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Mitotic Asters Separate, although Chromosomes Do Not Separate at Slightly Acidic pHi in the Fertilized Egg of the Sea Urchin, *Hemicentrotus pulcherrimus*

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ABSTRACT—The effect of slightly acidic intracellular pH (pHi) on the development of the sea urchin, *Hemicentrotus pulcherrimus* was investigated. At first cleavage, the fertilized eggs were treated with artificial sea water containing sodium acetate (Ac-pHSW) at pH 6.8 or 7.0 at the onset of nuclear envelope breakdown, and their pHi decreased from 7.30 to 6.68 or 6.78, respectively. When the eggs were observed after fixation by indirect immunofluorescence and differential interference contrast microscopy, the mitotic stage of the treated eggs was arrested at metaphase and the mitotic apparatus was maintained until more than 50 min after the treatment, although it was smaller in size than that of non-treated eggs. On the other hand, the number of the mitotic asters increased from 2 to 3-4, and further to 6-8 following prolonged exposure, suggesting that the centrosomes had divided and replicated. These results suggest that the centrosome cycle advanced at slightly acidic pHi, even when the mitotic cycle did not advance beyond metaphase.

INTRODUCTION

The centrosome is considered to be the organizing center of the mitotic apparatus in an animal cell. The centrosomes and chromosomes closely coordinate and cooperate in the formation of the mitotic apparatus. However, Mazia (1974) demonstrated that chromosome cycles turn on in unfertilized sea urchin eggs in the absence of the mitotic apparatus when they were exposed to sea water (SW) containing NH_4OH at alkaline pH, which is believed to result in increase of intracellular pH (pHi) of the eggs. Recently, we have found that the chromosome cycle did not advance beyond metaphase and the mitotic apparatus appeared to be stable in fertilized sand dollar eggs when the pHi was slightly lowered by treatment with SW containing acetate (Watanabe *et al.*, 1997). These reports indicate that the change in pHi is able to influence the chromosome cycle. However, it is unknown whether the centrosome cycle is affected by pHi.

In this study, we have investigated the effect of acidic pHi on cell division in the sea urchin egg, following prolonged treatment with SW containing acetate. We observed that the mitotic stage was arrested at metaphase in terms of the chromosome behavior, unless the mitotic apparatus became multipolar from bipolar, suggesting that the centrosome cycle advances at slightly acidic pHi, whereas the chromosome cycle stops.

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MATERIALS AND METHODS

Gametes of the sea urchin, *Hemicentrotus pulcherrimus* were obtained by injection of SW containing 1 mM acetylcholine into the body cavity. Eggs were washed three times with artificial SW (Jamarin-U, Jamarin Laboratory, Osaka, Japan), kept at 15°C, and used within four hr of shedding. Sperm was collected as "dry", kept at 4°C in a refrigerator, and diluted just before use. Fertilized eggs were deprived of both the fertilization envelope and hyaline layer by treating them with 1 M urea solution for 1.5 min shortly after insemination and incubated in Ca-free SW (Ca-free Jamarin-U, Jamarin Lab., Osaka) at 20°C. The fertilized eggs were treated at the onset of nuclear envelope breakdown with SW containing 20 mM acetate at pH 6.5, 6.8, 7.0, and 7.3 (Ac-pHSW). At an appropriate time after the treatment, the eggs were extracted using a microtubule-stabilizing solution; 10 mM EGTA (ethyleneglycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid), 25 mM MES (2-(*N*-morpholino)ethanesulfonic acid), 0.55 mM $MgCl_2$, 25% glycerol, 1% Nonidet P-40, adjusted to pH 6.7 with KOH. These eggs were used for morphological measurements either directly or after immunofluorescence staining.

Indirect immunofluorescence was carried out using an anti-tubulin antibody as described earlier (Oka *et al.*, 1994). The fertilized eggs were extracted, attached to polylysine-coated coverslip, and fixed with 100% cold methanol. They were stained with the antibody, DM1A (Amersham, England) for microtubule observation and then stained with 0.1 μg/ml of DAPI (4',6-diamidino-2-phenylindole; Sigma Chemical Co., St. Louis, USA) for chromosome observation.

The mitotic stage of the cell was determined by observing chromosomes with fluorescence and differential interference contrast microscopes and the populations of the cells were categorized into three groups; prophase/prometaphase, metaphase, and anaphase/telophase. The number of asters was determined by counting mitotic or-

ganizing centers with a fluorescence microscope.

