Evidence that cAMP-dependent Protein Kinase and a Protein Factor Are Involved in Reactivation of Triton X-100 Models of Sea Urchin and Starfish Spermatozoa

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ABSTRACT A fraction obtained from detergent-extract of sea urchin or starfish spermatozoa using DEAE-cellulose chromatography reactivated Triton X-100 models of the spermatozoa in a cAMP-dependent manner. The DEAE fraction contained cAMP-dependent protein kinase with a high level of specific activity. Rabbit muscle inhibitor protein highly specific for cAMP-dependent protein kinases inhibited the ability of the DEAE fraction to induce reactivation of Triton X-100 models. This inhibition paralleled inhibition of cAMP-dependent protein kinase activity of the DEAE fraction, suggesting participation of the enzyme in the cAMP-dependent reactivation of Triton X-100 models. However, cAMP-dependent protein kinase further purified from the DEAE fraction was incapable of reactivating these models by itself. A protein factor which was separated from the protein kinase in the course of purification of the enzyme was found to also be necessary for the reactivation. When cAMP-dependent protein kinase was pretreated with protein kinase inhibitor before addition of the protein factor, the reactivation of Triton X-100 models was no longer detected. However, after the protein factor had been incubated with cAMP and cAMP-dependent protein kinase, protein kinase inhibitor did not repress reactivation of Triton X-100 models. We propose that the reactivation needs phosphorylation of the protein factor by cAMP-dependent protein kinase.
Materials and Methods

Spermatozoa of sea urchins (Anthocidaris crassispina, Pseudocentrotus depressus, and Hemicentrotus pulcherrimus) and those of starfish (Asterias amurensis) were used as experimental materials. The sea urchin spermatozoa were collected by injecting 0.5 M KCl into the coelom of the animal, and were stored as dry sperm until use. The starfish spermatozoa were obtained according to the method described by Mabuchi et al. (17).

Preparation of Sperm TXM and Definition of the Index of Motility

Dry sperm were diluted with 20 vol of Ca-free sea water at 25°C to make a stock sperm suspension, which was used within 4 h. A 30-μl portion of this suspension was added to 0.5 ml of an extraction medium (0.04% Triton X-100 and 1 mM DTT) containing 10 mM Tris-HCl, 2 mM MgSO4, 150 mM KCI, 0.1 mM EDTA, pH 8.1 (TMKE) at room temperature and gently swirled for 30 s. A 30-μl portion of the extracted sperm suspension was diluted with 3 ml of an ATP-containing medium (1 mM ATP and 1 mM DTT in TMKE), immediately followed by observation of TXM in 30 μl of the above ATP-containing medium without a cover slip. Dark-field illumination was used for the measurement of motility, an index of which was determined as % motility, defined as % motility = (the number of motile TXM/total TXM) × 100. When the effects of some fractions on the motility of TXM were studied, those dialyzed against TMKE containing 0.1 mM DTT were added to 2 vol of TXM suspension in the ATP-containing medium, and % motility was calculated. % Motility in the absence of cAMP ([–]cAMP) was a value at 5 min after each fraction was added, and % motility in the presence of cAMP ([+]cAMP) was that at 3 min after the final addition of cAMP, unless otherwise indicated.

Extraction of Soluble Materials from Spermatozoa and Preparation of DEAE-adsorbed Fraction

Unless otherwise mentioned, preparations were performed at 0-4°C. Spermatozoa were washed twice with Ca-free sea water. Packed wet spermatozoa were then homogenized with eight strokes in 2 vol of a solution containing 0.2% Nonidet P-40, 0.1 mM ATP, 0.1 mM DTT, 1 mM EGTA, and TMKE, followed by centrifugation at 12,000 g for 40 min. The supernate was stored at −80°C until use. The thawed supernate was dialyzed against two changes of a buffer solution (40 vol) consisting of 0.1 mM DTT and 20 mM Tris·HCl (pH 8.1), and then centrifuged at 100,000 g for 1 h to remove aggregates. The 100,000 g supernate called sperm extract was charged on a DEAE-cellulose column pre-equilibrated with the same buffer solution. After the column was thoroughly washed with the same solution to remove Nonidet P-40, the adsorbed materials were eluted with 0.5 M KCl containing 0.1 mM DTT and 20 mM Tris·HCl (pH 8.1) to obtain the DEAE-adsorbed fraction.

