Regulatory mechanisms of fowl sperm motility: possible role of endogenous myosin light chain kinase-like protein

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Original version	2nd version
A member of Editorial Board	
about 'statistics'	Indicating by referee, the statistical analysis was altered (Page 9 lines 17-20, Legends for figures 1-5).
about 'analysis of motility'	Actually, not only % motility, but also other parameters (e.g. velocity, beat frequency etc.) are interesting. We have a computerized motility analyzer (Hamilton Thorn, HTM-S). However, we did not use it. There are two reasons: (1) this expensive machine could not be reliable for fowl spermatozoa, since fowl spermatozoa have stick-like head, not like mammals. So, the optimization of the main gates (size, contrast, intensity etc.) was quite difficult. We have tried many times for the optimization, but the machine often counts one spermatozoon as two or three, and sometimes as zero. Therefore, the data are so variable, even though from the same sample. Now, we recognize that the counting by human using video monitor is more accurate for % motility, although it takes a long time. (2) fowl sperm motility is interesting, especially at the temperature-dependent motility. At this time, the % of moving sperm is almost zero or above 60%. Furthermore, fowl spermatozoa do not show hyperactivation even though after getting capacitation. So, we think that '% motility' is enough for the assessment in this experiment.
Referee	
about 'statistics'	According to your kind suggestion, statistical methods were changed. The data was transformed using arc sine transformation. The results were analyzed by ANOVA test and then by the multiple range tests (Page 9 lines 17-20, Legends for figures 1-5).

title	'prtein' to 'protein'
Page 4 lines 4–6	We have already deleted many other articles. I think that these references are minimally necessary.
about 'evaluation of motility'	'How motility was assessed' These are written in page 7, lines 20–27. We measured '% motility'. So, as your kind suggestion, we changed the word (page 6, line 27, page 7, line 27).

Summary. The motility of both intact and demembranated fowl spermatozoa was vigorous at 30°C, but decreased markedly following the addition of 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1, 4-diazepine hydrochloride (ML-9), a specific inhibitor of myosin light chain kinase (MLCK). Furthermore, the presence of a MLCK substrate peptide also inhibited the motility of demembranated spermatozoa at 30°C. In contrast, the addition of N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-8) or N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004), specific inhibitors of cAMP-dependent protein kinase, did not appreciably affect the motility of either intact or demembranated spermatozoa. Cyclic AMP-dependent protein kinase substrate peptides were also ineffective for the inhibition of motility of demembranated spermatozoa at 30°C. Immunoblotting of sperm extract using an antibody to MLCK revealed two major cross-reacting proteins of 130 kDa and 61-64 kDa, which corresponded to the molecular mass of MLCK. In addition, immunogold particles which reacted with the anti-MLCK antibody were observed around/on the axoneme at the ultrastructural level. These results suggest that the phosphorylation of axonemal protein(s) by MLCK or a MLCK-like protein, rather than by cAMP-dependent protein kinase, may be involved in the maintenance of fowl sperm motility at 30°C.

Introduction

There is substantial evidence that protein phosphorylation-dephosphorylation plays a primary role in the second messenger regulatory mechanisms of sperm movement (for review, see Tash and

Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Tash, 1989; Majumder et al., 1990; Morisawa, 1993, 1994; Tash and Bracho, 1994). With regard to protein dephosphorylation, it has been proposed that inhibition of mammalian sperm motility by Ca^{2+} may be due to the activation of protein phosphatase type 2B (calcineurin), a Ca^{2+}/calmodulin-dependent protein phosphatase (Tash et al., 1988), and also that protein phosphatase type 1 may be involved in the inhibition of fowl sperm motility at 40°C (Ashizawa et al., 1994b).

