Effects of Tyrosine Kinase Inhibitor on the Motility and ATP Concentrations of Fowl Spermatozoa

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ABSTRACT The possible role of tyrosine kinase in the regulation of fowl sperm motility was investigated by using a stable analogue of erbstatin, methyl 2, 5-dihydroxycinnamate (2, 5-MeC), a specific inhibitor of tyrosine kinase. This inhibited the motility of intact spermatozoa at 30°C in a dose-dependent manner. In contrast, the motility of demembranated spermatozoa was not inhibited by the same concentrations of 2, 5-MeC. At 40°C, both intact and demembranated spermatozoa were almost immotile with or without 2, 5-MeC. Additionally, intact spermatozoa, stimulated by the addition of Ca^{2+} or calyculin A, a specific inhibitor of protein phosphatases, lost their motility with the subsequent addition of 2, 5-MeC at 40°C. However, unlike the motility, the ATP concentrations of spermatozoa were maintained in about 30-35 nmol ATP/10^9 cells during these incubation periods. The activity of tyrosine kinase of spermatozoa at 30°C, estimated by measuring the phosphorylation of a synthetic peptide substrate, RR-SRC, was 0.17 pmol/min/mg protein. This activity was lower than those of fowl testes and chick brain, but higher than that of chick liver. These results suggest that tyrosine kinase activity, which is not retained in the axoneme and/or accessory cytoskeletal components, may be involved in the maintenance of flagellar movement of fowl spermatozoa at 30°C.

Keywords: Spermatozoa, Protein phosphorylation/dephosphorylation, Tyrosine kinase, Motility

INTRODUCTION

Intracellular cascade systems for the regulation of sperm motility are triggered by several factors, mainly second messengers, such as cyclic nucleotides and Ca^{2+}, which seem to converge upon the phosphorylation and dephosphorylation of proteins through several kinds of protein kinases and phosphatases which would be related to axonemal movement (for review, see Morisawa, 1994). Based on the hydroxyamino acids phosphorylated in their substrates, protein kinases have been traditionally subdivided into two broard classes: the protein serine/threonine kinases and the protein tyrosine kinases (Berruti, 1994).

With regard to the protein serine/threonine kinases involved in the regulation of sperm motility, there is substantial evidence that protein phosphorylation by cAMP-dependent protein kinase is involved in the activation of sperm motility (for reviews, see Tash and Means, 1983; Lindemann and Kanous, 1989; Morisawa, 1994). The activation of myosin light chain kinase (MLCK) or MLCK-like protein also may be involved in the maintenance of fowl sperm motility, since the addition of MLCK substrate peptide inhibited the motility of demembranated spermatozoa at 30°C (Ashizawa et al., 1995b, c). Protein kinase C has been identified in human, bovine and fowl spermatozoa (Rotem et al., 1990a; Chaudhry and Casillas, 1992; Ashizawa et al., 1994a; respectively). Since phorbol esters and diacylglycerol analogues, the activators of protein kinase C, stimulated human sperm motility, protein kinase C may also be involved in regulating human sperm motility (Rotem et al., 1990a,b).

On the other hand, the involvement of protein tyrosine kinase in the initiation and activation of motility of trout and *Ciona* spermatozoa was suggested (Hayashi et al., 1987; Dey and Brokaw, 1991). With trout

spermatozoa, it was demonstrated that a 15 kDa axonemal protein was phosphorylated on tyrosine residues, and that cAMP-dependent phosphorylation of this protein in vitro was inhibited by a randam copolymer of tyrosine and glutamate (PGT) that can serve as a competitive substrate or competitive inhibitor of tyrosine phosphorylation (Hayashi et al., 1987). The addition of PGT also inhibited the motility of demembranated *Ciona* spermatozoa (Dey and Brokaw, 1991). A tyrosine kinase has been partially purified from boar spermatozoa, the first in mature mammalian spermatozoa to be partially characterized (Berruti and Porzio, 1992). Furthermore, recent work has demonstrated the identification of a 55 kDa soluble protein whose tyrosine phosphorylation varies directly with bovine sperm motility and has suggested that regulation of motility may involve cross talk between cAMP-dpendent protein kinase, Ca^{2+} and tyrosine kinase pathways (Vijayaraghavan et al., 1997).

Fowl spermatozoa display the unique phenomenon of reversible temperature-dependent immobilization: in simple salt solutions they become immotile at the avian body temperature of 40-41°C, but motility is immediately restored by decreasing the temperature (Munro, 1938; Ashizawa and Nishiyama, 1978; Thomson and Wishart, 1989, 1991). Even although they provide an excellent model for investigating the regulatory mechanisms of sperm motility, no information is available concerning the effects of tyrosine kinase on the regulation of sperm motility.

In the following experiment, therefore, attempts were made to investigate the effects of tyrosine kinase inhibitor on the motility and ATP concentrations of fowl spermatozoa.

MATERIALS AND METHODS

Animals and Preparation of Spermatozoa

Commercial White Leghorn roosters (Babcock strain, Akagi Poultry Breeding Farm, Miyazaki, Japan) were used throughout the study. All birds were housed in individual cages and fed ad libitum on a commercial breeder diet. They were exposed to a photoperiod of 14 hr light : 10 hr dark. New born chicks were also used for the analysis of protein tyrosine kinase activity.