Protein Kinase Assay

Protein kinase activity was assayed by the method reported by Murofushi (18) using calf thymus whole histones as substrate. 1 U of the activity was defined as the amount of enzyme which catalyzes transfer of 1 pmol 32P/min from ATP to histone in the standard assay system at 25°C.

Partial Purification of cAMP-dependent Protein Kinase

The sperm extract of Hemicentrotus (250 mg protein) was chromatographed on DEAE-cellulose column (2.3 x 41 cm) equilibrated with 20 mM Tris·HCl (pH 8.1) containing 0.1 mM DTT. By elution with a linear concentration gradient of KCl from 0 to 0.5 M, a single peak of the activity for cAMP-dependent protein kinase was detected at a position of 0.15 M. This fraction was dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM DTT, and further chromatographed on a hydroxyapatite column (1.5 x 26 cm) equilibrated with the same solution. Adsorbed materials were eluted with a linear gradient of phosphate buffer from 20 mM to 0.2 M. The enzyme was eluted with 120 mM phosphate buffer. For further purification, the fraction was condensed by ultrafiltration and with Aquacide, and subjected to gel filtration using an apparatus for high-speed liquid chromatography (HLC-803 system equipped with two G 3000 SW columns in series (each 7.5 x 600 mm), Toyo Soda Manufacturing Co.). The column was pre-equilibrated with TMKE containing 0.1 mM DTT. The enzyme emerged just after the void volume fraction and was used as a partially purified cAMP-dependent protein kinase. The purification steps are summarized in Table I.

Partial Purification of Protein Kinase Inhibitor

Protein kinase inhibitor was partially purified from rabbit skeletal muscle by the method of Walsh et al. (19), except that the final gel filtration step was omitted. 1 U of inhibitor activity was defined as the amount which inhibits 2 U of the protein kinase activity by half. The specific activity of the inhibitor finally obtained was 36,000 U/mg protein.

Others

The amount of protein was determined by the method of Lowry et al. (20) using bovine serum albumin as a standard. Hydroxyapatite was prepared following the method of Mains et al. (21). [γ-32P]ATP was prepared according to the method described by Glyn and Chappell (22). DEAE-cellulose column chromatography was performed using DE-52 from Whatman. A Diaflo membrane PM-10 from Amicon Corp. (Scientific Sys. Div., Lexington, Mass.) was used for ultrafiltration. ATP and cAMP were obtained from Kyowa Hakko Co. (Tokyo), 5'-AMP and cGMP were from Kohjin Co. (Tokyo) and Boehringer Mannheim Biochemicals (Indianapolis, Ind.), respectively. Trypsin, soybean trypsin inhibitor and calf thymus whole histones were purchased from Sigma Chemical Co. (St. Louis, Mo.). Aquacide was from Calbiochem-Behring Corp. (American Hoeschi Corp., La Jolla, Calif.).

Results

Cyclic AMP-dependent Reactivation of TXM

TXM prepared by the present procedure from Pseudocentrotus spermatozoa gradually ceased to undulate (Fig. 1). Cyclic AMP alone did not show any effect on motility of models. TXM quickly stopped beating when the DEAE-adsorbed fraction of the sperm extract was added, but began to beat after

Table I

| Purification of cAMP-dependent Protein Kinase from Sea Urchin Spermatozoa |
|--------------------------------------------------|
| Fraction                          | Total protein | Total activity | Specific activity | Yield (%) |
|-----------------------------------|---------------|---------------|------------------|----------|
| Sperm extract                     | 250           | 40,000        | 160              | 100      |
| DEAE-cellulose fraction           | 13            | 28,000        | 2,200            | 70       |
| Hydroxyapatite fraction           | 3.4           | 11,000        | 3,200            | 28       |
| High-speed liquid chromatography fraction | 0.31          | 2,900         | 9,400            | 7        |

Protein kinase activity was measured using the standard assay mixture.

