Effects of Acylpeptide K-26 on the Motility of Sea Urchin Sperm Model. I. Inhibition of the Motility of Both Live and Triton X-100-Extracted Sperm

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ABSTRACT. An acylpeptide called K-26, which was isolated from a culture filtrate of Bacillus sp. 503, inhibited motility of both live sea urchin sperm as well as Triton X-100-extracted sperm model. In each case, complete inhibition was observed at concentrations of more than 1 µM or 6 µM, respectively. This inhibitory effect of K-26 on the Triton-extracted sperm could not be reversed by reducing the concentration of K-26 in the reactivation medium containing 1 mM ATP. However, it was reversed when the ATP concentration in the reactivation medium was simultaneously reduced.

At concentrations below 4 µM, K-26 did not completely suppress the flagellar movement of the Triton-extracted sperm, but reduced the beat frequency in a competitive manner; the maximal beat frequency (Fmax) was 31.2 Hz whether in the absence or in the presence of 4 µM K-26, while Km for ATP was 0.19 mM or 0.28 mM, respectively. ATPase activity of isolated axonemes was increased severalfold in the presence of K-26. The sliding movement between the outer doublet microtubules in trypsin-treated axonemes was normal at K-26 concentrations as high as 20 µM.

From these results, it was suggested that K-26 affects the mechanism that converts the sliding movement between the outer doublet microtubules into a bending motion, and thereby inhibits the motility of the Triton-extracted sperm model.

The bending waves of cilia and flagella are believed to be generated by the sliding movement between the outer doublet microtubules of the axonemes (16, 18). It has strongly been suggested that the sliding movement is generated by the interaction of the dynein arms on each A-subfiber of the doublet microtubules with the B-subfiber of the adjacent doublet, which is coupled with the hydrolysis of ATP (for reviews, see 8, 11, 14, 19).

Recently, Kobayashi et al. (12) isolated an acylpeptide, called K-26, from a

Abbreviations used: cAMP, adenosine 3',5'-cyclic monophosphate; DTT, dithiothreitol; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol bis-(β-aminoethylrether)-N,N,N',N'-tetraacetic acid; EHNA, erythro-9-[3-(2-hydroxynonyl)] adenine; FSW, filtrated sea water; NEM, N-ethylmaleimide; PEG, polyethylene glycol.
culture medium of *Bacillus sp.* 503. K-26 is composed of a heptapeptide (Glu-Leu-Leu-Val-Asp-Leu-Leu) and a \( \beta \)-hydroxy fatty acid residue. A lactone linkage is formed between the \( \beta \)-hydroxy group of the fatty acid and the carboxyl group of C-terminal Leu. An amido bond linkage is formed between the carboxyl group of the fatty acid and the amino group of N-terminal Glu. Consequently, K-26 shows a cyclic structure (Fig. 1). K-26 inhibited the fertilization in sea urchin eggs by affecting the spermatozoa (12). In the present study, we investigated the effects of K-26 on the motility of sea urchin spermatozoa. We found that the flagellar motility of both intact and Triton X-100-extracted sperm was inhibited, but both the sliding movement between the outer doublets and the ATPase activity of axonemes were not inhibited.

**MATERIALS AND METHODS**

*Materials.* Following biochemicals were purchased: ATP from Yamasa Shoyu Co., Tokyo, trypsin (Type II) from Sigma Chem. Co., St Louis, MO., U.S.A., soybean trypsin inhibitor from Cooper Biomedical Inc., Worthington Biochemicals, Freehold, N.J., U.S.A.

*Purification of K-26.* K-26 was purified from the culture medium of a soil bacterium *Bacillus sp.* 503 (12). Purified K-26 was dissolved in dimethyl sulfoxide (DMSO) at 1–2 mg/ml.

