Effects of Detergents Used to Demembranate the Sea-Urchin Spermatozoa on the Reactivated Flagellar Movement, with Special Reference to the Rotatability of the Plane of Flagellar Beat

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ABSTRACT. Spermatozoa of the sea urchin, *Hemicentrotus pulcherrimus*, were demembranated with solutions containing various detergents and then reactivated with ATP. The reactivation rates of sperm demembranated with 0.05% (w/v) saponin, Brij 58, or CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) were less than 10%; increasing the concentration of detergent up to 0.1% (w/v) did not improve the reactivation rate. High reactivation rates were obtained with 0.04% (w/v) Triton X-100, 0.05% Nonidet P-40, 0.04% CHAPS+0.01% Triton X-100, or 0.04% CHAPS+0.01% Nonidet P-40. The flagellar waveform as well as the relationship between the beat frequency and the ATP concentration did not vary among these four preparations. Unlike the live sperm, Triton-extracted sperm failed to undergo rotation of the flagellar beat plane for more than one and a half revolutions when lateral vibration was imposed on the head in a plane that rotated around the head axis. However, the sperm demembranated with a solution containing 0.04% CHAPS and 0.01% Nonidet P-40 responded to the imposed rotatory vibration by rotating their flagellar beat plane through more than 10 cycles. Electron microscopic studies showed that the membrane were completely removed from around the axoneme by treatment with Triton X-100 or CHAPS+Nonidet P-40. These results indicate that the rotation of the beat plane does not require the presence of the plasma membrane, and also that the mechanism controlling the beat plane is independent of that controlling other parameters of flagellar movement.

Cells permeabilized or demembranated with detergents have been used for the study of cell movement in many cell types. In cilia and flagella, the use of demembranated 'models' has been particularly successful in the study of motility and various detergents have been used for this purpose including glycerol (10), saponin (14), Triton X-100 (4), Nonidet P-40 (1), and Brij 58 (13). For experiments with sea urchin sperm, demembranation procedures using a solution containing 0.04% (w/v) Triton X-100 have been widely used as they produce a very high (nearly 100%) reactivation rate and the reactivated sperm swim in a manner resembling that of the live sperm (4).

Recently, it has been reported that when the head of a live sea urchin sperm is held with a sinusoidally vibrating micropipette and the plane of pipette vibration is

gradually rotated, the beat plane of the flagellum rotates along with the plane of vibration of the pipette for several revolutions. Surprisingly, cessation of the imposed vibration is followed by a spontaneous 'unwinding' in which the beat plane of the flagellum rotates back through the same number of revolutions as that of the imposed 'winding' (6). With a view to investigating the mechanism of this interesting phenomenon, we have been applying similar vibration to the head of a reactivated sperm. Our preliminary experiments, however, indicated that, unlike the live sperm, the sperm demembranated with Triton X-100 showed little rotation of the beat plane.

The failure of the Triton-demembranated sperm to rotate their flagellar beat plane might indicate that the plasma membrane is somehow involved in the rotation seen in the live sperm. Alternatively, it is possible that the rotatability of the beat plane is not dependent on the presence of the plasma membrane but has been lost during demembranation with Triton X-100. To investigate these possibilities, we studied the motility and rotatability of the beat plane of sea urchin sperm that had been demembranated with various detergents. One of our objectives was to see if it was possible to develop a method for obtaining reactivated sperm that would show rotation of the flagellar beat plane as seen in the live sperm. We found that sperm demembranated with detergents other than Triton X-100 could also be reactivated and that sperm demembranated with a solution containing both CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and Nonidet P-40 show rotation of the beat plane for several revolutions.

In this report, we compare the motility and ultrastructure of sperm that were reactivated after being demembranated with various detergents. We also describe the results of rotation experiments carried out using several kinds of reactivated sperm.

MATERIALS AND METHODS

Spermatozoa obtained from the sea urchin, *Hemicentrotus pulcherrimus*, were suspended in Ca^{2+} -free artificial sea water and the suspension was transferred to a demembranation solution containing 0.2 M potassium acetate, 2 mM MgSO_4 , 2 mM EGTA, 1 mM dithiothreitol, 20 mM Tris-HCl (pH 8.2) and one or two of the following detergents: Triton X-100, Nonidet P-40, saponin, Brij 58, and CHAPS. After 30–90 sec, the resultant suspension of demembranated spermatozoa was diluted into a reactivating solution containing 0.2 M potassium acetate, 2 mM MgSO_4 , 2 mM EGTA, 20 mM Tris-HCl (pH 8.2), 1 mM dithiothreitol and 2% (w/v) polyethyleneglycol. ATP was added immediately before each experiment and observation and recording of the reactivated flagellar movement were carried out at room temperature (19–24°C).