Figure 1

Time-course of changes in % motility of TXM in the presence or absence of DEAE-adsorbed fraction and motility reactivation by cAMP. O, control, without addition of DEAE-adsorbed fraction. ●, 20 μg of DEAE-adsorbed fraction was contained in 30 μl of TXM suspension. Cyclic AMP (10 μM) was added at the point indicated by the arrow.
further addition of CAMP at a final concentration of 10 μM. When TXM were added to the mixture of the DEAE-adsorbed fraction and cAMP, a high level of % motility was maintained. Cyclic AMP-induced reactivation was also observed in TXM of *Anthocidaris*, *Hemicentrotus*, and *Asterias* spermatozoa. The DEAE-adsorbed fraction from *Pseudocentrotus* or *Asterias* spermatozoa could cross-react with TXM of *Hemicentrotus* spermatozoa, suggesting that the factors involved in the DEAE-adsorbed fraction are not species-specific. The DEAE-adsorbed fraction used in the experiments described below was from *Pseudocentrotus* spermatozoa, and the TXM mainly from *Hemicentrotus* spermatozoa.

**Figure 2** Effect of the concentration of DEAE-adsorbed fraction on % motility in the presence of 10 μM CAMP. (a) Time-course of % motility after CAMP addition. Figures on each curve indicate the amount of protein in DEAE-adsorbed fraction added in μg/30 μl. (b) Relationship between the plateau levels of % motility and concentrations of DEAE-adsorbed fraction. The plateau levels of % motility vs. concentrations of DEAE-adsorbed fraction were plotted from the data shown in a.

It should be mentioned here that TXM of sea urchin sperm flagella without the head and middle piece were also reactivated to undulate by CAMP in the presence of the DEAE-adsorbed fraction. The plateau level of % motility after CAMP addition was found to be dependent on the amount of the DEAE-adsorbed fraction added to the models (Fig. 2a). When the plateau levels were plotted vs. the amounts of the DEAE-adsorbed fraction, a linearity was observed up to 15 μg of the DEAE-adsorbed fraction (Fig. 2b), although the full activation level changed from one batch of spermatozoa to another. The data shown in Fig. 2 indicate that the mode of the reactivation caused by the DEAE-adsorbed fraction with cAMP was stoichiometric rather than catalytic. Fig. 2a also shows that the addition of >15 μg of the fraction causes decline of % motility after reaching maximal levels.

Cyclic AMP at low concentration preferentially elevated % motility (Fig. 3). Half-maximal reactivation occurred at 0.3 μM. Cyclic guanosine monophosphate (cGMP) increased % motility only at higher concentrations, revealing the concentration for the half-maximal reactivation to be on the order of 10 μM. 5'-AMP was inactive. It is evident that the reactivation is specific for cAMP.

When the movement of TXM was repressed in the presence of the DEAE-adsorbed fraction, the addition of 1 mM ATP never initiated the beating. The occurrence of the motionless states of TXM was not due to exhaustion of ATP in the

**Figure 3** Nucleotide specificity for the reactivation of TXM. % motility was determined in the presence of 39 μg DEAE-adsorbed fraction/30 μl at 3 min after the addition of various concentrations of the following nucleotide: CAMP (●); cGMP (○); 5'-AMP (△); and ATP (□).

**Figure 4** Effect of protein kinase inhibitor on protein kinase activity in DEAE-adsorbed fraction (a) and on % motility (b). Protein 329 kinase activity was measured in the same condition as that used for kinase activity was measured in the same condition as that used for motility determination, except for the addition of histones as substrate and the omission of TXM. Protein kinase inhibitor activity represents the values in 30 μl of the assay system. O, (−) CAMP; ●, (+) CAMP.

**Figure 5** DEAE-cellulose column chromatography of sperm extract. Sperm extract of *Hemicentrotus* (508 mg protein) was applied on a DEAE-cellulose column (3.4 × 30 cm) equilibrated with 20 mM Tris-HCl (pH 8.1) containing 0.1 mM DTT. After unadsorbed materials were washed out with ~400 ml of the same solution, elution was carried out by a linear concentration gradient of KCl from 0 to 0.5 M in 20 mM Tris-HCl (pH 8.1) and 0.1 mM DTT, total 300 ml, collecting fractions of 9.5 ml. (a) Elution profile. Protein kinase activity was assayed using the standard assay mixture. A90 (k = −x). KCl concentration (x = −). Protein kinase activity (−−−). (b) Protein kinase activity in 30 μl of assay solution for TXM reactivation system. (c) Activity of each fraction in enhancing % motility. □, (−) CAMP; ○, (+) CAMP.
Participation of cAMP-dependent Protein Kinase in Reactivation of TXM