The composition of the pHSW used was as follows: Normal-pHSW; 436 mM NaCl, 9 mM KCl, 34 mM $MgCl_2$, 16 mM $MgSO_4$, 1 mM EGTA, and 5 mM PIPES (1,4-piperazinediethanesulfonic acid), Ac-pHSW; 416 mM NaCl, 20 mM sodium acetate, 9 mM KCl, 34 mM $MgCl_2$, 16 mM $MgSO_4$, 1 mM EGTA, and 5 mM PIPES. These pH was adjusted to 6.5, 6.8, 7.0 and 7.3 with NaOH.

pHi was measured using a fluorescent pH indicator dye, pyranine (1-hydroxypyrene-3,6,8-trisulfate; Tokyo Kasei Kogyo Ltd., Tokyo, Japan), by the method described by Hamaguchi *et al.* (1997). Measurement of pHi was performed before treatment and 6-10 min after treatment with Ac-pHSW at 6.5, 6.8, 7.0, and 7.3, and Normal-pHSW at pH 6.5, 6.8, and 7.3.

RESULTS AND DISCUSSION

Stability of the mitotic apparatus and the mitotic stage at slightly acidic pH

The eggs treated at the onset of nuclear envelope breakdown with Ac-pHSW at pH 7.3 divided, but after treating the eggs with Ac-pHSW at pH 6.5, 6.8, and 7.0, most eggs did not divide (data not shown). When the eggs were treated with Normal-pHSW at pH 6.5, 6.8, 7.0, and 7.3 as controls, all eggs divided (data not shown). According to previous reports (Hamaguchi *et al.*, 1997; Watanabe *et al.*, 1997), the division appeared to be inhibited by acidification of the pHi. To estimate the acidification, pHi was determined during the treatment. The pHi of the fertilized egg was 7.30 ± 0.04 (n = 48). The pHi gradually decreased during treatment with Ac-pHSW and reached a plateau 6-10 min after the treatment. At 6-10 min of the treatment with Ac-pHSW at pH 6.5, 6.8, 7.0, and 7.3, the pHi of the eggs were estimated to be 6.53 ± 0.03 (n = 7), 6.68 ± 0.03 (n = 15), 6.78 ± 0.07 (n = 10), and 6.89 ± 0.01 (n = 6), respectively. On the other hand, the pHi of the egg treated with Normal-pHSW at pH 6.5, 6.8, and 7.3 were estimated to be 7.14 ± 0.04 (n = 6), 7.27 ± 0.03 (n = 11), and 7.29 ± 0.02 (n = 8), respectively. Furthermore, to understand how the division was inhibited, the mitotic figure was examined. The eggs of *H. pulcherrimus* were extracted using a microtubule-stabilizing medium after 5-17 min of treatment with Ac-pHSW at pH 6.5, 6.8, or 7.0, when cell cycle advanced from prometaphase to telophase in control eggs, and stained with the anti-tubulin antibody. At pH 6.5, the mitotic apparatus was not organized, but chromosomes were condensed (data not shown). suggesting that cell division was inhibited on the way to organization of the mitotic apparatus. At pH 6.8 and 7.0, during treatment, many eggs developed from prometaphase to metaphase (Fig. 1). The mitotic stage of each egg was determined, and then the rates of the eggs at prophase/prometaphase, metaphase, and anaphase/telophase were measured after the treatment with Ac-pHSW at pH 6.8 and 7.0. As shown in Fig. 2, the eggs at metaphase increased in number with time, but none at anaphase/telophase were observed. This result indicates that the treated eggs were arrested at metaphase, and confirms previous results (Watanabe *et al.*, 1997) in regard to the mitotic stage. Moreover, pHi decrease to 6.78 or less inhibited the advance of the mitotic stage, but a decrease to 6.89 in the eggs treated with Ac-pHSW at

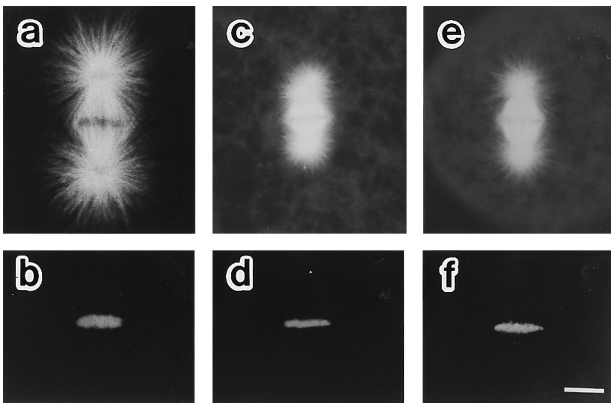

Fig. 1. Fluorescence micrographs of the mitotic apparatus and chromosomes in the fertilized egg at 11 min after treatment with Ac-pHSW. (**a, c, e**) Immunofluorescence micrographs of the mitotic apparatus. (**b, d, f**) Fluorescence micrographs of chromosomes stained with DAPI. (**a, b**) Control. (**c, d**) An egg treated with Ac-pHSW at pH 6.8. (**e, f**) An egg treated with Ac-pHSW at pH 7.0. Bar shows 10 μm.