The movement of the sperm was recorded with a high speed video system (nac MHS-200) with stroboscopic illumination. For the measurement of bend angle and wavelength, the recorded image of the flagellum was traced directly on the screen of the video monitor. The bend angle was measured as the angle between the tangents drawn to the flagellum on either side of the bend. The wavelength was defined as the distance between the midpoints of interbend regions along the flagellum (3, 7).

To study the rotation of the flagellar beat plane, the sperm head was held with a micropipette by gentle suction. The micropipette was oscillated by a piezoelectric driver and the direction of vibration was determined by the distribution of sinusoidal voltages applied to the lateral and vertical piezo elements. To rotate the plane of pipette vibration continuously, we used a computer to change the amplitude and phase of the voltages applied to two sets of

piezo element in a coordinated manner (6). The frequency of the vibration was set slightly higher than the original beat frequency (i.e., the frequency measured without applying vibration) of sperm. The amplitude of vibration was 15 μm and the frequency of rotation was about 0.2 Hz.

Sperm samples for the electron microscopic studies were prepared according to the methods of Luft (12) and Gibbons and Gibbons (4) with some modifications.

RESULTS

Reactivation rate. The motility of the sperm preparations obtained by using each of the different detergents was checked in a reactivation solution containing 2 mM ATP. Table 1 shows the rates of reactivation as percentages of motile sperm. The sperm preparation obtained by using a solution containing 0.05% saponin showed a reactivation rate of less than 5% and the sperm that were motile beat irregularly. Increasing the saponin concentration up to 0.1% did not affect the percent motility. Essentially the same result was obtained with Brij 58. When the sperm were permeabilized with a solution containing 0.05% CHAPS, 14% of the reactivated sperm swam like live sperm and about 40% showed flagellar beating only in the proximal half of the flagellum. The sperm permeabilized with 0.05% Nonidet P-40 showed nearly 100% reactivation. We also examined the effects of demembration solutions each containing two different detergents. The reactivation rate of the sperm permeabilized with a solution containing both 0.04% saponin and 0.01% Triton X-100 was almost the same as that of the sperm treated with a solution containing saponin alone. On the other hand, high reactivation rates were obtained when sperm were demembrated with a solution containing 0.04% CHAPS and 0.01% Triton X-100 or a solution containing 0.04% CHAPS and 0.01% Nonidet P-40.

Movement of the reactivated sperm. As shown above, high reactivation rates were obtained when the sperm were demembrated with 0.04% (w/v) Triton X-100, 0.05% Nonidet P-40, 0.04% CHAPS+0.01% Triton X-100, or 0.04% CHAPS+0.01% Nonidet P-40. We therefore examined the flagellar movement of the sperm reactivated following these four different demembration procedures.

The effects of ATP concentration on the flagellar beat frequency were studied within the range from 10 μM to 2 mM. Fig. 1 shows the relationship between the

TABLE 1. PERCENT MOTILITY OF REACTIVATED SPERM.

% (w/v) Detergent	Percent motility
0.04% Triton X-100	about 100%
0.05% saponin	< 5%
0.1% saponin	< 5%
0.05% Brij 58	< 5%
0.05% CHAPS	14%
0.05% Nonidet P-40	about 100%
0.04% saponin+0.01% Triton X-100	< 5%
0.01% Nonidet P-40+0.04% CHAPS	> 90%
0.01% Triton X-100+0.04% CHAPS	> 90%

Sperm were reactivated in a solution containing 1.3 mM MgATP (2 mM ATP and 2 mM MgSO_4).

Fig. 1. Relationship between the ATP concentration in the reactivating solution and the flagellar beat frequency of sperm demembrated with a solution containing 0.04% CHAPS and 0.01% Nonidet P-40. The reactivating solution contained 2 mM MgSO_4 and the beat frequency was measured at room temperature (19–24°C). Each point represents the mean $\pm$ S.D. of 15–20 measurements made with sperm swimming near the bottom surface. The regression line is drawn to best fit the data above 20 μM ATP.

beat frequency and the ATP concentration in sperm permeabilized with the solution containing CHAPS and Nonidet P-40 as plotted double reciprocally. The relationship is highly linear except for the data at the very low (10 μM) ATP concentration. (The regression line in Fig. 1 was drawn so as to best fit the data except at 10 μM .) Essentially the same results were obtained in other reactivated sperm. These results confirm the previous reports that the beat frequency of Triton-demembrated sperm shows Michaelis-Menten type dependence on the ATP concentration, and also show that the same relationship exists in the other sperm preparations. The corresponding values of the apparent Michaelis constant for the beat frequency, k_m , and for the maximum frequency, $f_{\max}$, are summarized in Table 2. There were no significant differences in k_m and $f_{\max}$ between the four sperm preparations.