The action of cAMP is generally mediated through cAMP-dependent protein kinase (23). This suggested to us that the cAMP-dependent reactivation of TXM was also the case. In fact, the DEAE-adsorbed fraction contained this enzyme with a high level of specific activity (177 U/mg), and the medium condition for TXM reactivation strongly supported the enzyme activity. In addition, the reactivation profiles obtained by changing concentration of cAMP and cGMP (Fig. 3) closely resembled those obtained for cAMP-dependent protein kinases from sperm flagella of sea urchin (9) and from Tetrahymena cilia (18).

To confirm that this cAMP-dependent reactivation was driven by cAMP-dependent protein kinase, we employed an inhibitor protein which is highly specific for protein kinases from a variety of sources (19). When the inhibitor protein was added to the DEAE-adsorbed fraction, % motility of TXM did decrease in parallel with inhibition of the cAMP-dependent protein kinase activity in the DEAE-adsorbed fraction (Fig. 4). The parallel relation strongly indicates that the enzyme actually participates in the reactivation. It should be noted here that protein kinase inhibitor by itself did not affect the motility of TXM at all.

We partially purified cAMP-dependent protein kinase from the sperm extract of Hemicentrotus (Materials and Methods) to obtain further evidence on the role of this enzyme in TXM reactivation. When 17 U of the kinase (high-speed liquid chromatography fraction) was added to TXM suspension in a total volume of 30 μl, % motility was 12% for (−) cAMP, and 10% for (+) cAMP. This indicated clearly that the partially purified enzyme fraction alone was not capable of reactivating TXM.

Participation of Regulatory Factor in Reactivation of TXM

Since the partially purified enzyme was incapable of reactivating the models, it was reasonable to assume that some factor other than cAMP-dependent protein kinase was further necessary for the reactivation and that it was removed in the course of purification of the enzyme. This possibility was also suggested by the data indicating that the mode of action was not catalytic but stoichiometric (Fig. 2). Upon measuring the ability of all the enzyme fractions obtained at each purification step to enhance the motility of TXM in a cAMP-dependent manner, it became clear that the enzyme fraction obtained at the hydroxyapatite column chromatography step no longer reactivated TXM in the presence of CAMP. Therefore, we focused on the fractions obtained by DEAE-cellulose chromatography to find some regulatory factor for reactivation of TXM. Fig. 5 indicates the data at the step of DEAE-cellulose chromatography. The activity of cAMP-dependent protein kinase emerged from fraction 71 to fraction 80 as a single peak, which was divided into left and right halves (fractions 71–75 and fractions 76–80). The former half, showing the highest activity of the enzyme, reactivated TXM with about 1.7% motility in the presence of cAMP. But the latter half, showing the second highest enzyme activity, reactivated TXM with about 0.09% motility in the absence of cAMP.

| Hyaluronate fraction | I | II | III | IV | V |
|----------------------|---|----|-----|----|---|
| Protein kinase activity* | 0.01 | 0.03 | 0.17 | 0.06 | 0.01 |
| Activity for inducing reactivation (% motility) | 12 | 13 | 14 | 10 | 19 |
| Activity for inducing reactivation by supplementing fraction III‡ (% motility) | 24 | 23 | 35 | 39 | 66 |

* Values in 30 μl of assay system for motility. ‡ Contained 3.7 U of protein kinase activity.

Figure 6 Hydroxyapatite column chromatography of fractions 71–75 obtained by DEAE-cellulose column chromatography. Fractions 71–75 (40 mg) were loaded on a hydroxyapatite column (1.5 × 26 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM DTT. After the column was washed with 75 ml of the same buffer solution, adsorbed materials were eluted with a linear concentration gradient of phosphate buffer (from 20 mM to 0.2 M, total 60 ml) and then with 0.5 M phosphate buffer. Each fraction was 5 ml. The roman numerals in the figure indicate fractions combined. A280 (x-x). Phosphate buffer concentration (−−−−−−). Protein kinase activity (●●●●●●).
enzyme fraction, were obtained (Fig. 6). However, none of these fractions had the ability to reactivate Triton models (Table II). This was consistent with the above result, that the enzyme purified by hydroxyapatite chromatography did not reanimate the movement of TXM. We concluded from this result that the kinase (fraction III) was separated from the factor at this step. Recombination experiments revealed that it was fraction V which showed the most pronounced reactivation of TXM in the presence of fraction III (Table II). In addition, fraction V was sensitive to both proteolytic and heat treatments (Table III). These results showed that reactivation required two factors present in fractions from the DEAE-cellulose column: a cAMP-dependent protein kinase in fraction III and a protein factor in fraction V.