On the other hand, it is well recognized that protein phosphorylation by cAMP-dependent protein kinases is involved in the activation of sperm motility (see above reviews). For example: phosphorylation of a 15 kDa axonemal protein by a cAMP-dependent protein kinase is essential for the initiation of trout sperm motility (Morisawa and Hayashi, 1985); a 55 kDa protein that is phosphorylated in a cAMP-dependent manner, is apparently related to the motility state of bovine spermatozoa (Brandt and Hoskins, 1980); axokinin, a soluble 56 kDa phosphoprotein, seems to play a key role in mediating the cAMP response in dog spermatozoa (Tash et al., 1984, 1986); and the type II regulatory subunit of cAMP-dependent protein kinase has been identified as the major cAMP-dependent phosphoprotein in bovine spermatozoa (Noland et al., 1987; Paupard et al., 1988). Therefore, it seems likely that cAMP is indispensable for the initiation and activation of sperm motility of a variety of species including mammals (for review, see Morisawa, 1993, 1994; Tash and Bracho, 1994). However, unlike that of mammalian spermatozoa, the motility of demembranated fowl spermatozoa could not be restored by the addition of cAMP at 40°C (Ashizawa et al., 1989b, 1992b).

Fowl spermatozoa become immotile at the avian body temperature of 40–41°C. Motility is restored at 30°C or by the addition of Ca^{2+} at 40°C (Munro, 1938; Ashizawa and Nishiyama, 1978; Takeda, 1982; Ashizawa and Okauchi, 1984; Wishart and Ashizawa, 1987; Ashizawa et al., 1989a; Thomson and Wishart, 1989, 1991). Ca^{2+} is the major stimulatory factor in body fluids such as seminal plasma, or the fluid of female ovarian pocket taken at the time of ovulation, in which spermatozoa can maintain their motility even at 40°C (Ashizawa and Wishart, 1987, 1992). Additionally, the motility of demembranated fowl spermatozoa is inhibited in Ca^{2+}–free medium at 30°C (Ashizawa et al., 1992a). Furthermore, the motility of intact spermatozoa loaded with an intracellular Ca^{2+} chelator, 1, 2–bis (2–aminophenoxy) ethane–N, N, N', N'–tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) is also negligible at 30°C, but can be instantly restored by the subsequent addition of Ca^{2+} (Ashizawa et al., 1994a). Even in the presence of Ca^{2+}, however, the addition of calmodulin antagonists such as N–(6–aminohexyl)–5–chloro–1–naphthalenesulfonamide hydrochloride (W–7) and trifluoperazine inhibited the motility of intact spermatozoa at 30°C and 40°C (Ashizawa et al., 1994a). Thus, intracellular free Ca^{2+} seems to be essential for the maintenance of fowl sperm motility, and calmodulin is a prominent candidate as the signal transducer in Ca^{2+}–stimulated motility. Moreover, it may be assumed that the phosphorylation by calmodulin–dependent protein kinase, rather than cAMP–dependent protein kinase is likely to be a regulatory step in the maintenance of fowl sperm motility. However, the intracellular molecular cascades involved in the maintenance of motility have remained unsolved.

In this study, attempts have been made to clarify what kind of

protein kinase is involved in the regulation of fowl sperm motility. We have proposed here that myosin light chain kinase (MLCK), one of calmodulin-dependent protein kinases, or a MLCK-like protein might be involved in the maintenance of fowl sperm motility at the axonemal level.

Materials and Methods

Animals and preparation of spermatozoa

Commercial White Leghorn roosters (Babcock strain, Akagi Poultry Breeding Farm, Miyazaki) 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 14 h light: 10 h dark cycle.

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 mmol NaCl l^{-1} with 20 mmol TES (N-Tris-[hydroxymethyl]-methyl-2-aminoethanesulphonic acid) l^{-1} at pH 7.4 and centrifuged at 700 g for 13 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^{-1}. Samples of 3-4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Chemicals

Protein kinase inhibitors, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-8), N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004) and 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1, 4-diazepine hydrochloride

(ML-9) were purchased from Seikagaku Co., Ltd. (Tokyo). Protein kinase substrate peptides, Arg-Arg-Lys-Ala-Ser-Gly-Pro, Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) and Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys (Syntide 2) were obtained from Sigma Chemical Co. (St Louis, MO), and Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2 was from Peninsula Laboratories, Inc. (Belmont, CA). ATP, bovine serum albumin (BSA), 3, 3'-diaminobenzidine tetrahydrochloride (DAB), dithiothreitol, potassium glutamate, TES and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO). Avidin-biotin peroxidase complex and protein A-gold complex were obtained from Vector Laboratories (Burlingame, CA) and Funakoshi Co., Ltd. (Tokyo), respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased from Life Technologies, Inc. (Gaithersburg, MD). Calyculin A was from Wako Pure Chemical Industries, Ltd. (Osaka) and other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto).