Semen was collected by the method of Bogdonoff and Shaffner (1954). Samples of semen pooled from four to six males were diluted approximately tenfold in 150 mM NaCl with 20 mM TES (N-Tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) at pH 7.4 and centrifuged at 700 ***g*** for 15 min at room temperature (20-25°C). The washed spermatozoa were reconstituted in the same buffer to give a final concentration of approximately 1 x 10^9 cells/ml. Samples of 3-4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Chemicals

Methyl 2, 5-dihydroxycinnamate (2, 5-MeC), a stable analogue of erbstatin, was purchased from Kyowa Medex Co., Ltd., Tokyo, Japan. Calyculin A was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Adenosine 5'-triphosphate (ATP), aprotinin, desiccated firefly tails, dithiothreitol (DTT), β-glycerophosphate, leupeptin, potassium glutamate, a random copolymer of tyrosine and glutamate (poly-Glu-Tyr [4:1], or PGT), TES and Triton X-100 were purchased from Sigma Chemical Co., St Louis,

MO. Protein tyrosine kinase assay system kit was obtained from Life Technologies, Inc., Gaithersburg, MD, and bicinchoninic acid (BCA) protein assay regent was from Pierce Chemical Co., Rockford, IL. Other chemicals were of reagent grade from Nacalai Tesque Inc., Kyoto, Japan.

Measurement of Motility of Intact and Demembranated Spermatozoa

Sperm samples were pre-incubated aerobically in a shaking water bath at 30°C or 40°C for 10 min. After the pre-incubation, the dose-response and time course of motility of intact spermatozoa were measured at 30°C or 40°C after addition of various concentrations of 2, 5-MeC, a membrane permeable specific inhibitor of tyrosine kinase (Umezawa et al., 1990). The effects of the addition of $CaCl_2$ or calyculin A, a inhibitor of protein phosphatase-type 1 and -type 2, before the addition of 2, 5-MeC were also examined at 30°C and 40°C to be compared with those of inhibitor alone. Stock solutions of 2, 5-MeC and calyculin A were made in dimethyl sulfoxide and were stored at -20°C. Diluent for the measurement of intact sperm motility was TES/NaCl buffer described above.

Demembranation and reactivation of spermatozoa were performed at 30°C and 40°C according to the method described previously (Ashizawa et al., 1989b). The extraction medium used consisted of 0.1% (v/v) Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM DTT and 20 mM Tris-HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM ATP, 200 mM sucrose, 25 mM potassium glutamate, 1.5 mM $MgSO_4$, 1 mM DTT and 20 mM Tris-HCl buffer (pH 7.9). To examine the effects of typrosine kinase inhibitors, various concentrations of 2, 5-MeC and PGT were added to the reactivation medium.

The suspension of intact or demembranated spermatozoa was placed into a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a thermostatically-controlled warm plate, and the motility of spermatozoa was recorded by videomicroscopy (magnification on the 12-inch black and white monitor was approximately x 600) at 30°C or 40°C (Katz and Overstreet, 1981). Measurements were made on a total of 200-300 spermatozoa, distributed uniformly among three or more fields, to determine the percentage of vigorously motile spermatozoa.

Measurement of ATP Concentrations of Intact Spermatozoa

ATP content in spermatozoa was assayed by firefly bioluminescence in a boiled extract (Wishart, 1982). Numbers of spermatozoa were estimated by the method of Wishart and Ross (1985), using a double-beam spectrophotometer (Shimadzu, Model UV-150-02, Kyoto, Japan). The concentration of ATP was expressed in terms of nmol ATP/10^9 spermatozoa.

Measurment of Tyrosin Kinase Activity

Washed spermatozoa were suspended in the extraction buffer consisted of 50 mM HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), pH 7.4, 50 mM β-glycerophosphate, 25 mM NaF, 150 mM NaCl, 15 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 20 mM EGTA (ethylene glycol bis (β-aminoethylether)-N, N, N', N'-tetraacetic acid), 1 mM DTT, 25μg/ml leupeptin, 25 μg/ml aprotinin. The suspension was sonicated for 60 sec on ice with an ultrasonic processor with a microtip (Sonics & Materials, Inc., Danbury, CT) at 50 W. After removing cellular debris by centrifuging at 16,000 ***g*** for 20

min at 0°C, the supernatant was collected as a sample for the enzyme activity assay. New born chick brain and liver and rooster testes were also treated as the same manner, except the homogenization; in these cases teflon glass homogenizer was used. Protein concentration was determined using BCA protein assay reagent and bovine serum albumin as a standard.

