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Increase in intracellular pH induces phosphorylation of axonemal proteins for activation of flagellar motility in starfish sperm

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Summary

Increased intracellular pH ($[pH]_i$) activates dynein in sea urchin and mammalian sperm and induces activation of flagellar motility. It is thought that cAMP-dependent protein phosphorylation is associated with motility activation through increasing $[pH]_i$, but little attention has been given to the cAMP-independent phosphorylation also induced by the $[pH]_i$ increase. The present study demonstrates that the increase in $[pH]_i$ in starfish sperm induces the phosphorylation of axonemal proteins and activation of flagellar motility independently of cAMP. Flagellar motility of intact sperm was activated when the $[pH]_i$ was raised by addition of NH_4Cl. Histidine, which is known to activate motility of starfish sperm, also raised the $[pH]_i$ during the motility activation. In addition, motility of demembranated sperm flagella was activated in a pH-dependent manner without cAMP. These results indicate that in starfish sperm it is the increase in $[pH]_i$ that induces activation of flagellar motility. Moreover, phosphorylation of axonemal proteins (of molecular mass 25, 32 and 45 kDa) was observed during the pH-dependent and cAMP-independent motility activation of demembranated sperm. This suggests that the increase in $[pH]_i$ regulates flagellar motility *via* cAMP-independent phosphorylation of axonemal proteins.

Key words: flagellar motility, regulation, axoneme, cAMP-independent, protein phosphorylation, intracellular pH, sperm, starfish.

Introduction

Eukaryotic cilia and flagella are found on various types of cells, from single-cell protozoa to epithelial cells of multicellular organs. Most have a well-conserved structure called the axoneme, which generates ciliary and flagellar movements. The axoneme is a microtubule-based, highly organized structure composed of more than 250 kinds of proteins. Molecular motors, called the dyneins, exert force to drive the active sliding of microtubules, which is converted into the bending wave of cilia and flagella. It is known that the phosphorylation of axonemal proteins plays a crucial role in the regulation of motility (Inaba, 2003).

An increase in intracellular pH ($[pH]_i$) is reported to be involved in the activation of sperm flagellar motility in sea urchin (Christen et al., 1982; Lee et al., 1983), marine teleosts (Oda and Morisawa, 1993) and mammals (Wong et al., 1981; Babcock et al., 1983). It is thought that cAMP-dependent protein phosphorylation is associated with motility activation through increased $[pH]_i$ (Brokaw, 1987; Goltz et al., 1988; Carr and Acott, 1989), but little attention has been given to the cAMP-independent phosphorylation induced by the increased $[pH]_i$. According to Carr and Acott (1989), two proteins are phosphorylated in intact bull sperm during motility activation induced by an increase in $[pH]_i$, and the phosphorylation is probably not due to changes in cAMP levels. However, it is not clear whether these phosphoproteins are associated with the signaling pathway induced by an increase in $[pH]_i$ nor whether the phosphorylation occurs independently of cAMP. In addition, these phosphoproteins are located in the membrane (Carr and Acott, 1989).

In the present study, we demonstrate in starfish sperm that an increase in $[pH]_i$ induces phosphorylation of axonemal proteins and activation of flagellar motility independently of cAMP. It is reported that starfish testicular sperm are generally immotile in seawater, but motility is activated by histidine, which has a strong zinc-binding capacity (Fujii et al., 1955). EDDA and TPEN, high-affinity chelators of Zn^{2+}, are also effective in activating motility (Mohri et al., 1990; Morisawa, et al., 2004). Therefore the liberation of Zn^{2+} from the sperm is thought to be a key factor for motility activation in starfish (Fujii et al., 1955; Mohri et al., 1990; Morisawa et al., 2004), but downstream events in the signaling pathway for motility activation remain unknown. As the first step, we show that an increase in $[pH]_i$ is also involved in the activation of intact starfish sperm motility. We then demonstrate pH-dependent but

cAMP-independent activation of demembranated sperm motility and associated phosphorylation of axonemal proteins.

Materials and methods

Materials

Starfish *Asterina pectinifera* L. were collected from Suruga Bay in Japan in May 2003 and 2004. The testes were removed from the body cavity, blotted on paper to remove the body fluids, and placed in a glass dish on ice. The sperm exuding from the testes on the dish were collected in a tube and kept on ice, which were used for the measurement of sperm motility and intracellular pH. The testes were then transferred from the dish on to a nylon mesh (pore size 48 μm). The sperm squeezed through the mesh were used for the detection of protein phosphorylation. No significant differences in motility were found between the sperm exuded from the testes and the sperm squeezed from the testes.

Carboxy SNARF-1 AM and nigericin were purchased from Molecular Probes (Carlsbad, CA, USA). [γ-^{32}P]ATP was obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). All other chemicals were of analytical grade.

All the experiments were carried out at room temperature except where noted.

pH measurement of seminal plasma

The testes prepared as described above were immediately transferred to a 1.5 ml Eppendorf tube, covered with mineral oil, and then centrifuged at approximately 17 000 ***g*** for 5 min at 4°C. The supernatant excluding mineral oil was used as seminal plasma. The pH of seminal plasma was measured using a pH meter.