Antibodies

Immune mouse ascites fluid against purified fowl gizzard myosin light chain kinase (a monoclonal IgG2b) was purchased from Sigma Chemical Co. (St Louis, MO). Biotinylated anti-mouse immunoglobulins goat serum was obtained from Dako Japan (Kyoto).

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 protein kinase inhibitors (H-8, HA1004 or ML-9). The effects of the addition of $CaCl_2$ before the addition of inhibitors were also examined at 30°C and 40°C to be compared with those of inhibitor alone. 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% Triton X-100, 200 mmol sucrose l^{-1}, 25 mmol potassium glutamate l^{-1}, 1 mmol $MgSO_4$ l^{-1}, 1 mmol dithiothreitol l^{-1} and 20 mmol Tris-HCl buffer l^{-1} (pH 7.9). The reactivation medium consisted of 0.5 mmol ATP l^{-1}, 200 mmol sucrose l^{-1}, 25 mmol potassium glutamate l^{-1}, 1.5 mmol $MgSO_4$ l^{-1}, 1 mmol dithiothreitol l^{-1} and 20 mmol Tris-HCl buffer l^{-1} (pH 7.9). To examine the effects of protein kinase inhibitors, various concentrations of H-8, HA1004 or ML-9 were added to the reactivation medium. Addition of calyculin A, a protein phosphatase inhibitor, or various protein kinase substrate peptides to demembranated spermatozoa was also performed.

The suspension of intact or demembranated spermatozoa was placed into a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo) 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 the three or more fields, to determine the % motility.

Western immunoblot analysis

Demembranated spermatozoa were mixed with equal volumes of concentrated (twofold) Laemmli (1970) sample buffer and were boiled for 5 min. Samples containing approximately 40 μg protein were loaded on to 7.5% SDS-polyacrylamide slab gel, and electrophoresed. Western blotting was performed basically according to the protocol of Towbin et al. (1979), but with some modifications. Briefly, the proteins were transferred electrophoretically to an polyvinylidene difluoride membrane sheet (Atto Co., Ltd., Tokyo, AE-6660). Following transfer, nonspecific sites on the membranes were blocked by incubating them for 1 h at room temperature (20-25°C) in phosphate buffered saline (PBS) containing 5% skimmed milk powder. The blots were then incubated overnight at 4°C with antibody to MLCK (1:200 dilution with 1% BSA in PBS). For control, the blots were incubated in PBS containing 1% BSA alone. The blots were further incubated for 1 h at 37°C with biotinylated anti-mouse immunoglobulins goat serum (1:100 dilution with 1% BSA in PBS) and then avidin-biotin peroxidase complex. Following each incubation, the membranes were rinsed extensively in PBS containing 0.05% Tween 20. Finally, 0.05% DAB/0.01% H_2O_2 solutions in 50 mmol Tris-HCl l^{-1} (pH 7.5) were applied to visualize the immunoreactive materials.

Immunocytochemistry

The samples of spermatozoa were routinely processed for postembedding immunogold procedures. Briefly, washed spermatozoa described above were centrifuged to be a pellet, placed in periodate-

lysine-paraformaldehyde fixative (McLean and Nakane, 1974), washed with 0.1 mol phosphate buffer l^{-1} (pH 7.4), mixed with 150 mmol NaCl l^{-1} containing 1% agar, dehydrated through a graded series of ethanols and embedded in Lowicryl K4M resin. Thin sections were collected on uncoated 200 mesh nickel grids. Thin sections were blocked by incubating them in PBS containing 1% BSA and then treated with anti-MLCK antibody (1:25 dilution with 1% BSA in PBS) overnight at 4°C. The sections were further incubated for 2 h at room temperature (20-25°C) with protein A conjugated approximately 10 nm gold particles (1:10 dilution with 1% BSA in PBS). Following each incubation, the sections were rinsed extensively in PBS. The grids were stained with 5% uranyl acetate/2% methyl cellulose complex solution (9:1) before electron microscopic examination using Hitachi H-800 at 100 kV. Control omitting primary antibody was also performed to assess the specificity of the immunostainings.