*Preparation of Triton X-100-extracted sperm model and measurement of the motility and beat frequency.* Spermatozoa were obtained from a sea urchin *Anthocidaris crassispina* by the injection of 0.5 M KC1 or 2 mM acetylcholine•Cl into the body cavity, and were stored on ice as “dry” sperm, which were used within 10 h. The “dry” sperm were diluted with 20 vol of chilled filtrated sea water (FSW) to make a stock sperm suspension, which was used within 4 h. Ten \( \mu \)l of this suspension was added to 490 \( \mu \)l of an extraction medium consisting of 0.04 \% Triton X-100, 0.15 M KC1, 2 mM MgSO\(_4\), 2 mM CaCl\(_2\), 0.5 mM EDTA, 1 mM dithiothreitol (DTT) and 10 mM Tris-HCl (pH 8.0), and gently swirled at room temperature (19–23 °C). After 1 min, 10 \( \mu \)l of the extracted sperm suspension was added to 490 \( \mu \)l of the reactivation medium consisting of 1 mM ATP, 0.15 M KC1, 2 mM MgSO\(_4\), 0.2 mM ethyleneglycol bis-(\( \beta \)-aminoethylether)-N,N',N'-tetraacetic acid (EGTA), 1 mM DTT, 2 \% (w/v) polyethylene glycol (PEG, M.W. 20,000), and 10 mM Tris-HCl (pH 8.0). One-twentieth vol of K-26 solution was added to the reactivation medium prior to the addition of

![Fig. 1. Chemical structure of K-26.](image-url)
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Triton-extracted sperm suspension. Since K-26 was dissolved in DMSO, the final DMSO concentration in the reactivation medium was adjusted to 5% (v/v) in all experiments. In some experiments, the order of addition of the Triton-extracted sperm suspension, K-26 or ATP, or the concentration of K-26 or ATP was varied as described in RESULTS.

Forty µl of the reactivation medium containing the Triton-extracted sperm was mounted on a glass slide and the sperm motility was examined after 30 sec under a Nikon Optiphot microscope equipped with a dark field condenser (Nippon Kogaku K.K., Tokyo, Japan). When the effects of K-26 on the motility of the intact sperm were investigated, 5 µl sperm suspension that was made by the dilution of the "dry" sperm with 500 vol of chilled FSW, was added to 495 µl FSW containing K-26. The % motility of the intact sperm or Triton-extracted sperm was defined as the percentage of swimming sperm in the total number of the sperm. A sperm wagging its head but not beating its flagellum was counted as a non-motile one.

Beat frequency of the sperm tails was measured with a stroboscopic flash unit according to the method of Hosoya et al. (10).

Measurement of the ATPase activity of axonemes and observation of sliding. Preparation of axonemes and trypsin-treated axonemes from sperm tails was carried out as described previously (23). ATPase activity of axonemes was measured at 20°C in the reactivation medium devoid of PEG. The amount of Pi liberated was determined by the method of Fiske and SubbaRow (5). Sliding of outer doublet microtubules was induced in the same medium for the ATPase assay, and was observed with an electron microscope (JEM 1200EX, JEOL, Ltd., Tokyo) or the dark field microscope. For monitoring the sliding movement, the turbidity of the axonemal suspension was measured at 350 nm using a Shimadzu UV-240 spectrophotometer (Shimadzu Seisakusho Co., Ltd., Kyoto). Specimens for electron microscopy

Fig. 2. Effect of K-26 concentration on the % motility of intact sperm. The % motility was assayed as described in MATERIALS AND METHODS, except that the K-26 concentration was varied.
was prepared as described previously (23).

Protein determination. Protein concentration was determined by the method of Lowry et al. (13) using bovine serum albumin as a standard.

RESULTS

Effect of K-26 on the motility of the intact or the Triton-extracted sperm. In the presence of K-26 in FSW, the motility of intact sperm was reduced in a dose dependent manner and was completely inhibited at concentrations above 1 µM (Fig. 2). The concentration of K-26 that was required to attain 50 % inhibition of the motility was 0.88 µM.