Motility measurement of intact sperm

Sperm were diluted into 40 μl of an experimental solution on a glass slide (approximately $1–2\times10^7$ sperm cells ml^{-1}). Movement of sperm was recorded using a video recorder (HR-B12; Victor, Yokohama, Japan) and a CCD camera (CV-10; Video device, Tokyo, Japan) mounted on a phase contrast microscope (Optiphoto; Nikon, Tokyo, Japan). Recording of sperm movement was performed without a coverslip because *A. pectinifera* sperm were found to adhere to the glass surface. The percentage of motile sperm was calculated from the video recordings. Sperm were counted as motile when they exhibited progressive movement. In case the sperm head was attached to the glass slide, sperm exhibiting spontaneous flagellar beating were also counted as motile.

The effect of histidine on sperm motility was examined using artificial seawater (ASW: 430 mmol l^{-1} NaCl, 9 mmol l^{-1} $CaCl_2$, 9 mmol l^{-1} KCl, 23 mmol l^{-1} $MgCl_2$, 25 mmol l^{-1} $MgSO_4$ and 10 mmol l^{-1} Hepes-NaOH, pH 8.2) and Na-free ASW (430 mmol l^{-1} choline chloride, 9 mmol l^{-1} $CaCl_2$, 9 mmol l^{-1} KCl, 23 mmol l^{-1} $MgCl_2$, 25 mmol l^{-1} $MgSO_4$ and 10 mmol l^{-1} Hepes-KOH, pH 8.2), both of which contained varying concentrations (0–40 mmol l^{-1}) of histidine. Choline chloride solutions (CC solution, 0.5 mol l^{-1} choline chloride and 10 mmol l^{-1} Hepes-KOH, pH 8.2) containing 0–20 mmol l^{-1} NH_4Cl were used to examine the effect of NH_4Cl.

Measurement of intracellular pH by fluorescence spectrophotometer

$[pH]_i$ was measured using a fluorescent pH indicator, carboxy SNARF-1 AM. The emission spectrum of carboxy SNARF-1 undergoes a pH-dependent wavelength shift, causing changes in the ratio of the fluorescence intensities at two emission wavelengths, resulting in more accurate determination. In the present study, carboxy SNARF-1 was excited at 514 nm and monitored at 580 nm and 640 nm. The ratio of fluorescence intensities measured at the two wavelengths (580 nm/640 nm) was then calculated.

Stock solution (1 mmol l^{-1}) of carboxy SNARF-1 AM was made in dimethyl sulfoxide (DMSO). Sperm (20–40 μl) were diluted with 5 volumes of loading buffer (0.5 mol l^{-1} choline chloride, 10 mmol l^{-1} Hepes-KOH, pH 7.0 and 50 μmol l^{-1} carboxy SNARF-1 AM) and incubated at 4°C in the dark. After overnight incubation, the sperm were washed with CC solution adjusted to pH 7.0 (0.5 mol l^{-1} choline chloride and 10 mmol l^{-1} Hepes-KOH, pH 7.0). The sperm were resuspended in the original volume (100–200 μl) of CC solution, pH 7.0 and kept on ice. 12.5 μl of this suspension were added to a cuvette containing 2 ml of experimental solution: (i) Na-free ASW, (ii) ASW or (iii) CC solution. The emission spectrum of carboxy SNARF-1 was monitored using a fluorescence spectrophotometer (F-4500; Hitachi, Tokyo, Japan). After monitoring, either 10 mmol l^{-1} histidine (i and ii) or 20 mmol l^{-1} NH_4Cl (iii) was added and emission spectra monitored again. Carboxy SNARF-1 loaded sperm retained normal motility during the measurement of $[pH]_i$.

It is known that the fluorescence response is significantly different when the dye is loaded in cells. *In situ* calibration can be performed by using the K^+/H^+ ionophore, nigericin, in the presence of 120–130 mmol l^{-1} K^+ (approximately equivalent to the intracellular K^+ concentration) to equilibrate $[pH]_i$ with the controlled extracellular pH ($[pH]_o$; Thomas et al., 1979; Babcock, 1983; Negulescu and Machen, 1990). 12.5 μl of the suspension containing carboxy SNARF-1 loaded sperm was added to a cuvette containing 2 ml of calibration solution (120 mmol l^{-1} KCl, 380 mmol l^{-1} choline chloride and 20 mmol l^{-1} Hepes-KOH, pH 6.8, 7.0, 7.25, 7.5, 7.75, 8.0 or 8.2). Nigericin (10 μmol l^{-1}) was then added and emission spectra monitored. A stock solution (10 mmol l^{-1}) of nigericin was made in ethanol. $[pH]_i$ was calculated from a calibration curve of the ratio of fluorescence intensities measured at two wavelengths (580 nm/640 nm) *vs* $[pH]_o$ ($\approx[pH]_i$).