Statistical analysis

Percent motility was transformed using arc sine transformation. The results were analyzed by ANOVA test (Snedecor and Cochran, 1980) and then by the multiple range tests (Duncan, 1955).

Results

Effects of inhibitors and substrate peptides of various protein kinases on the motility of intact and demembranated fowl spermatozoa

The percent of motility of intact spermatozoa at 30°C was inhibited in a dose-dependent manner by the addition of ML-9, a

specific inhibitor of MLCK. In contrast, no inhibition of motility was observed following the addition of the cAMP-dependent protein kinase inhibitors, H-8 or HA1004, within the range 0-1000 μmol l^{-1}. At 40°C, none of the above inhibitors appreciably affected the motility of intact spermatozoa which remained almost negligible (Fig. 1).

Fig. 1 shows the motility at 30 min after the addition of the inhibitors. The time-course of motility in the presence of Ca^{2+} before the addition of drugs at 30°C and 40°C is shown in Fig. 2. Even in the presence of Ca^{2+} before the addition of ML-9 could not prevent the inhibition of motility of intact spermatozoa at 30°C (Fig. 2a). At 40°C, the motility of intact spermatozoa was restored instantly after the addition of 1 mmol $CaCl_2$ l^{-1}, but the subsequent addition of ML-9 inhibited the motility again (Fig. 2b). In contrast, inhibition of control or Ca^{2+}-supplemented sperm motility was not observed after the addition of H-8 or HA1004 at 30°C and 40°C.

No stimulation or inhibition of motility of demembranated spermatozoa was observed following the addition of H-8 or HA1004, within the range 0-1000 μmol l^{-1} at 30°C and 40°C. In contrast, the motility of demembranated spermatozoa was markedly affected by the addition of ML-9 at 30°C: as the concentrations of ML-9 were increased, the motility was reduced gradually (Fig. 3a-c).

Fig. 3a shows the inhibition of motility at 5 min after the addition of ML-9. The effect of more prolonged exposure at 30°C is shown in Fig. 4. Inhibition by ML-9 was reversible, since the motility of demembranated spermatozoa could be restored by the dilution (6 fold) in ML-9 free assay medium to decrease ML-9 concentrations after 10 min of exposure.

The presence of the protein phosphatase inhibitor, calyculin A permitted reactivation of demembranated spermatozoa at 40°C, and this effect was maintained by the following addition of H-8 or HA1004 as well as those of the control (no addition). However, stimulation of motility by calyculin A was not observed by the subsequent addition of ML-9 (Fig. 5).

Furthermore, inhibition of motility of demembranated spermatozoa at 30°C was observed by the addition of Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2, a MLCK substrate peptide. In contrast, the addition of Arg-Arg-Lys-Ala-Ser-Gly-Pro and Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide), cAMP-dependent protein kinase substrate peptides, or Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys (Syntide 2), a calmodulin-dependent protein kinase II substrate peptide did not appreciably affect the motility of demembranated spermatozoa (Table 1).

Immunoblot and immunoelectron microscopic identification of MLCK in fowl spermatozoa

Following SDS-PAGE of sperm extracts, a band of a 82 kDa protein was detected non-specifically in both control and anti-MLCK antibody-treated lanes, even although the visible immunoreactive band was faint. However, bands of 130 kDa and 61-64 kDa proteins were specifically recognized by the anti-MLCK antibody (denoted by arrow) (Fig. 6).

Immunolabeling with the anti-MLCK antibody showed a light distribution of gold particles on the neck and tail regions (Fig. 7), whereas no appreciable particles was detected in the control.

Discussion

Substantial evidence exists suggesting a role for cAMP and cAMP-dependent protein kinase as a positive effectors for the activation of sperm motility (for review, see Tash and Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Tash, 1989; Majumder et al., 1990). Tash et al. (1986) reported that reactivation of the motility of demembranated dog spermatozoa produced by ATP alone was inhibited with H-8, a specific inhibitor of cAMP-dependent protein kinase. At this time, half-maximal inhibition of motility was achieved with 50 μmol l^{-1} of drug. A heat-stable protein inhibitor of cAMP-dependent protein kinase purified from rabbit skeletal muscle or rat testis also inhibited reactivation of demembranated mammalian and sea urchin spermatozoa (Ishiguro et al., 1982; Tash et al., 1984). Thus, the reactivation of sperm motility of a variety of species may be entirely dependent on cAMP-dependent protein phosphorylation (Tash, 1989).