The motility of Triton X-100-extracted sperm, which preserve the motile apparatus devoid of plasma membranes, was also inhibited by K-26 (Fig. 3). However, the concentration of K-26 that gave either a complete inhibition or 50 % inhibition of motility, was higher than that observed for the intact sperm (> 6 µM or 3.6 µM, respectively). When K-26 was added to the Triton-extracted sperm 1 min after induction of activation by ATP, the motility was inhibited in a manner similar to the case where K-26 was included in the reactivation medium prior to the initiation of the motility (Fig. 3).

Fig. 4 shows dark field micrographs of the Triton-extracted sperm. In the absence of K-26 (in the presence of 5 % DMSO), the Triton-extracted sperm swam in a circular pattern. However, in the presence of 6 µM K-26, they became quiescent and

Fig. 3. Effect of K-26 concentration on the % motility of Triton-extracted sperm. The % motility was assayed as described in MATERIALS AND METHODS. Triton-extracted sperm suspension was added to the reactivation medium before (closed symbols) or after (open symbols) the addition of K-26 to the reactivation medium.
showed a peculiar form where the proximal region of the flagellum was bent and the rest of the flagellum was scarcely curved.

The reversibility of the inhibitory effect of K-26 on the motility of the Triton-extracted sperm was investigated. The Triton-extracted sperm were first preincubated in the reactivation medium containing 1 mM ATP and various concentrations of K-26 for various times and then diluted with 10 vol of the reactivation medium that did not contain K-26. The motility was measured 1 min after the dilution. The inhibition with 4 μM K-26 was fully reversible only when the preincubation was for 20 sec. It was completely irreversible after a 120 sec preincubation. The reversibility became less with the increase in the K-26 concentration (Fig. 5). A similar result was obtained when % motility was measured 5 min after the dilution with 10 vol of the reactivation medium or 1 min after dilution with 50 to 100 vol of the medium (data not shown).

Effect of ATP concentration on the inhibition of motility by K-26. The inhibitory effect of K-26 was found to be dependent on the ATP concentration in the reactivation medium. For instance, 6 μM K-26 completely suppressed the motility of the Triton-extracted sperm in the presence of 1 mM ATP (Fig. 3). However, 86.0 % motility was observed when the ATP concentration was 20 μM. Fig. 6 shows the concentration of K-26 that was required to attain 50 % inhibition of the motility when the ATP concentration was varied. With the decreasing ATP concentration, the concentration of K-26 that was required to attain 50 % inhibition of the motility was increased.

Fig. 4. Dark field micrographs of Triton-extracted sperm in the absence (A) and in the presence (B) of 6 μM K-26. Exposure times were: A, 2 sec; B, 1 sec. The scale represents 10 μm.
Fig. 5. Measurement of % motility after dilution to reduce the concentration of K-26. The Triton-extracted sperm were pretreated with 4 μM (○), 6 μM (△), or 8 μM (☐) K-26 in the reactivation medium for periods indicated in abscissa, and were diluted with 10 vol of the reactivation medium which did not contain K-26. The % motility was assayed 1 min after the dilution as described in MATERIALS AND METHODS.

Fig. 6. Effect of ATP concentration on the inhibition of the motility by K-26. The K-26 concentrations which were required to obtain a half maximal inhibition of the motility of the Triton-extracted sperm are plotted versus the ATP concentration in the reactivation medium. The % motility was assayed as described in MATERIALS AND METHODS, except that the ATP concentration in the reactivation medium was varied.
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Furthermore, the inhibitory effect of K-26 on the motility was canceled by dilution of the reactivation medium containing 20 μM ATP as follows. The Triton-extracted sperm were pretreated in the reactivation medium containing both 6 μM K-

Fig. 7. Double reciprocal plots of the beat frequency versus ATP concentration. Measurements were carried out in the absence (○), or in the presence of 2 μM (△), or 4 μM (●) K-26. Vertical bars represent the standard deviations.