Measurement of $[pH]_i$ by fluorescence microscope

Sperm were loaded with carboxy SNARF-1 as described above. The loaded sperm suspension was added to 19 volumes of CC solution, pH 7.0, and kept on ice. 1 μl of this suspension

was diluted into 40 μl of CC solution containing 0 or 20 mmol l^{-1} NH_4Cl on a glass slide. The diluted sperm were covered with a coverslip and observed using fluorescence microscopy (S. Kamimura, manuscript in preparation). We used micrographs representing the fluorescent intensity at 640 nm with 470–550 nm excitation.

Motility measurement of demembranated sperm

The effect of pH on reactivation of demembranated sperm was examined. Glass slides and coverslips were coated with 1% (w/v) bovine serum albumin (BSA) to prevent sperm from adhering to the glass surface. Sperm were suspended in 80 volumes of experimental solution: (i) CC solution, (ii) CC solution containing 20 mmol l^{-1} NH_4Cl or (iii) ASW containing 10 mmol l^{-1} histidine. 5 μl of the sperm suspension was gently diluted into 40 μl of demembranation solution [150 mmol l^{-1} KCl, 2 mmol l^{-1} $MgCl_2$ 1 mmol l^{-1} dithiothreithol (DTT), 1 mmol l^{-1} EDTA, 0.04% (w/v) NP-40 and 20 mmol l^{-1} Hepes-KOH, pH 8.0] and kept on ice for 3 min. After demembranation, 2 μl of this suspension was gently diluted into 40 μl reactivation solution (200 μmol l^{-1} ATP, 150 mmol l^{-1} KCl, 2.2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} DTT, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA and 20 mmol l^{-1} Hepes-KOH, pH 7.0, 7.2, 7.4, 7.6, 7.8 or 8.0) on a glass slide. The effect of cAMP and cGMP was examined as follows. Sperm suspended in CC solution were demembranated and then diluted into the reactivation solution (pH 7.0 or 8.0) containing 10 μmol l^{-1} cAMP or cGMP. The concentration of Mg-ATP^{2-} in the reactivation solution was calculated as 158 μmol l^{-1}, sufficient to reactivate the demembranated sperm. Movement of the demembranated sperm was recorded as described above, and reactivation rate (percentage of demembranated motile sperm) was calculated.

Labeling of axonemal proteins with ^{32}P

Sperm were diluted with 20 volumes of experimental solution: (i) CC solution for preparation of immotile sperm or (ii) CC solution containing 20 mmol l^{-1} NH_4Cl for motile sperm preparation, and centrifuged at 3000 ***g*** for 5 min at 4°C. The sperm pellet was resuspended in 5 volumes of the fresh ice-cold experimental solution mentioned above, and homogenized using a Teflon homogenizer for 20 strokes to separate the heads and the flagella. The separation of heads and flagella was checked by phase contrast microscopy. The homogenized sperm suspension was centrifuged at 800 ***g*** for 5 min at 4°C to remove sperm heads, and the upper part of the supernatant was carefully collected in order to prevent the contamination of the pellet (sperm heads). Collected supernatant was checked for head contamination using a phase contrast microscope and centrifuged at 15 000 ***g*** for 5 min at 4°C to recover the isolated flagella. The pellet of flagella was demembranated with demembranation solution on ice for 10 min and centrifuged at 15 000 ***g*** for 5 min at 4°C. The pellet of axonemes was diluted with the ice-cold, ATP-free reactivation solution (pH 7.0–8.0; 2–3 mg ml^{-1}) and [γ-^{32}P]ATP (2 MBq ml^{-1}) was added to the suspension, mixed and kept at room temperature for 10 min. After this period, the suspension was centrifuged at 15 000 ***g*** for 5 min at 4°C. The pellet was diluted with SDS-sample buffer, heated in boiling water for 2 min and kept at 4°C until used.

Detection of phosphoproteins

Axonemal proteins were separated by SDS-PAGE according to Laemmli (1970) and the phosphoproteins detected by autoradiography. SDS-PAGE was performed on 5%–15% polyacrylamide gradient gels. The gels were stained with Coomassie Brilliant Blue R-250, dried and exposed to X-ray film for 3 days.

Results

Activation of flagellar motility in intact sperm

Histidine is reported to activate the flagellar motility of starfish testicular sperm in seawater (Fujii et al., 1955). The effect of histidine on the motility activation of sperm flagella was examined further using *A. pectinifera*. Sperm were suspended in ASW or Na-free ASW, both of which contained 0-40 mmol l^{-1} histidine. In ASW, sperm motility (percentage of motile sperm) increased in a histidine-dependent manner (Fig. 1A), whereas sperm showed little motility in Na-free ASW, even in the presence of histidine (Fig. 1B). These results suggest that the extracellular Na^+ was necessary for the flagellar motility activation by histidine in *A. pectinifera*.