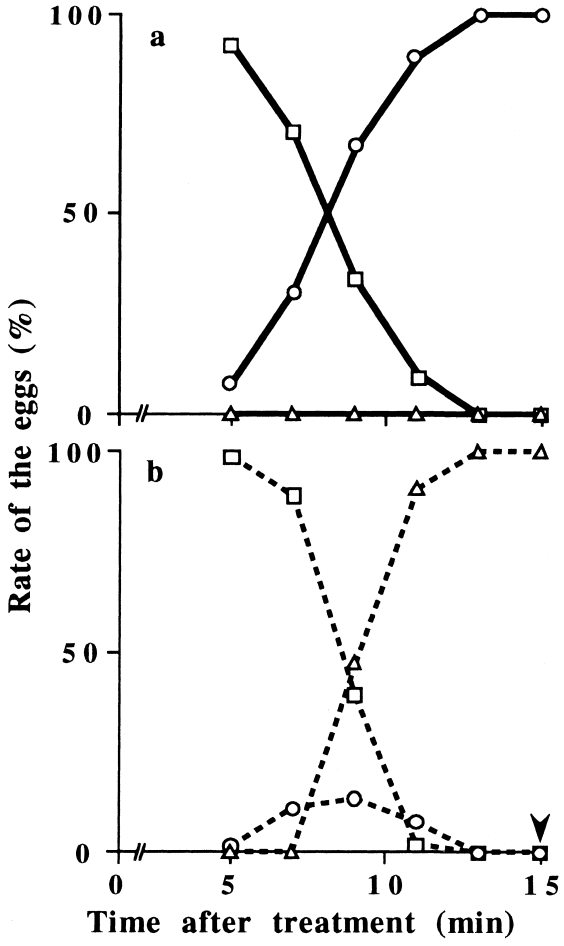

Fig. 2. The rates of mitotic stage in the fertilized eggs treated with Ac-pHSW at pH 7.0 (**a**) and without treatment (**b**). Squares, circles, and triangles represent the rates of pro-/prometaphase, metaphase, and ana- /telophase, respectively. Arrowhead shows the time of the onset of the first cleavage in non-treated eggs.

pH 7.3 was insufficient to arrest mitosis at metaphase, which concurred with the previous findings (Watanabe *et al*., 1997). It has not been reported previously whether mitotic arrest is caused by pHi shift to acidic values, although mitotic arrest at metaphase is well-known during maturation division in various animals (Masui, 1985). Although pHi was not constant during cell division (Grandin and Charbonneau, 1990; Lee and Steinhardt, 1981a,b; Webb and Nuccitelli, 1981), the pHi change was much less than the pHi shift during the mitotic arrest in this study.

The microtubules appeared to be stable by treatment with Ac-pHSW at pH 6.8 and 7.0, but the pole-to-pole length of the mitotic apparatus at metaphase gradually reduced by 20-30% 11-17 min after the treatment with Ac-pHSW at pH 6.8, 7.0, compared to the pole-to-pole length at metaphase in non-treated eggs. Treatment with a microtubule stabilizing agent, increases the number of microtubules in the cell but does not always induce microtubule elongation. Recently, plus- and minus-end dynamics of kinetochore microtubules are reported to be differentially sensitive to taxol; plus-ends were inactivated by taxol, whereas minus-ends were not, resulting in spindle shortening (Waters *et al*., 1996). If such inactivation of microtubules occurred during stabilization by the treatment with Ac-pHSW in *H. pulcherrimus*, the mitotic apparatus may reduce in size. In addition, in the eggs arrested with Ac-pHSW, a large number of small cytasters were formed in the cytoplasm outside the mitotic apparatus. This may result in the depletion of free tubulin dimers in the cytoplasm, which may cause the reduction in size of the mitotic apparatus. A similar phenomenon was reported following treatment of eggs with hexylene glycol, another microtubule stabilizing agent (Endo *et al*., 1990).