Using a synthetic peptide substrate with the sequence Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, referred to as RR-SRC, specific for tyrosine kinase, the activity of the enzyme was measured by the method described in the protocal of protein tyrosine kinase assay system kit. Briefly, 10 µl of sample described above and 10 µl of substrate solution containing 1 µCi [γ-^{32}P]ATP, 60 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.2 mM DTT, 40 µM EDTA (ethylenediaminetetraacetic acid), 50 µg/ml bovine serum albumin, 0.3% (v/v) Nonidet P-40, 140 µM sodium orthovanadate, 120 µM ATP and with or without 1 mM RR-SRC peptide were mixed and incubated at 30°C for 15 min. The reaction was stopped by the addition of 20 µl of 10% (w/v) trichloroacetic acid. Following centrifugation (16,000 ***g***, 20 min), the supernatant was spotted on a piece of phosphocellulose paper and washed twice in 1% (v/v) acetic acid, three times in distilled water for 3-5 min, respectively. The papers were then placed into scintillation vials with scintillation fluid, and counted for peptide-incorporated ^{32}P.

Statistical Analysis

Percentage motility was transformed using arc sine transformation. The results were analyzed by Duncan's multiple-range tests (Duncan, 1955).

RESULTS

Effects of Tyrosine Kinase Inhibitor on the Motility of Intact and Demembranated Fowl Spermatozoa

The vigorous motility of intact spermatozoa at 30°C was inhibited in a dose-dependent manner by the addition of tyrosine kinase inhibitor, 2, 5-MeC. At 40°C, the addition of inhibitor did not appreciably affect the motility of intact spermatozoa, which remained almost negligible (Fig. 1a).

On the other hand, inhibition of motility by the addition of 2, 5-MeC was not observed in demembranated and reactivated spermatozoa at 30°C within the range 1-100 μM (Fig. 1b), and the motility was maintained as well as those of the control (no addition of 2, 5-MeC). In addtion, PGT, a synthetic substrate of tyrosine kinase, did not appreciably affect the motility (Fig. 1b). PGT was not used for intact spermatozoa due to the impermeability to the plasma membrane (M.W. of 20,000-50,000).

Figure 1 shows the inhibition of motility at 10 min after the addition of drugs. The time-course of motility at 30°C and 40°C is shown in Fig. 2. Even the presence of Ca^{2+} before the addition of 2, 5-MeC could not prevent the inhibition of motility of intact spermatozoa at 30°C (Fig. 2a). At 40°C, the motility of intact spermatozoa was negligible, but the motility was restored immediately after the addition of 1 mM $CaCl_2$. However, the subsequent addition of 2, 5-MeC inhibited the motility again (Fig. 2b).

The inhibition of motility of intact spermatozoa was also observed at 30°C by the addition of 2, 5-MeC, even the presence of protein phosphatase inhibitor, calyculin A (Fig. 3a). The presence of calyculin A permitted restoration of motility of intact spermatozoa at 40°C, and this effect was

maintained for at least 30 min. However, calyculin A-stimulated motility was inhibited by the subsequent addition of 2, 5-MeC (Fig. 3b).

Effects of Tyrosine Kinase Inhibitor on the ATP Concentrations of Intact Fowl Spermatozoa

The ATP concentration of intact spermatozoa following exposure to 200 μM 2, 5-MeC decreased slightly at both 30°C and 40°C (32.9±2.8 and 38.9±4.9 nmol/10^9 spermatozoa, respectively; the mean±SEM of five samples) compared with those of untreated spermatozoa (control) (37.2±2.9 at 30°C and 49.7±5.2 at 40°C), but there was no significant difference between them.

Additinally, the ATP concentrations of intact spermatozoa decreased slightly at 30°C and 40°C after the addtion of $CaCl_2$ or calyculin A. The subsequent addition of 2, 5-MeC did not induce the decrease of ATP concentrations compared with those of the controls (Figs. 4, 5).

Protein Tyrosine Kinase Activity of Fowl Spermatozoa and Different Tissues

The presence of 100 μM 2, 5-MeC inhibited the activity of sperm tyrosine kinase (0.0121±0.010 pmol/min/mg protein; the mean±SEM of five samples) significantly ($P<0.05$) compared with those of untreated spermatozoa (control) (0.174±0.015). Among 4 kinds of cells, the highest activity was obtained in the chick brain (0.994±0.025 pmol/min/mg protein; the mean± SEM of five samples). The enzyme activity of spermatozoa (0.173±0.012) was lower than those of brain and fowl testes (0.348±0.019), but significantly higher than that of chick liver (0.068±0.010) ($P<0.05$).

DISCUSSION

Protein phosphorylation is one of the major mechanisms for controlling cell functions in response to changing physiological conditions, and this posttranslational modification is carried out by protein kinases (Berruti, 1994). Although we did not demonstrate the molecular identity of tyrosine kinase of fowl spermatozoa directly, using immunoblotting and/or immunoprecipitation procedure, our results indicated that fowl spermatozoa possessed protein tyrosine kinase activity, by measuring the phosphorylation of a synthetic peptide substrate, RR-SRC: the activity was lower than those of fowl testes and chick brain, but higher than that of chick liver. In general, proliferative cells have relatively high levels of tyrosine kinase activity in adult rat tissues (Kobayashi et al., 1989), and it has been suggested that the primary function of tyrosine kinase is the regulation of cell proliferation (Swarup et al., 1983). However, participation of tyrosine kinase activity in several sperm functions of mammalian spermatozoa has already been reported. For example, tyrosine phosphorylation of sperm proteins is increased with capacitation and in response to physiological stimulation of acrosome reaction with zona pellucida proteins in mouse and human spermatozoa (Leyton and Saling, 1989; Naz et al., 1991; Leyton et al., 1992; Duncan and Fraser, 1993; Burks et al., 1995; Luconi et al., 1995; Visconti et al., 1995a, b).