In sea urchin sperm, an increase in $[pH]_i$ induced through the Na^+/H^+ exchanger activates motility (Lee, 1985). It is possible that an increase in $[pH]_i$ through the Na^+/H^+ exchanger is also involved in the activation of flagellar motility in starfish sperm. The effect of NH_4Cl on the activation of sperm flagella motility was examined to verify this possibility, since NH_4Cl is known to increase $[pH]_i$. When sperm were suspended in CC solutions containing 0–20 mmol l^{-1} NH_4Cl, the motility of sperm increased in a NH_4Cl-dependent manner as shown in Fig. 2. Maximum motility of 85% was attained with 10 mmol l^{-1} NH_4Cl. It is likely that the increase in $[pH]_i$ was involved in the motility activation of starfish sperm flagella.

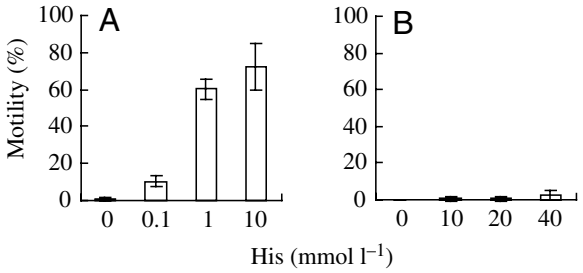

Fig. 1. Effect of histidine (His) on the flagellar motility activation of *Asterina pectinifera* sperm. Sperm were suspended in (A) ASW containing 0–10 mmol l^{-1} histidine or (B) Na-free ASW containing 0–40 mmol l^{-1} histidine, and the percentage of motile sperm was calculated. Values are means ± S.D.; *N*=3.

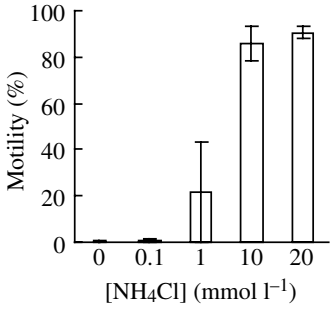

Fig. 2. Effect of increased $[pH]_i$ on the activation of *A. pectinifera* sperm flagellar motility. Sperm were suspended in CC solutions containing 0–20 mmol l^{-1} NH_4Cl, which is known to increase $[pH]_i$. The percentage of motile sperm was calculated. Values are means ± S.D.; *N*=7.

Measurement of $[pH]_i$

The flagellar motility of starfish sperm was activated by NH_4Cl (Fig. 2). Therefore, we measured the $[pH]_i$ of starfish testicular sperm under various conditions to confirm the involvement of increased $[pH]_i$ in the motility activation. As the first step, the $[pH]_i$ of testicular sperm in loading buffer (pH 7.0) was measured, and was approximately 7.0. Taking the pH value of seminal plasma (6.4±0.1) into consideration, the *in situ* $[pH]_i$ of testicular sperm might be lower.

Next, the effect of histidine and NH_4Cl on sperm $[pH]_i$ were examined. Carboxy SNARF-1 loaded sperm were suspended in (i) Na-free ASW, (ii) ASW or (iii) CC solution, and then histidine (i and ii) or NH_4Cl (iii) was added. Fig. 3A shows the $[pH]_i$ measured before and after the addition of histidine or NH_4Cl. The pH of Na-free ASW, ASW and CC solution was 8.2. When sperm were suspended in the solutions, the $[pH]_i$ increased to approximately 7.3–7.5, but flagellar motility was not activated. Motility activation was caused when histidine or NH_4Cl raised the $[pH]_i$ approximately to 7.8–8.1. These results suggest that the activation of sperm flagella motility was caused when the $[pH]_i$ increased up to 7.8, and also that histidine as well as NH_4Cl raised the $[pH]_i$ of sperm during the motility activation. However, histidine raised the $[pH]_i$ to only approximately 7.5 in Na-free ASW, and sperm flagellar motility was generally not activated, as shown in Fig. 1B. It is possible that histidine requires extracellular Na^+ to increase the $[pH]_i$ of *A. pectinifera* sperm.

The change in $[pH]_i$ during the activation of sperm flagella motility was also observed using fluorescence microscopy. The fluorescence intensity of carboxy SNARF-1 at 640 nm, which increases with pH, was higher in the sperm activated by NH_4Cl than in immotile sperm (Fig. 3B). These results confirm that the $[pH]_i$ of starfish sperm increased with motility activation.

Effect of pH on reactivation of demembranated sperm

The experiments described here using intact testicular sperm with NH_4Cl and histidine suggest that the increase in $[pH]_i$ of sperm was associated with the activation of sperm flagellar