Furthermore, the inhibitory effect of K-26 on the motility was canceled by dilution of the reactivation medium containing 20 μM ATP as follows. The Triton-extracted sperm were pretreated in the reactivation medium containing both 6 μM K-

Fig. 8. Effect of K-26 on the ATPase activity of axoneme. The ATPase activity was assayed as described in MATERIALS AND METHODS. The concentration of axonomes in the reaction mixture was 26 μg/ml (○) or 106 μg/ml (●). The ATP concentration was 1 mM. Activities relative to that in the absence of K-26 were plotted.
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26 and 1 mM ATP for 4 min. Under these conditions, the motility was completely suppressed (Fig. 3). When the suspension was diluted with 50 vol of the reactivation medium containing 1 mM ATP but no K-26, the motility was not restored, as described above (Fig. 5). However, when the suspension was diluted with 50 vol of the reactivation medium containing 20 μM ATP but no K-26, the motility was restored (% motility, 90.0 %). When the initial K-26 concentration was 4 μM, 95.4 % motility was obtained after the dilution.

Effect of K-26 on the beat frequency. The beat frequency of the Triton-extracted sperm was measured as a function of ATP concentration (Fig. 7). Even at concentrations of K-26 where the motility was not completely suppressed, the beat frequency was reduced by K-26 in a competitive manner. The Km for ATP was increased from 0.19 mM to 0.22 mM or 0.28 mM in the presence of 2 or 4 μM K-26, respectively. However, Fmax (31.2 Hz) was not affected in the presence of 2 or 4 μM K-26.

Effect of K-26 on the ATPase activity of the axonemes. It was possible that K-26 inhibited the dynein ATPase activity and thereby suppressed the motility of the Triton-extracted sperm, just like vanadate (6), erythro-9-[3-(2-hydroxynonyl)] adenine (EHNA) (2) or antibodies against dynein (15) are known to do. The ATPase activity of axonemes isolated from sperm tails, was measured in the presence of K-26 (Fig. 8). K-26 did not inhibit the ATPase activity of the axonemes, but activated it. A similar extent of activation of the axonemal ATPase activity was observed for two different concentrations of the axonemes in the reactivation medium. In the range of K-26 concentration where the inhibition of the motility of the Triton-extracted sperm in the presence of 1 mM ATP was observed, the axonemal ATPase activity was slightly activated.

Effect of K-26 on the sliding movement of the outer doublet microtubules in the trypsin-treated axonemes. If K-26 inhibited the interaction of dynein arms with outer doublet microtubules in the axoneme, the motility of the Triton-extracted sperm would be suppressed. To examine this possibility, the effect of K-26 on the sliding of outer doublet microtubules in the trypsin-treated axonemes in the presence of ATP, was investigated. Fig. 9 shows the electron micrographs of negatively stained axonemes. Three seconds after the addition of ATP to the trypsin-treated axonemes, the sliding movement between outer doublet microtubules was observed both in the absence and in the presence of 5, 10, or 20 μM K-26. Furthermore, the extent of the turbidity drop of the axonemal suspension, which occurred as a consequence of the sliding movement, was not affected by the presence of 20 μM K-26 (data not shown).

DISCUSSION

Acylpeptide K-26 inhibited the flagellar motility of both intact and Triton X-100-extracted sperm at low concentrations. In the case of the intact sperm, the half maximal inhibition was decreased to less than 1 μM K-26. K-26 is a kind of the surfactin derivative (22), which has a feature of a surfactant (1). Tsukagoshi et al. (22)

Fig. 9. Electron micrographs of trypsin-treated axonemes. Trypsin-treated axonemes were first suspended in the ATP-depleted reaction medium containing A, 0; B, 5 μM; C, 10 μM; D, 20 μM K-26, and then ATP(final concentration of 0.1 mM) was added. After 3 seconds, the samples were stained negatively with 2 % uranyl acetate. Final concentration of trypsin-treated axonemes was 76.7 μg/ml. The bar represents 2 μm.
reported that "surfactin" was able to lyse a protoplast prepared from *Bacillus megaterium* KM at concentrations less than 10 μM. It is likely that K-26 damaged the plasma membrane of the sperm to some extent as a detergent and thereby inhibited the motility of the sperm.

