of a complex with a half-life of approximately 1.0 hour and 14% having a half-life of 250 hours. The complex formed with cAMP is heat sensitive; all binding activity is lost after 5 minutes at 75°C. These results are consistent with the binding agent being one or more proteins.

The soluble components we have implicated in the motility response are very likely the same ones previously described by Garbers et al [1973]. They reported that soluble protein kinases could be isolated from bull sperm which complexed with a cAMP binding factor. Very recently Tash and Means [1981, 1982] identified specific subunits of the sperm that are phosphorylated when cAMP is added to a detergent
extracted model system. One of the phosphorylated components is tubulin, a molecule that plays an integral part in the force producing mechanism of the flagellar axoneme. If the site phosphorylated is vital to the dynein-tubulin interaction responsible for microtubular sliding, it would explain how cAMP production could change motility.

Our results indicate that the cAMP binding factor we have identified by gel filtration is involved in the motility response. Our evidence suggests that the primary interaction of cAMP with a soluble intermediate directly effects motility. Apparently the chemical pathway leading to motility activation is still functional in the modeled sperm system in spite of the extensive dilution of the cellular constituents after the cell membranes have been disrupted. Direct binding of cAMP to the flagellar structure or to the plasma membrane appears to be less important to the motile response.

ACKNOWLEDGMENTS

We wish to thank Dr. Berlin Kagy and the staff at NOBA Inc. for their help, patience, and cooperation. We also wish to thank Dr. Arun K. Roy who suggested we use gel filtration to identify cAMP binding components of our system. This work was supported by NICHD grant number HD 12535 and a Biomedical Science support grant from Oakland University.

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