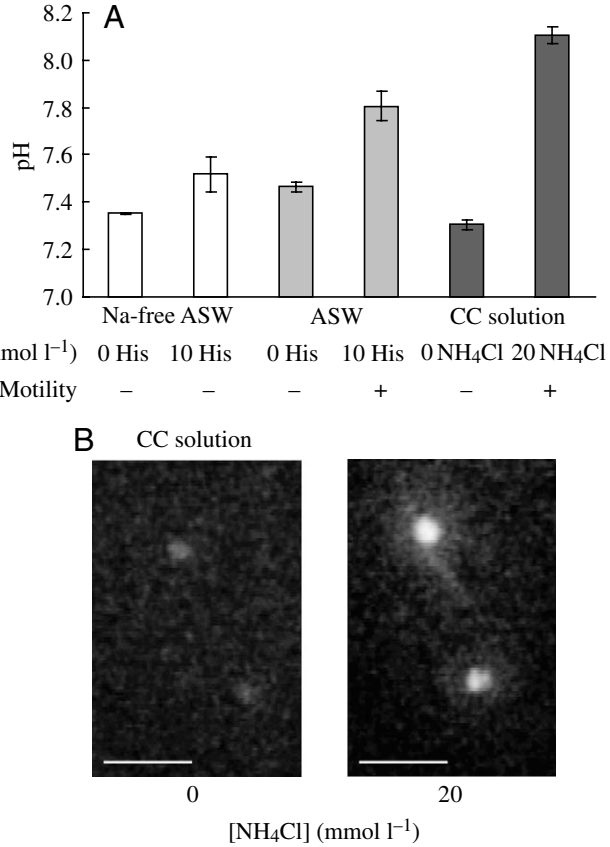

Fig. 3. Measurement of $[pH]_i$ using a fluorescent pH indicator, carboxy SNARF-1. (A) The effect of histidine and NH_4Cl on sperm $[pH]_i$. Carboxy SNARF-1 loaded sperm were suspended in Na-free ASW containing 0 or 10 mmol l^{-1} histidine (white bars), ASW containing 0 or 10 mmol l^{-1} histidine (gray bars), or choline chloride (CC) solution containing 0 or 20 mmol l^{-1} NH_4Cl (black bars). Values are means ± S.D.; *N*=2. Sperm motility (percentage of motile sperm) in each solution is given under the graph; – and + indicate generally immotile and >60% motile, respectively. (B) Fluorescence micrographs of carboxy SNARF-1 loaded sperm suspended in CC solution containing 0 (left) or 20 mmol l^{-1} (right) NH_4Cl. Micrographs represent the fluorescent intensity at 640 nm with 470–550 nm excitation. Bars, 10 μm.

motility. In order to confirm the involvement of $[pH]_i$ increase in the motility activation, the effect of pH on the reactivation of demembranated sperm was examined. Sperm were suspended in CC solution alone (CC treatment), CC solution containing 20 mmol l^{-1} NH_4Cl (NH_4Cl treatment) or ASW containing 10 mmol l^{-1} histidine (histidine treatment), and then demembranated. The demembranated sperm were diluted into reactivation solutions of several different pH values (7.0–8.0). Regardless of the treatment given to sperm before demembranation, the ratio of the reactivated sperm increased in a pH-dependent manner. When sperm showed no motility activation before demembranation (CC treatment), the optimal pH range for reactivation was 7.8 or above, which was almost the same as that reported previously in sea urchin (Gibbons and Gibbons, 1972) and bull (Goltz et al., 1988). However, sperm

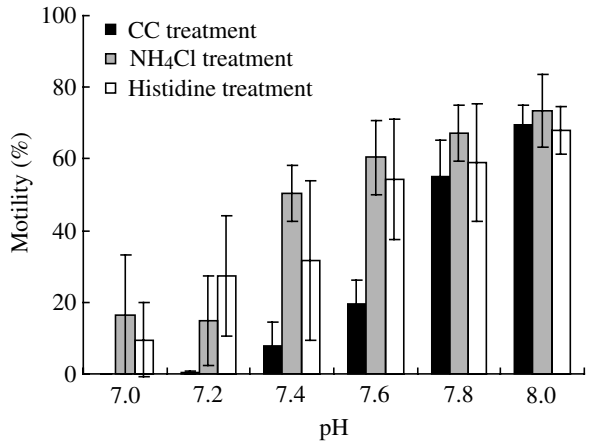

Fig. 4. Effect of pH on the reactivation of demembranated *A. pectinifera* sperm. Sperm were suspended in CC solution (CC treatment, black bars), CC solution containing 20 mmol l^{-1} NH_4Cl (NH_4Cl treatment, gray bars), or ASW containing 10 mmol l^{-1} histidine (histidine treatment, white bars), and then demembranated. The demembranated sperm were diluted with the reactivation solution of various pH (ranging 7.0–8.0), and the percentage of motile sperm calculated. Values are means ± S.D.; *N*=5 (CC treatment and NH_4Cl treatment); *N*=3 (histidine treatment).

demembranated after motility activation (NH_4Cl-treated sperm and histidine-treated sperm) exhibited higher reactivation rate in the lower pH range (7.0–7.6), resulting in a wider optimal range of pH (Fig. 4). These results indicate that the increase in $[pH]_i$ induced activation of flagellar motility. It also suggests that the activation of flagellar motility before demembranation modified the flagellar axoneme, which enabled the demembranated sperm to be motile at the lower pH. The effect of cAMP on the reactivation was examined at pH 7.0 and 8.0, but the percentage of motile demembranated sperm was not improved at either pH value. Cyclic GMP also did not improve the motility (data not shown).