However, the motility of demembranated fowl spermatozoa could not be restored by the addition of cAMP at 40°C, even though spermatozoa are naturally immotile at this temperature (Ashizawa et al., 1989b, 1992b). Moreover, the present study showed that the addition of even 1 mmol l^{-1} H-8 or HA1004 did not appreciably affect the motility of either intact or demembranated fowl spermatozoa at 30°C. Cyclic AMP-dependent protein kinase substrate peptides were also ineffective as inhibitors of the motility of demembranated spermatozoa at 30°C. In contrast, the motility of intact and demembranated spermatozoa at 30°C decreased considerably following the addition of

ML-9, a specific inhibitor of MLCK. Furthermore, the presence of a MLCK substrate peptide also caused inhibition of the motility of demembranated spermatozoa at 30°C, whilst syntide 2, a calmodulin-dependent protein kinase II substrate peptide, did not. These results strongly suggest that the protein phosphorylation of axonemal protein(s) by MLCK or MLCK-like protein, rather than by cAMP-dependent protein kinase, may be dominant in the involvement for the maintenance of fowl sperm motility at 30°C.

It has been shown that MLCK, a Ca^{2+}/calmodulin dependent protein kinase, is localized in the various types of non-muscle cells as well as muscle cells and suggested that MLCK is involved in the regulation of cell motility by Ca^{2+}/calmodulin (Guerriero et al., 1981). Preliminary results from Tash's laboratory have also demonstrated that mammalian spermatozoa contain MLCK as assessed by immunofluorescence microscopy and by immunoblots of electrophoretically-separated sperm proteins, providing preliminary evidence for a role of MLCK in mediating flagellar motility consistent with its presence in flagella (Tash and Means, 1983). The data reported here also show that endogenous MLCK or a MLCK-like protein may be present in fowl sperm axoneme, since immunogold particles which reacted with the anti-MLCK antibody were observed around/on the axoneme at the ultrastructural level, even though the reaction deposits were scattered. In addition, immunoblotting of sperm extract using an antibody to MLCK revealed a major cross-reacting proteins of 130 kDa and 61-64 kDa. The molecular weight of MLCK from various fowl tissues including brain heart, skeletal muscle and gizzard were estimated to be approximately 130 kDa (Guerriero et al., 1981).

Proteolysis of the 130 kDa MLCK from fowl gizzard produces a 64 kDa fragment, that neither binds to Ca^{2+}/calmodulin nor exhibits catalytic activity, and a 61 kDa peptide that is active in the absence of Ca^{2+}/calmodulin (data sheet of Sigma Immuno Chemicals). In the present work, we have found immunoreactive proteins in fowl spermatozoa coresponding to both molecular weight ranges (see Fig. 6).

If MLCK or MLCK-like protein affects the maintenance of fowl sperm motility, then the question of its target and precise mechanisms of the action is raised. Myosin, a substrate for MLCK, has been identified in bovine spermatozoa (Tamblyn, 1981), and in the human spermatozoa it has been localized in the neck region (Virtanen et al., 1984). Myosin is also detected on the mitochondrion and in the tail of ascidian spermatozoa (Lambert and Lambert, 1984). However, until now there has been no information demonstrating its presence in fowl spermatozoa. Additional studies are therefore necessary to examine: (1) whether myosin is present in fowl spermatozoa; and (2) which protein(s), i.e., myosin and/or other axonemal protein(s), are phosphorylated by MLCK or a MLCK-like protein during the activation of fowl sperm motility.

Using immunoelectron microscopy, Yamamoto (1985) reported that the reaction deposits against anti-calmodulin antibody were observed on the cytoplasmic surface of plasma membrane, the outside of fibrous sheath and along the axial filaments of the guinea pig sperm tail. We have assumed that most of the free calmodulin in fowl spermatozoa is located in the soluble fraction after demembranation and not retained in the axoneme, since inhibition of motility of demembranated spermatozoa was not observed by the addition of calmodulin

antagonists such as W-7 and trifluoperazine (Ashizawa et al., 1994a). From the present results, however, it is possible that some of calmodulin molecules may be bound to substrate proteins such as MLCK within the axoneme, since the presence of ML-9 or MLCK substrate peptide inhibited the motility of demembranated spermatozoa.