With regard to sperm motility, phosphorylation of a tyrosine residue with a 15 kDa axonemal protein, called a flagellar movement-initiating phosphoprotein (MIPP) (Jin et al., 1994), by tyrosine kinase has been found to play a role in the initiation of flagellar movement of quiescent spermatozoa of the rainbow trout (Morisawa and Hayashi, 1985; Hayashi et al., 1987). In

addition, it is suggested that in this system the activation of tyrosine kinase might be activated by a cAMP-dependent protein kinase (Hayashi et al., 1987). Furthermore, Dey and Brokaw (1991) reported that the activation of motility of demembranated *Ciona* spermatozoa by incubation with cAMP can be completely inhibited by the addition of PGT at a relatively low concentration of 20 μg/ml. Since no inhibition of activation was observed with a control polymer, PGA (a random 3:2 copolymer of glutamate and alanine), at concentrations up to 200 μg/ml, the PGT effect was interpreted as a tyrosine-specific effect, rather than a general polyanion effect (Dey and Brokaw, 1991). The PGT has also been shown to inhibit tyrosine phosphorylation in other systems (Braun et al., 1984). In this study, not only PGT, but also 2, 5-MeC, a stable analogue of erbstatin, was used as a specific inhibitor of tyrosine kinase. This analogue is permeable to the plasma membrane, about 4 times more stable than erbstatin in serum and specifically inhibits tyrosine kinase activity (Umezawa et al., 1990). Consequently, the addition of 2, 5-MeC inhibited the motility of intact fowl spermatozoa at 30°C in a dose-dependent manner. Furthermore, the activity of tyrosine kinase of fowl spermatozoa was also inhibited significantly by the addition of 2, 5-MeC. These results suggest that the activity of tyrosine kinase is involved in the regulation of fowl spermatozoa as well as in trout and *Ciona* spermatozoa.

Fowl spermatozoa display the unique phenomenon of reversible temperature-dependent immobilization: in simple salt solutions they become immotile at the avian body temperature of 40-41°C, but motility is immediately restored by decreasing the temperature (Munro, 1938; Ashizawa and Nishiyama, 1978; Thomson and Wishart, 1989, 1991). The axoneme and/or accessory cytoskeletal components itself appears to be directly involved in this regulatory system, since the motility of demembranated spermatozoa is, as with intact

spermatozoa, negligible at 40°C and restored at 30°C (Ashizawa et al., 1989a, b). The addition of Ca^{2+} (Wishart and Ashizawa, 1987; Ashizawa et al., 1989a, 1994b) or calyculin A (Ashizawa et al., 1995a) is effective for the restoration of motility of intact spermatozoa at 40°C, and it appears that protein phosphatase-type 1 (PP1) may play a dominant role in the temperature-dependent inhibition of flagellar movement of fowl spermatozoa (Ashizawa et al., 1994c).

In the study reported here, stimulation of the motility of intact spermatozoa at 40°C by Ca^{2+} or calyculin A, was inhibited by the subsequent addition of 2, 5-MeC. Since this inhibition occurred without change in intracellular levels of ATP, it appears that 2, 5-MeC is not simply inhibiting energy production in these spermatozoa, but may be acting on some part of the regulatory cascade initiated by Ca^{2+} or calyculin A - which thus appears to involve tyrosine phosphorylation. Since the motility of intact spermatozoa at 30°C in the absence of any effector was also inhibited by 2, 5-MeC, tyrosine phosphorylation also appears to involved in this 'intrinsic' regulatory process.

As described above, the addition of PGT inhibited the motility of demembranated *Ciona* spermatozoa (Dey and Brokaw, 1991). Therefore, tyrosine kinase involved in the regulation of motility is presumably retained in the axonemal systems. In contrast, Berruti (1994) has demonstrated that the enzyme of boar spermatozoa was cytosolic. Our results showed that the addition of 2, 5-MeC or even 1000 μg/ml PGT had no effect on the motility of demembranated fowl spermatozoa, either at 30°C or at 40°C. Thus, at least, tyrosine kinase may be present in the cytoplasmic matrix and/or plasma membrane, but not retained in the axoneme and/or accessory cytoskeletal components. However, the location of the substrate(s) for this enzyme still remains unclear: whether in the cytoplasmic matrix and/or plasma membrane

or in the axoneme and/or accessory cytoskeletal components. Earlier studies have clearly shown that the stimulatory effects of temperature or PP1 inhibitors on fowl sperm motility act on demembranated spermatozoa - i.e. on the axoneme and/or accessory cytoskeletal components (Ashizawa et al., 1989a, b, 1994c). It seems therefore that in intact fowl spermatozoa, stimulation of motility by these effectors/conditions depends on the 'permissive' effect of a protein(s) 'upstream' of the axoneme and/or accessory cytoskeletal components, which is active only on phosphorylation of tyrosine residues. The location of this protein(s) or whether it is involved in regulation of motility per se, or in the transfer of ATP to the contractile apparatus, requires further investigation.