Detection of axonemal phosphoproteins associated with motility activation induced by an increase in $[pH]_i$

The experiments with the demembranated sperm suggested that flagellar axonemal proteins could be modified during the motility activation. It is known that the activation of flagellar motility is regulated by the phosphorylation of proteins comprising the flagellar axoneme (Inaba, 2003). Therefore, phosphorylation of axonemal proteins during motility activation induced by the $[pH]_i$ increase was detected using [γ-^{32}P]ATP. Flagella were isolated from the sperm before activation (CC-treated sperm) and after activation (NH_4Cl-treated sperm), and then demembranated. The demembranated sperm flagella were diluted with reactivation solutions (pH 7.0–8.0) containing [γ-^{32}P]ATP and the ^{32}P-labeled axonemal proteins were detected.

If phosphorylation of certain axonemal proteins is associated with motility activation induced by a $[pH]_i$ increase, the following can be assumed. When the immotile sperm in CC solution were demembranated and reactivated in reactivating solution at high pH, ^{32}P would have been incorporated into the axonemal proteins during the reactivation (CC treatment). By contrast, the incorporation should have been prevented when sperm were demembranated after motility activation (NH_4Cl treatment), since the axonemal proteins would have already been phosphorylated before demembranation. However, the prevention of incorporation may not be observed when the phosphorylated proteins are dephosphorylated before reactivation.

Fig. 5A indicates that incorporation of ^{32}P into several axonemal proteins (25, 32 and 45 kDa) occurred in a pH-dependent manner. As previously mentioned, the proportion of reactivated sperm also increased in a pH-dependent manner (Fig. 4). It is plausible that the pH-dependent phosphorylation of axonemal proteins reflects the pH-dependent reactivation of the demembranated sperm. Fig. 5B indicates that less ^{32}P was incorporated into axonemal proteins in sperm flagella demembranated after motility activation (NH_4Cl treatment). It is likely that the phosphorylation of the axonemal proteins occurred with the motility activation before demembranation and avoided the incorporation of ^{32}P as suggested above.

The intensity of the 25, 32 and 45 kDa bands was quantitated by densitometry, and the relative band intensities are shown in Fig. 5C,D,E. The relative intensity of the 25 kDa band (Fig. 5C) exhibited a pH-dependent increase in CC-treated sperm axonemes, while no incorporation was observed in NH_4Cl-treated sperm axonemes. These results strongly suggest that the phosphorylation of the 25 kDa protein induced by $[pH]_i$ increase is associated with flagellar motility activation. The relative intensity of the 32 kDa band shown in Fig. 5D exhibited a similar pattern, but weak incorporation was observed in NH_4Cl-treated sperm axonemes. On the other hand, the pattern of the 45 kDa band (Fig. 5E) was different. Most of the incorporation seen in CC-treated sperm axoneme was also observed in NH_4Cl-treated sperm axonemes.

Discussion

In this study, we have shown (1) an increase in $[pH]_i$ is involved in the motility activation of intact starfish sperm (Figs 2, 3); (2) demembranated sperm are reactivated in a pH-dependent but cAMP-independent manner (Fig. 4); and (3) several axonemal proteins (25, 32 and 45 kDa) are phosphorylated during the pH-dependent and cAMP-independent reactivation (Fig. 5).

Activation of flagellar motility by histidine

The effect of histidine was first reported by Fujii et al. (1955) and has been assumed to be *via* chelation of Zn^{2+}, since histidine has a strong zinc-binding capacity and EDTA and EDDA, metal chelators, have a similar effect (Fujii et al., 1955; Mohri et al., 1990). However, the details of the mechanism are not clear. The present experiments reveal that flagellar motility activation by histidine depends on extracellular $[Na^+]$ (Fig. 1) and involves a $[pH]_i$ increase (Fig. 3A). These results suggest