The angles of the principal and the reverse bends are plotted as a function of the distance from the flagellar base in Fig. 2. The bend angle increased constantly from the base to the point about 8 μm away from the base; thereafter the bend propagated toward the tip with an almost constant bend angle. The plateau values of bend angle were defined as the maximum bend angle, and their values averaged over 20 sperm were shown in Table 3. For both principal and reverse bends, no significant difference was found between the four preparations by F-test ($P < 0.05$). Also, we found no significant difference in wavelength between the four sperm prepara-

TABLE 2. THE APPARENT MICHAELIS CONSTANTS FOR BEAT FREQUENCY (K_m) AND FOR THE MAXIMUM FREQUENCY ($f_{\max}$).

	Triton X-100	Nonidet P-40	Triton X-100 +CHAPS	Nonidet P-40 +CHAPS
K_m (μM^{-1})	137.3	133.3	137.8	132.9
$f_{\max}$ (Hz)	35.2	35.1	35.3	34.9

These values were calculated from the Michaelis-Menten equation so as to fit the data above 20 μM ATP.

Fig. 2. Bend angles of propagating principal (P) and reverse (R) bends, plotted against the distance from the base, measured along the flagellum, of the midpoint of each bend. Sperm were demembrated with a solution containing: (a) 0.04% Triton X-100; (b) 0.05% Nonidet P-40; (c) 0.04% CHAPS plus 0.01% Triton X-100; and (d) 0.04% CHAPS plus 0.01% Nonidet P-40. The principal and reverse bends actually have opposite signs, but the absolute values were plotted here.

tions (data not shown).

Rotation of the beat plane. We studied the response of reactivated sperm to the rotation of the beat plane using a method similar to that used by Gibbons *et al.* (6). As Table 4 shows, the beat plane of the sperm demembrated with Triton X-100 did not rotate by more than one and a half revolutions. Substitution of Nonidet P-40 for Triton X-100 increased the proportion of the sperm showing rotation, but the plane could rotate through only a few cycles. In sperm demembrated with 0.05% CHAPS, though the reactivation rate was low, most of the motile sperm showed rotation of the beat plane. As shown above, the reactivation rate was higher when CHAPS was used with Nonidet P-40. By changing the ratio of Nonidet P-40 to CHAPS, while keeping the total concentration of detergents at 0.05% (w/v), we found that the sperm demembrated with a solution containing 0.04% CHAPS and 0.01% Nonidet P-40 showed the best results with about 85% of the reactivated sperm responding to the imposed rotatory vibration by rotating their flagellar beat

TABLE 3. MAXIMUM BEND ANGLES.

	Bend angle (radians)	
	Principal bend	Reverse bend
Triton X-100	2.45 ± 0.10	1.47 ± 0.12
Nonidet P-40	2.46 ± 0.11	1.48 ± 0.14
Triton X-100+CHAPS	2.44 ± 0.13	1.44 ± 0.17
Nonidet P-40+CHAPS	2.45 ± 0.10	1.47 ± 0.12

The maximum bend angles are the plateau values as shown in Fig. 2. Data from 20 sperm are averaged. Mean $\pm$ S.D.

TABLE 4. PERCENTAGES OF SPERM THAT SHOWED ROTATION OF THE FLAGELLAR BEAT PLANE.

Number of revolutions of the beat plane	Triton X-100	Nonidet P-40	CHAPS	Nonidet P-40 + CHAPS
<0.5	60%	45%	0%	0%
0.5–1.0	20%	38%	27%	15%
>1.0	20%	17%	73%	85%
Maximum	1.5	3	7	10

Concentrations of detergents: 0.04% Triton X-100; 0.05% Nonidet P-40; 0.05% CHAPS; 0.04% CHAPS plus 0.01% Nonidet P-40.