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Legends for figures

Fig. 1. (a) Motility of intact fowl spermatozoa after addition of various concentrations of 2, 5-MeC at (●) 30°C and (○) 40°C, and (b) motility of demembranated fowl spermatozoa after addition of various concentrations of (●,○) 2, 5-MeC or (■,□) a random copolymer of tyrosine and glutamate (PGT) at (●,■) 30°C and (○,□) 40°C. Each point represents the mean (± SEM) of five samples of spermatozoa. Values with different superscripts differ significantly ($P<0.05$) from each other. NS: not significant.

Fig. 2. Motility of intact fowl spermatozoa after addition (at arrows) of (●) 1 mM $CaCl_2$ and (○) 100 μM 2, 5-MeC at (a) 30°C and (b) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. *$P<0.05$ compared with value when no drug was added (control) at each period.

Fig. 3. Motility of intact fowl spermatozoa after addition (at arrows) of (●) 100 nM calyculin A and (○) 100 μM 2, 5-MeC at (a) 30°C and (b) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. *$P<0.05$ compared with value when no drug was added (control) at each period.

Fig. 4. ATP concentration of intact fowl spermatozoa after addition (at arrows) of (●) 1 mM $CaCl_2$ and (○) 200 μM 2, 5-MeC at (a) 30°C and (b) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. At both 30°C and 40°C, no significant difference was observed between in the presence and absence of 2, 5-MeC at each period.

Fig. 5. ATP concentration of intact fowl spermatozoa after addition (at arrows) of (●) 100 nM calyculin A and (○) 200 μM 2, 5-MeC at (a) 30°C and (b) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. At both 30°C and 40°C, no significant difference was observed between in the presence and absence of 2, 5-MeC at each period.

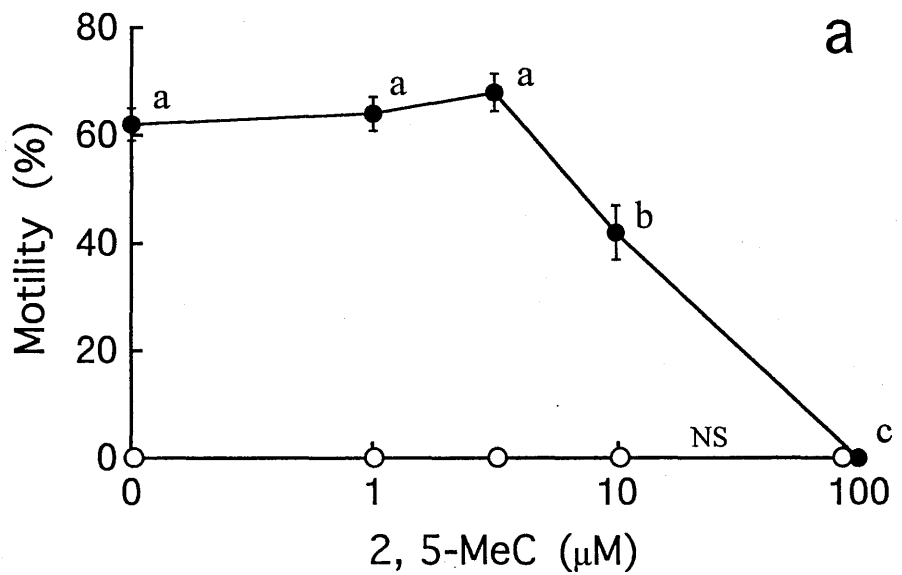

Fig. 1a

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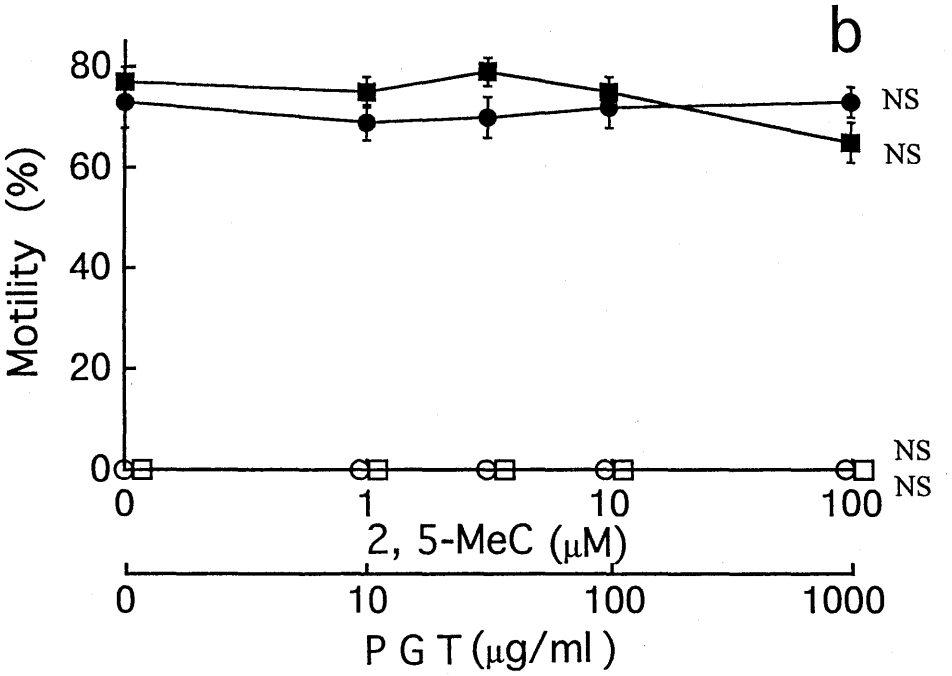