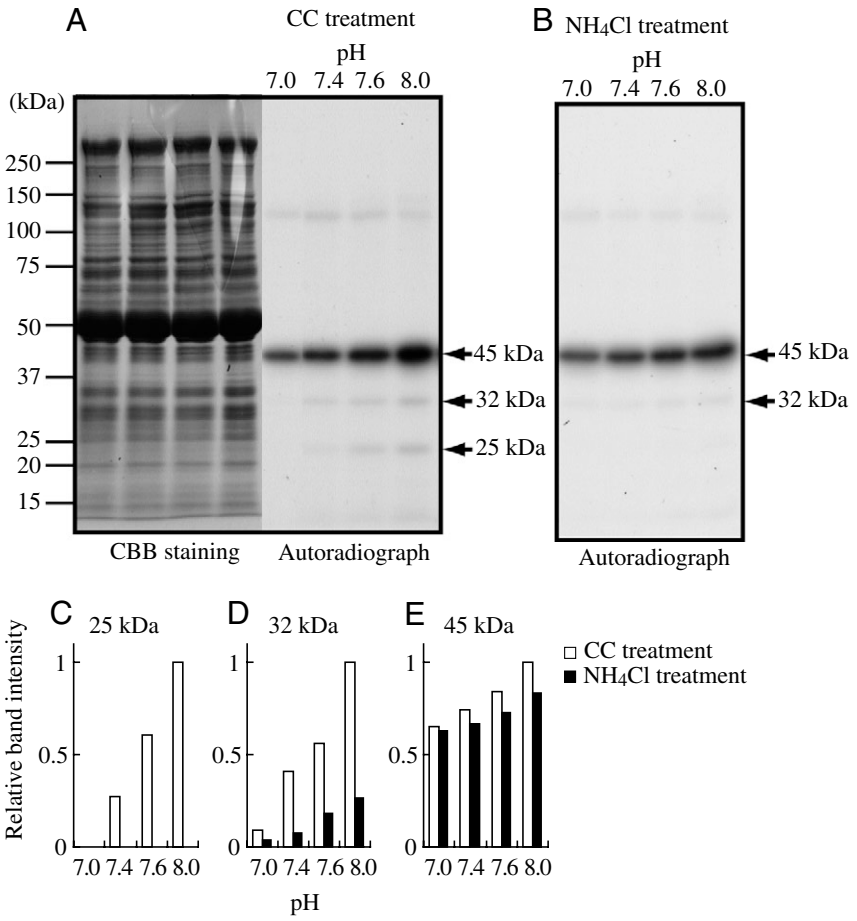

Fig. 5. Incorporation of ^{32}P during motility activation of demembranated flagella (axonemes). Sperm were suspended in CC solution (CC treatment) or CC solution containing 20 mmol l^{-1} NH_4Cl (NH_4Cl treatment) before the preparation of axonemes. The axonemes were diluted with the reactivation solutions (pH range 7.0–8.0) containing [γ-^{32}P]ATP, and axonemal proteins labeled with ^{32}P were detected. (A) ^{32}P-labeled proteins in the CC-treated sperm axonemes. (B) ^{32}P-labeled proteins in the NH_4Cl-treated sperm axoneme. An equal amount of protein (15 μg) was loaded on each lane in A and B. Numbers on the left of the gel show the positions of molecular mass markers (kDa). (C) Relative intensity of the 25 kDa band. The band intensities in the CC-treated sperm axoneme (white bars) and in the NH_4Cl-treated sperm axoneme (black bars) were compared. Band intensity was quantitated by densitometry and the maximum intensity of the band was taken as 1. Relative intensities of the 32 kDa band (D) and the 45 kDa band (E) are also shown.

the possibility that histidine induces the $[pH]_i$ increase through the Na^+/H^+ exchanger and causes the flagellar motility activation of starfish sperm. Therefore, the liberation of Zn^{2+} from the sperm can be assumed to induce the $[pH]_i$ increase through the Na^+/H^+ exchanger.

The motility activation of sea urchin sperm involves an increase in $[pH]_i$ through the Na^+/H^+ exchanger (Christen et al., 1982; Lee et al., 1983; Lee, 1985). Zn^{2+} is also involved in the regulation (Clapper et al., 1985), but the mechanism of action is different. Sea urchin sperm are known to initiate their motility when suspended in seawater. According to Clapper et al. (1985), EDTA delays the initiation of motility and Zn^{2+} prevents the inhibition. In addition, EDTA depresses sperm $[pH]_i$ and Zn^{2+} reverses the $[pH]_i$. It is likely that in sea urchin sperm the liberation of Zn^{2+} does not increase the $[pH]_i$ but, in contrast, depresses it.

The effect of histidine on the motility activation of flagella decreased at the end of the breeding season (data not shown), suggesting that there is an association of Zn^{2+} with the maturity of sperm. The effect of EDTA also decreases with maturity in starfish *Asterias amurensis* (Mohri et al., 1990). It is possible that the content of Zn^{2+} in starfish sperm or the association with Zn^{2+} decreases as they reach maturity. The Zn^{2+} content of starfish sperm should be measured throughout the breeding season to examine this possibility.

Effect of $[pH]_i$ on flagellar motility activation

The flagellar motility of intact sperm was activated when histidine or NH_4Cl raised the $[pH]_i$ up approximately to 7.8–8.1 (Fig. 3A). These results were confirmed by the experiments with demembranated sperm. Sperm demembranated before motility activation (CC-treated sperm) exhibited a relatively high reactivation rate (more than 50%) at pH 7.8 or more (Fig. 4). It could be inferred from these results that the range of $[pH]_i$ inducing the flagellar motility activation of *A. pectinifera* sperm is approximately 8.0±0.2.

It has been reported that intracellular alkalization raises the enzymatic activity of dynein (Christen et al., 1983; Inaba, 2003). The results shown in Fig. 4 suggest that the activation of flagellar motility before demembranation (NH_4Cl and histidine treatment) modifies the flagellar axoneme and enables the demembranated sperm to be motile at the lower pH. The modification(s) on axonemes caused during the motility activation might be associated with the regulation of dynein.