Multiplication of asters in the treated eggs at slightly acidic pH

Until more than 50 min after the treatment with Ac-pHSW, the eggs were extracted at intervals of a few minutes. As shown in Figs. 3 and 4, in spite of the chromosomes in the spindle indicating metaphase, the asters had divided from two to three or four 25-50 min after treatment, when control eggs had already cleaved into two cells. Furthermore, after the second cleavage of control eggs, the asters of treated eggs increased even further. In Fig. 3g and h, at least 7 asters were observed. This implies that the centrosomes replicated during mitotic arrest in treated eggs. The multiplication of the asters or centrosomes was reported without nuclear division in the sea urchin eggs treated with DNP (dinitrophenol), a metabolic inhibitor (Kojima and Czihak, 1990) and 2-mercaptoethanol, a reducing agent (Paweletz *et al*., 1984). According to their results, the spindle in treated eggs persisted but the asters were divided into from two to three or four. Although the pK of DNP is acidic like that of acetic acid, the effect was probably not caused directly as a result of acidification by the permeation of DNP, because the concentration of DNP was much lower than that of acetate in this study. However, it may be possible that pHi decreased as a result of the metabolic inhibition during the treatment with DNP. There are some differences between these reports and the present results. In this study, the chromosomes were arranged in the equator of the spindle (Fig. 3). On the other hand, in the case of the eggs treated with DNP or 2-mercaptoethanol, two or three spindles were formed similar to the case in poly-spermic eggs and the cen-

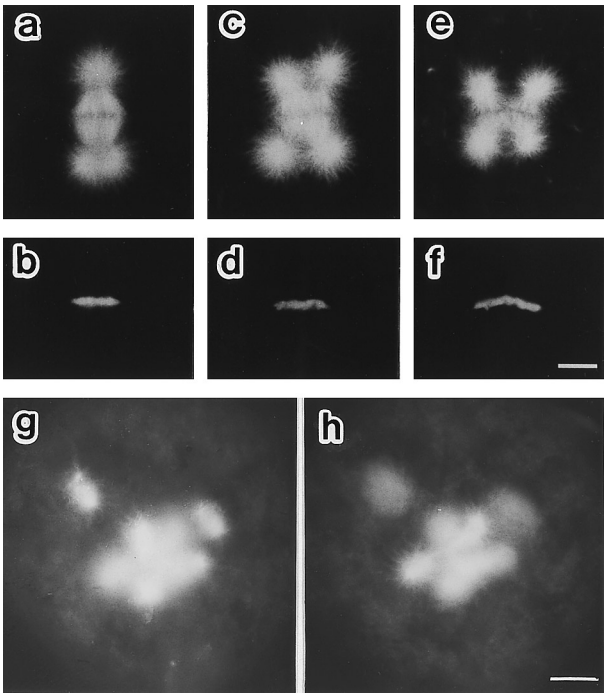

Fig. 3. Division of the mitotic asters in the fertilized egg treated with Ac-pHSW at pH 6.8. (**a, c, e, g, h**) Immunofluorescence micrographs of the mitotic apparatus. (**b, d, f**) Fluorescence micrographs of the chromosome stained with DAPI. (**a, b**) 26 min, (**c, d**) 36 min, (**e, f**) 49 min and (**g, h**) 65 min after the treatment. **h** is the same specimen as **g** at a focal plane different from **g**. Bars show 10 μm.

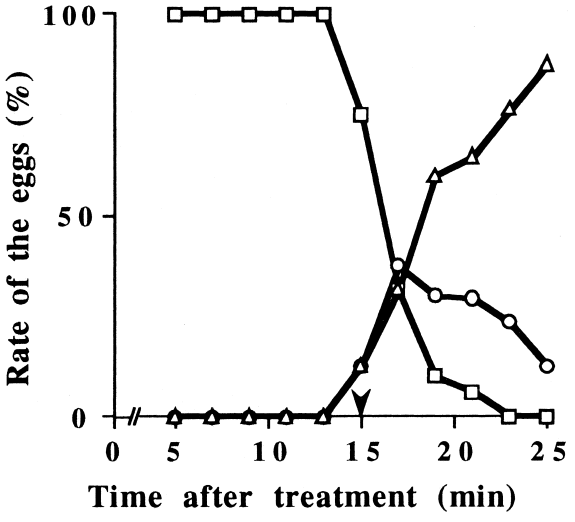

Fig. 4. The number of mitotic asters after treatment with Ac-pHSW at pH 7.0. The rate of eggs with bi- (square), tri- (circle) and tetra-polar (triangle) asters within 25 min after the treatment. Arrowhead shows the time of the onset of the first cleavage in control eggs.

trosomes did not divide any more. Nevertheless, three studies including ours demonstrate that the mitotic asters can separate despite the lack of chromosome movement. Accordingly, the separation mechanism of the mitotic aster seems to be clearly different from that of chromosomes.

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