K-26 suppressed the motility of the Triton-extracted sperm in a dose dependent manner, indicating that K-26 also affected directly the flagellar motile apparatus in sperm. The flagellar wave of motility-arrested sperm in the presence of K-26 showed a peculiar form. Gibbons and Gibbons (7) have found a similar shape in the flagellum of a Ca²⁺ induced non-motile *Tripneustes gratilla* sperm model and called this phenomenon "Ca-induced quiescence". However, some differences were observed between the two cases. In the Ca-quiescence, the bend remained in the proximal region of the flagellum and corresponded to the principal bend (7). On the other hand, in the case of K-26-quiescence, the bend angle in the proximal region was smaller than that of Ca-quiescence and it was considered to correspond to the reverse bend (24). Therefore, target regions in the sperm for Ca²⁺ and K-26 may be different from each other. Further analysis will be required to compare the two forms of quiescence, including experiments on various sea urchin species to check whether there is any species difference.

The flagellar movement of the Triton-extracted sperm that was inhibited by K-26, could not be resumed by reducing the concentration of K-26 to below the critical inhibitory level. However, by reducing the concentration of both K-26 and ATP in the reactivation medium, the suppression of the motility could be reversed. Moreover, the inhibitory effect of K-26 on the motility was dependent on the ATP concentration in the reactivation medium: that is, the inhibitory effect by K-26 was reduced when the ATP concentration became lower. Therefore, the inhibitory effect of K-26 is reversible, provided that the ATP concentration in the reactivation medium is appropriate. A similar effect has been observed in the case of the Ca-quiescence (7) in the presence of 0.1 mM Ca²⁺, in which flagella are non-motile at ATP concentrations around 1 mM while they are motile at around 0.1 mM. In the case of protein phosphatase, the inhibitory effect on flagellar movement of Triton-extracted sea urchin sperm was also observed to be sensitive to the ATP concentration in the reactivation medium (3).

At lower concentrations of K-26, where the motility of Triton-extracted sperm was scarcely inhibited, the beat frequency was reduced in a competitive manner. The reason for this is not known at present.

In contrast to the inhibitors for flagellar motility such as vanadate (6, 17), EHNA (2) or antibodies against dynein (15), K-26 did not inhibit the ATPase activity of axonemes, but rather activated it. Therefore the cessation of the flagellar movement in the presence of K-26 was not due to the inhibition of the dynein ATPase. Cosson et al. (4) reported that a prolonged treatment of the Triton-extracted sperm with N-ethylmaleimide (NEM) at a concentration of 10 μM, where the dynein ATPase activity was not inhibited, caused a complete loss of both motility and ATP-induced sliding of outer doublets. In the present case, however, the sliding movement in the trypsin-treated axonemes could be induced by the addition of ATP in the presence of 5–20 μM K-26 under which the motility of the Triton-extracted sperm was completely inhibited. Based on these facts, the interaction of dynein arms with outer doublet microtubules was not affected directly by K-26. It is speculated that K-26 affects the regulatory process to convert the sliding into flagellar movement rather than affecting the structural elements of axonemes such as outer doublet
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It has been reported that an inhibitor of cAMP-dependent protein kinase (9, 21) or protein phosphatase (3, 20) is able to suppress the flagellar movement of Triton-extracted sperm without affecting the sliding between the outer doublet microtubules. It is possible that K-26 affects the flagellar movement as an inhibitor of cAMP-dependent protein phosphorylation. This possibility will be discussed in a subsequent paper.

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REFERENCES


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