Furthermore, it was suggested that cAMP-dependent phosphorylation of the protein factor supported the reactivation of the TXM. The following experiment (Fig. 7) was carried out to study this possibility. Fraction III was first mixed with ATP, cAMP, and Mg²⁺, then mixed with fraction V or kinase inhibitor and incubated for a period of 30 s; the inhibitor or fraction V, respectively, was then added. A TXM suspension was finally added to this mixture. Three combinations of the experiment are shown in Fig. 7 in comparison with a control ([-] cAMP). (1) When TXM were added to the mixture of fraction III and V without addition of the kinase inhibitor, a pronounced reactivation was consistently observed. (2) Addition of the kinase inhibitor to fraction III before mixing in fraction V suppressed to a great extent the reactivation of TXM successively added. (3) In contrast, when the addition of kinase inhibitor was preceded by mixing fraction V with fraction III, significant reactivation was detected— that is, the inhibitor no longer greatly decreased the beating of TXM. We can assume that phosphorylation of the protein factor in fraction V was maintained in the first case; that, in contrast, phosphorylation of the protein factor was kept inhibited in the second case; and that phosphorylation of the protein factor had occurred before the kinase inhibitor was added in the third case. This also suggested that cAMP-dependent phosphorylation of some components in the TXM itself was not needed for the reactivation.

DISCUSSION

Here we report motionless TXM of sea urchin and starfish spermatozoa which showed cAMP-dependent reactivation. We point out that our TXM are essentially the same as the quiescent ones reported by Gibbons and Gibbons (24): (a) its low % motility was not due to exhaustion of ATP; (b) the microscopic image of the motionless models before reactivation by cAMP resembled the quiescent ones reported by Gibbons and Gibbons; and (c) potentially asymmetric TXM prepared by their method also showed cAMP-dependent reactivation in the presence of the DEAE-adsorbed fraction (data not shown).

The cAMP-dependent reactivation of TXM seems to be mediated by cAMP-dependent protein kinase, because (a) the DEAE-adsorbed fraction contained this enzyme with a specific activity of 177 U/mg, (b) the model reactivation was highly specific for cAMP, (c) the medium condition for the reactivation supported the enzyme activity, and (d) % motility of TXM was depressed in parallel with inhibition of the cAMP-dependent protein kinase activity by protein kinase inhibitor.

Moreover, the results indicated below support the conclusion that a factor other than the enzyme is also necessary for the reactivation: (a) The mode of action of the DEAE-adsorbed fraction seems to be not catalytic but stoichiometric. (b) Partially purified enzyme fraction alone is not capable of reactivating TXM. (c) When mixed back with the protein factor fraction free from cAMP-dependent protein kinase activity, the enzyme fraction regained the ability to reactivate TXM in a cAMP-dependent manner. The experiments using the protein kinase inhibitor present the hypothesis that phosphorylation of the regulatory protein factor in fraction V results in reactivation of TXM. Purification and identification of the protein factor and the mode of its action remain to be solved.

We have noticed that cAMP sometimes does not affect TXM prepared from very fresh spermatozoa. This is consistent with the results obtained by Gibbons and Gibbons (1), who reported that addition of cAMP had no effect on the motility of sea urchin sperm TXM. However, it is suggested that preincubation of spermatozoa at room temperature made their TXM susceptible to cAMP-dependent reactivation for beating. This might be due to dephosphorylation of the protein factor to some extent during preparation of TXM, which leads to quiescent state of the models.

Studies of ciliary and flagellar movement have hitherto focused only on the axoneme, especially on the interactions between microtubules and dynein accompanying sliding among groups of outer fibers. In contrast, this paper gives evidence that detergent solubilizes factors necessary for the regulation of flagellar movement.

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