Fig. 1b.
Ashizawa et al.

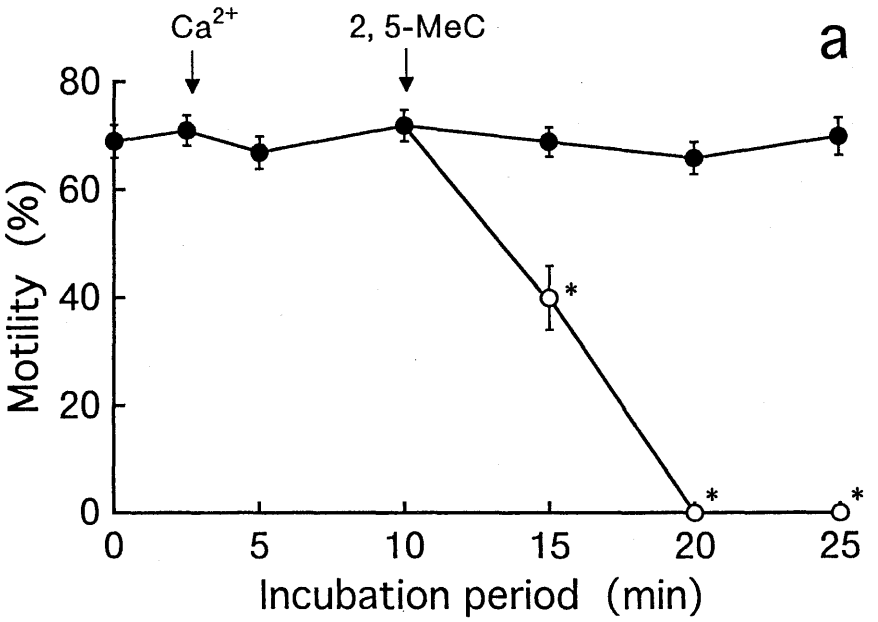

Fig. 2a.
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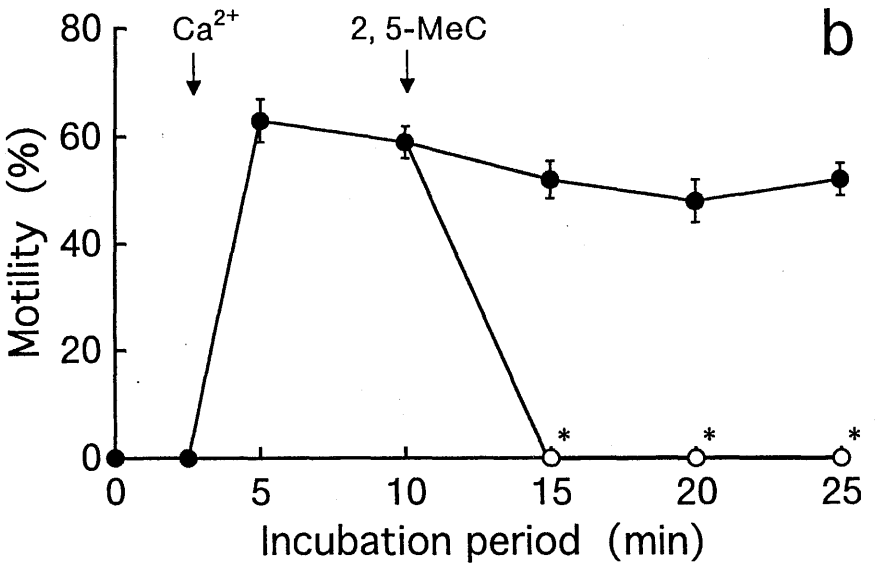

Fig. 2b.
Ashizawa et al.