Fig. 3. Electron micrographs of cross sections of flagella: (a) fixed without demembranation, (b) demembranated with a solution containing 0.04% Triton X-100, and (c) demembranated with a solution containing 0.04% CHAPS and 0.01% Nonidet P-40. Bar indicates 100 nm.

plane through as many as 10 cycles of revolution. Upon cessation of the rotatory vibration, these sperm also showed spontaneous reverse rotation ('unwinding'). When the concentration of Nonidet P-40 was above 0.013% (w/v) the beat plane rotated for a few revolutions at most and when it was below 0.008% the reactivation rate was low. A detailed study of the rotation of the beat plane in reactivated sperm will be reported on elsewhere.

Electron microscopic studies. As shown above, sperm permeabilized with Triton X-100 and those permeabilized with Nonidet P-40 and CHAPS behaved differently with respect to the rotation of the beat plane. To see if there is any structural difference between these two sperm preparations, we examined their ultrastructure. As has previously been reported (4), 0.04% Triton X-100 completely removed the membrane from around the axoneme, which remained apparently unchanged in structure as compared with that of the live sperm (Fig. 3a, b). The same results were obtained with sperm prepared with 0.04% CHAPS and 0.01% Nonidet P-40. The membrane was thoroughly removed from the axoneme and the ultrastructure of the axoneme was not different from that of the live sperm or that of the Triton-extracted sperm (Fig. 3c).

DISCUSSION

One of the primary objectives of the present study was to understand why the conventional Triton-extracted sea-urchin sperm whose head was subjected to vibration in a rotating plane was incapable of rotating its flagellar beat plane for more than one and a half cycles whereas the live sperm could rotate its flagellar beat plane through more than ten cycles under similar conditions. In many respects, the move-

ment of Triton-extracted sperm closely resembles that of the live sperm (3, 4). It is probable that Triton X-100 which removes the cell membrane also affects the flagellar motile system or its regulatory mechanism by removing or modifying some of the axonemal components. With a view to developing a new demembration procedure, therefore, we tested various detergents which had been reported to give good results in the study of cell motility in other types of cell, or those which have been known to have little effect on enzymatic activities (9, 15). We found that CHAPS effectively demembrates sea-urchin sperm while keeping their ability to rotate the flagellar beat plane apparently unchanged. A sulfobetaine derivative of cholic acid, CHAPS is a zwitterionic detergent (9). It prevents artifactual aggregation of proteins, is nondenaturing and preserves the enzymatic activities (9, 15). Though it has been used to solubilize proteins from membrane-bound compartments (8, 11), to our knowledge it has not been used for the study of cell motility. We found that when CHAPS was used with Nonidet P-40 the demembrated sperm showed a high reactivation rate and their beat plane could be rotated as in the live sperm. No significant difference in other aspects of motility and in ultrastructure has been found between the sperm demembrated by the new procedure and those demembrated with Triton X-100. These results suggest that the sperm demembrated with CHAPS and Nonidet P-40 retain many characteristics of the live sperm, including the rotatability of the beat plane. It is expected that demembration with CHAPS and Nonidet P-40 will be a useful method for the study of flagellar movement of sea urchin sperm. It will also be applicable to the study of cell motility in other cell types.

In this study, we examined the relationship between the ATP concentration and the beat frequency in solutions containing potassium acetate instead of potassium chloride (5). We found that the effect of ATP concentration on the beat frequency showed a Michaelis-Menten type relationship above $20\text{ }\mu\text{M}$ and that at $10\text{ }\mu\text{M}$ the beat frequency deviated from the value expected from the Michaelis-Menten equation. It has been reported that the beat frequency falls below the calculated Michaelis-Menten curve below $50\text{ }\mu\text{M}$ in solutions containing chloride as the major anion (2). Thus, the present results suggest that the critical concentration of ATP, above which the beat frequency obeys the Michaelis-Menten type kinetics, is lower with acetate than with chloride. We also calculated k_m values from the data, excluding those at $10\text{ }\mu\text{M}$ ATP, and obtained the k_m values of $130\text{--}140\text{ }\mu\text{M}$ in all of the four preparations. Although these values are slightly smaller than those reported previously (2, 4), it is probably because the major anion in the reactivation solution was acetate and not chloride (5).

The present results indicate that the mechanism controlling the flagellar beat plane is independent of the mechanism controlling the flagellar beat frequency or that controlling the flagellar waveform, because the motility of the sperm demembrated with CHAPS and Nonidet P-40 is similar to that of the Triton-extracted sperm except for the ability of rotating the beat plane. It is hoped that by using the new demembration procedure we can further study the mechanism controlling the flagellar beat plane.

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