The motility of demembranated bull sperm also increases with pH, and is improved by addition of cAMP at the lower pH range. Cyclic AMP (10 μmol l^{-1}) improves motility at pH 6.8, from approximately 10% to 80%. This indicates that cAMP is provided by adenylyl cyclase in a pH-dependent manner and plays an important role in motility activation (Goltz et al., 1988). In this case, the pH-dependent motility

activation reflects the pH-dependent increase in cAMP level. Goltz et al. (1988) assumed that dynein was phosphorylated in a cAMP-dependent manner, and in that state dynein activity was not limited to an alkaline pH range. However, cAMP did not improve the motility of demembranated starfish sperm in the present experiments. It is possible that dynein is phosphorylated through a cAMP-independent mechanism in starfish.

pH-dependent and cAMP-independent protein phosphorylation

In salmonid fish and mammals, it is reported that cAMP is necessary for the reactivation of demembranated sperm (Morisawa and Okuno, 1982; Ishida et al., 1987) and that cAMP-dependent phosphorylation is associated with the flagellar motility of sperm (Tash et al., 1984; Morisawa and Hayashi, 1985; Hayashi et al., 1987; Si and Okuno, 1995; Inaba et al., 1998, 1999; Itoh et al., 2001; Fujinoki et al., 2001, 2003). In starfish *A. pectinifera*, pH-dependent reactivation of demembranated sperm was observed without cAMP (Fig. 4), and axonemal proteins (25, 32 and 45 kDa) were phosphorylated during the pH-dependent reactivation (Fig. 5). These results suggest that pH-dependent and cAMP-independent phosphorylation is involved in the motility activation of starfish sperm flagella.

It was thought that cAMP-dependent protein phosphorylation is associated with the motility activation through an increase in $[pH]_i$ (Brokaw, 1987; Goltz et al., 1988; Carr and Acott, 1989), but little attention has been given to the cAMP-independent phosphorylation induced by the $[pH]_i$ increase. Whether the increasing $[pH]_i$ induces the phosphorylation of dynein component(s) or other axonemal proteins independently of cAMP remains unknown. Two proteins in intact bull sperm are phosphorylated during motility activation induced by an increase in $[pH]_i$, and the phosphorylation is thought to be cAMP-independent because increasing $[pH]_i$ does not raise the cAMP level (Carr and Acott, 1984, 1989). However, it is not clear whether these phosphoproteins are associated with the signaling pathway induced by an increase in $[pH]_i$, since the $[pH]_i$ of intact sperm loaded with $^{32}PO_4$ is raised and the phosphoproteins labeled during the motility activation are detected. Therefore, independence from cAMP is also not clear. In addition, the phosphoproteins are located in the membrane (Carr and Acott, 1989). This is the first report describing the pH-dependent and cAMP-independent phosphorylation of axonemal proteins in motility activation.

The detergent-soluble fraction contains cAMP-dependent protein kinase and its potential substrate. They together give cAMP-sensitivity to the demembranated sea urchin and starfish sperm (Ishiguro et al., 1982). Whether cAMP-dependent protein kinase and/or the substrates were removed from the axoneme during the demembranation was not investigated. This study does not contradict the association of cAMP with motility activation induced by increasing $[pH]_i$, but proposes another, new mechanism regulating motility activation. It is conceivable that cAMP-dependent phosphorylation and pH-dependent phosphorylation regulate flagellar motility together. Actually, we detected cAMP-dependent phosphorylation in addition to pH-dependent phosphorylation when cAMP was added to reactivation solution (pH 7.0–8.0; A. Nakajima et al., manuscript in preparation). Cyclic AMP did not improve the percentage of motile demembranated sperm but might be associated with the regulation of other motility factors. How pH-dependent phosphorylation relates to cAMP-dependent phosphorylation in the regulation of flagellar motility will need to be examined.

Using another species of starfish, *A. amurensis*, the outer dynein arms were extracted from the axoneme using high salt solution and fractionated by centrifugation on sucrose density gradient. A protein in the dynein fraction, thought to be a light chain of the dynein, was phosphorylated during the pH-dependent and cAMP-independent motility activation of demembranated sperm flagella (A. Nakajima et al., manuscript in preparation). It has been reported that the outer dynein arms of human sperm performed pH-dependent activation, since reactivation of sperm lacking the outer dynein arms was not pH-dependent, in contrast to reactivation of normal sperm (Keskes et al., 1998). The present study suggests a possibility that the increase in $[pH]_i$ activates dynein through the pH-dependent and cAMP-independent phosphorylation of dynein component(s) and/or other axonemal proteins.

Conclusion

The present study demonstrates that an increase in $[pH]_i$ induces the phosphorylation of axonemal proteins and flagellar motility activation in starfish sperm. It is possible that an increase in $[pH]_i$ regulates the flagellar motility activation through phosphorylation. Moreover, the pH-dependent phosphorylation and motility activation are independent of cAMP, suggesting the involvement of a new mechanism for regulation of flagellar motility.

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