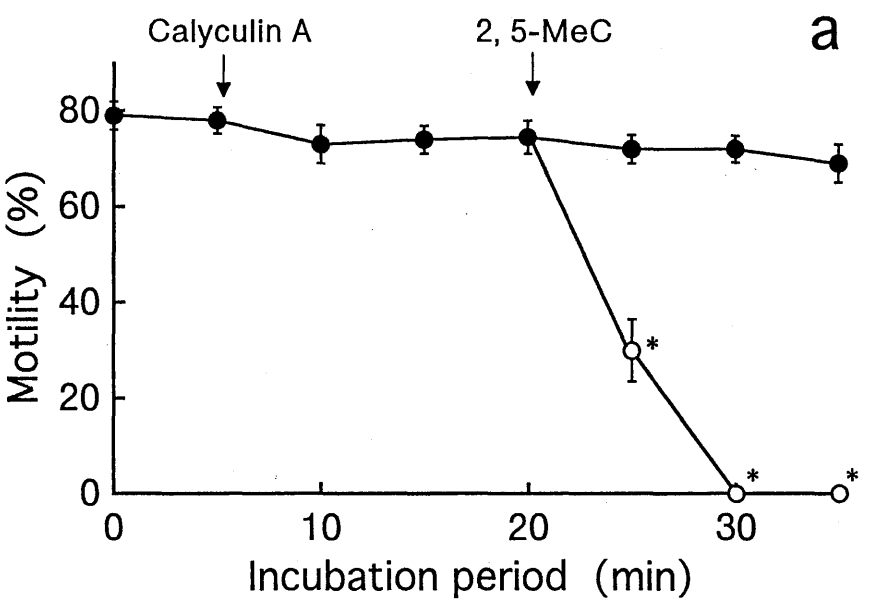

Fig. 3a.
Ashizawa et al.

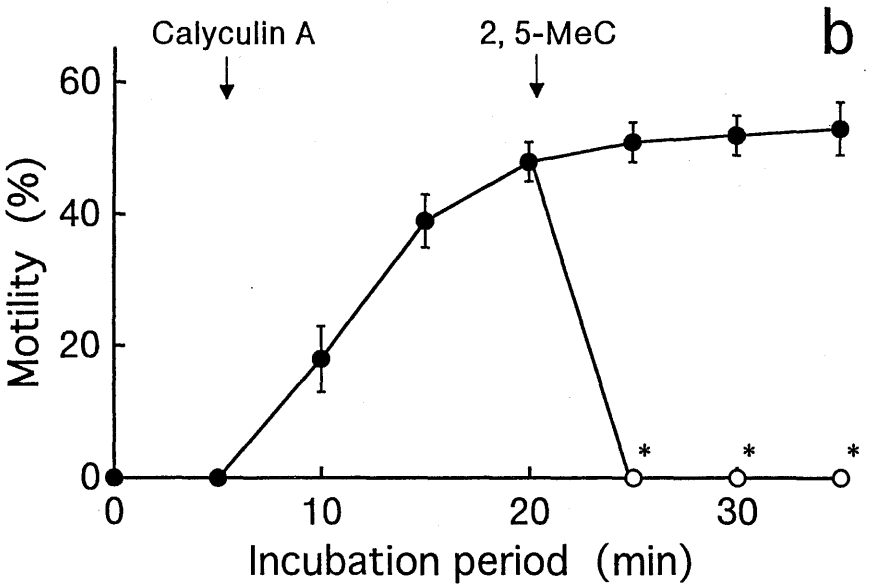

Fig. 3b.
Ashizawa et al.

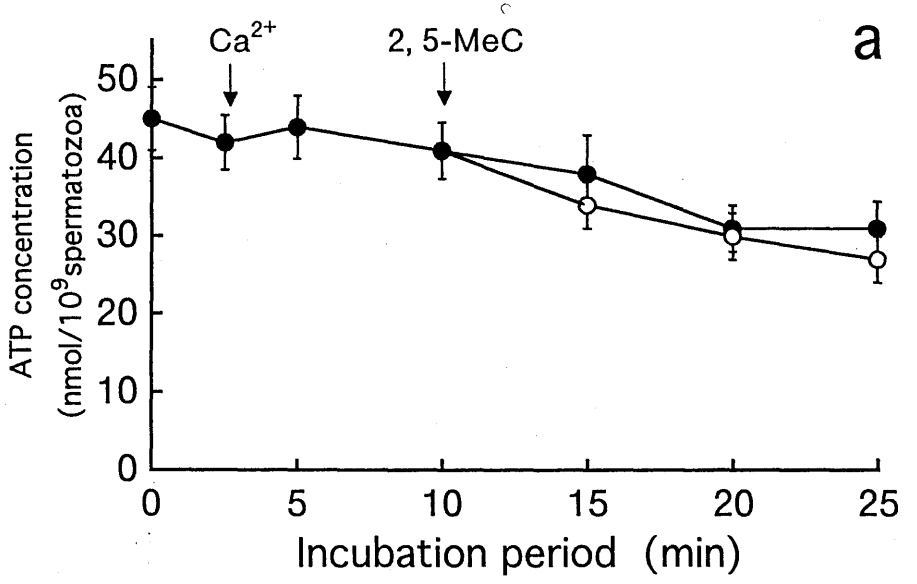

Fig. 4a.
Ashizawa et al.

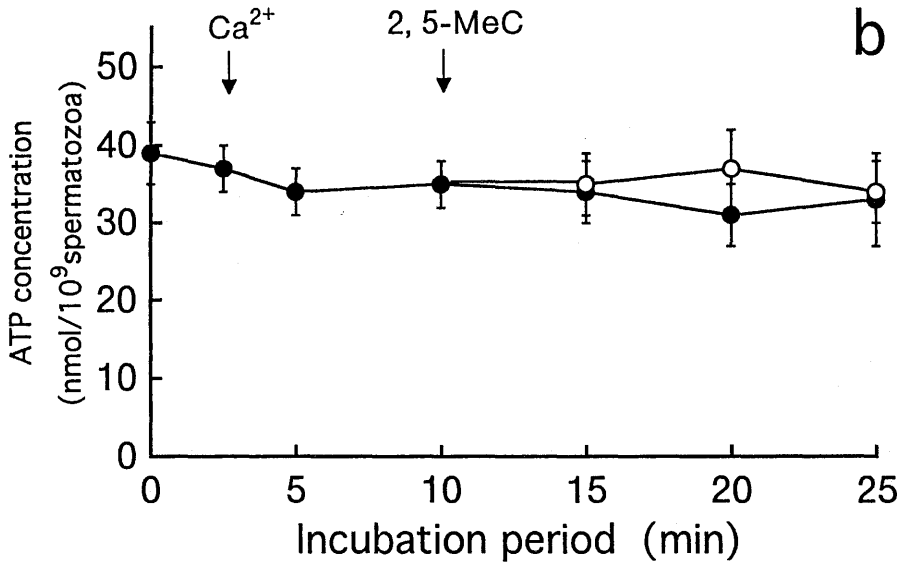

Fig. 4b.
Ashizawa et al.

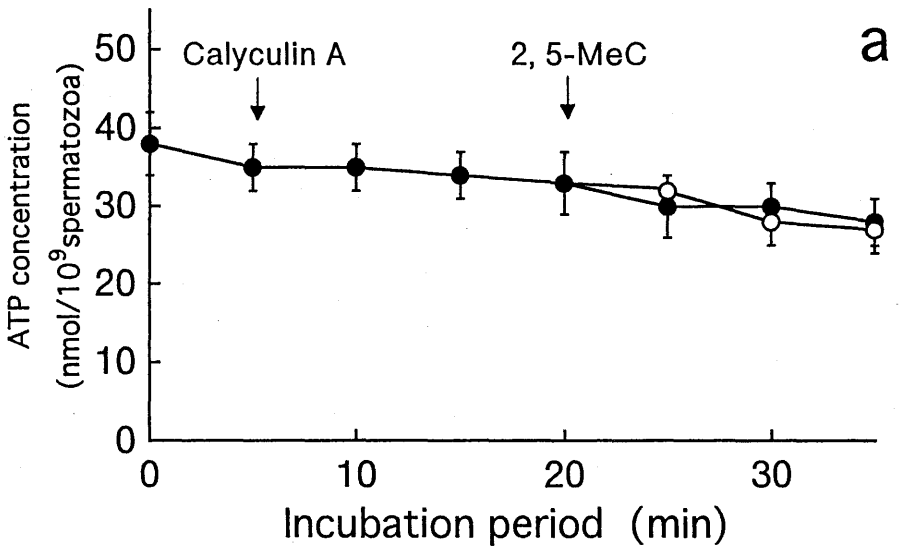

Fig. 5a.
Ashizawa et al.

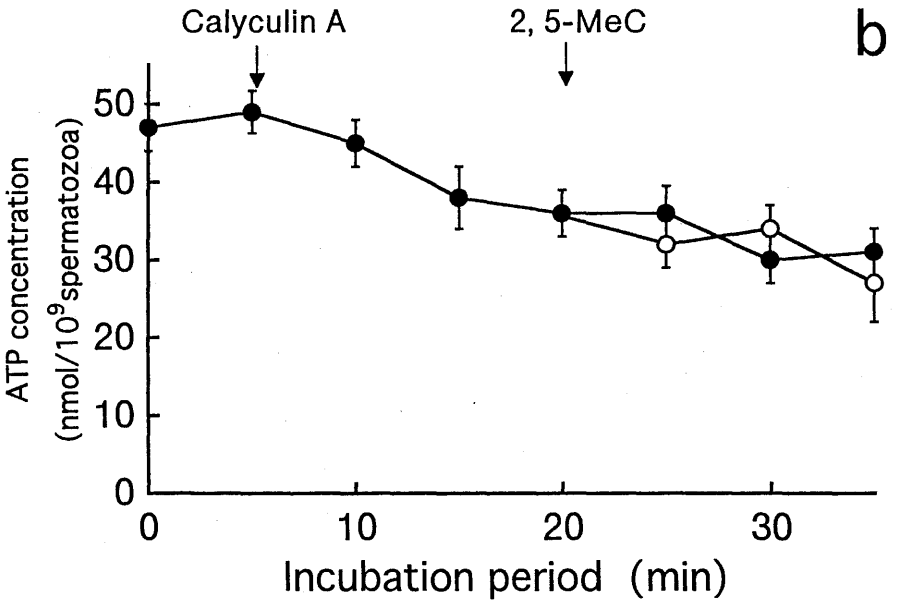

Fig. 5b.
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