It is suggested that protein phosphatase type 1 (PP1) present in the fowl sperm axoneme may be involved in the inhibition of fowl sperm motility at 40°C, since in addition to calyculin A and okadaic acid, inhibitors 1 and 2, which are specific inhibitors of PP1 (Cohen, 1989), also stimulated the motility of demembranated spermatozoa at 40°C (Ashizawa et al., 1994b). In the present work, demembranated fowl spermatozoa, stimulated by the addition of calyculin A at 40°C, lost their motility by the subsequent addition of ML-9, but not by the addition of H-8 or HA1004. These results suggest that the motility of fowl spermatozoa may be regulated by a balance of the phosphorylation-dephosphorylation system of axonemal protein(s) which are affected by, at least, endogenous MLCK or MLCK-like protein and PP1.

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Table 1. Effects of protein kinase substrate peptides on the motility of demembranated fowl spermatozoa at 30°C

Peptide	Motility (%)
None (control)	68.5±3.1[a]
Lys–Lys–Arg–Pro–Gln–Arg–Ala–Thr–Ser–Asn–Val–Phe–Ser–NH_2 (MLCK substrate)	5.1±1.0[b]
Pro–Leu–Ala–Arg–Thr–Leu–Ser–Val–Ala–Gly–Leu–Pro –Gly–Lys–Lys (Syntide 2; CaM–dependent kinase II substrate)	56.4±4.8[a]
Arg–Arg–Lys–Ala–Ser–Gly–Pro (cAMP–dependent kinase substrate)	63.4±2.0[a]
Leu–Arg–Arg–Ala–Ser–Leu–Gly (Kemptide; cAMP–dependent kinase substrate)	70.3±4.2[a]

Each peptide added in the reactivation medium was 0.4 mM. Each value represents the mean (±SEM) of five samples of spermatozoa. Values with different superscripts differ significantly ($P<0.01$) from each other.

Legends for figures

Fig. 1. Motility of intact fowl spermatozoa after addition of various concentrations of (a) ML-9, (b) H-8 and (c) HA1004 at (●) 30°C and (○) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. Values with different superscripts differ significantly ($P<0.01$) from each other. NS = not significant.

Fig. 2. Motility of intact fowl spermatozoa after addition (at arrows) of (●) 1 mmol $CaCl_2$ l^{-1}, (○) 500 μmol ML-9 l^{-1}, (■) 500 μmol H-8 l^{-1} and (□) 500 μmol HA1004 l^{-1} at (a) 30°C and (b) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. Value with * differs significantly ($P<0.01$) from other three values at each period.

Fig. 3. Motility of demembranated fowl spermatozoa after addition of various concentrations of (a) ML-9, (b) H-8 and (c) HA1004 at (●) 30°C and (○) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. Values with different superscripts differ significantly ($P<0.01$) from each other. NS = not significant.

Fig. 4. The time course of motility of demembranated fowl spermatozoa after addition of 500 μmol ML-9 l^{-1} at 30°C. At arrow, sperm sample was diluted (by a factor of approximately 6) in drug-free assay medium to decrease concentrations of drug. Each point represents the mean (±SEM) of five samples of spermatozoa. Values with different superscripts differ significantly ($P<0.01$) from each other.

Fig. 5. Motility of demembranated fowl spermatozoa after addition (at arrows) of (●) 100 nmol calyculin A l^{-1}, (○) 500 μmol ML-9 l^{-1}, (■) 500 μmol H-8 l^{-1} and (□) 500 μmol HA1004 l^{-1} at 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. Value with * differs significantly ($P<0.01$) from other three values at each period.

Fig. 6. Immunoblot analysis of fowl sperm MLCK. Lane 1: incubation with monoclonal antibody against purified fowl gizzard MLCK; lane 2: control (see Materials and Methods).

Fig. 7. Immunogold staining at the electron microscopic level of fowl spermatozoa. Immunogold particles are present on the tail region at cross section (left) and on the neck and tail regions at longitudinal section (right) treated with anti-MLCK antibody. Bar=0.1 μm.

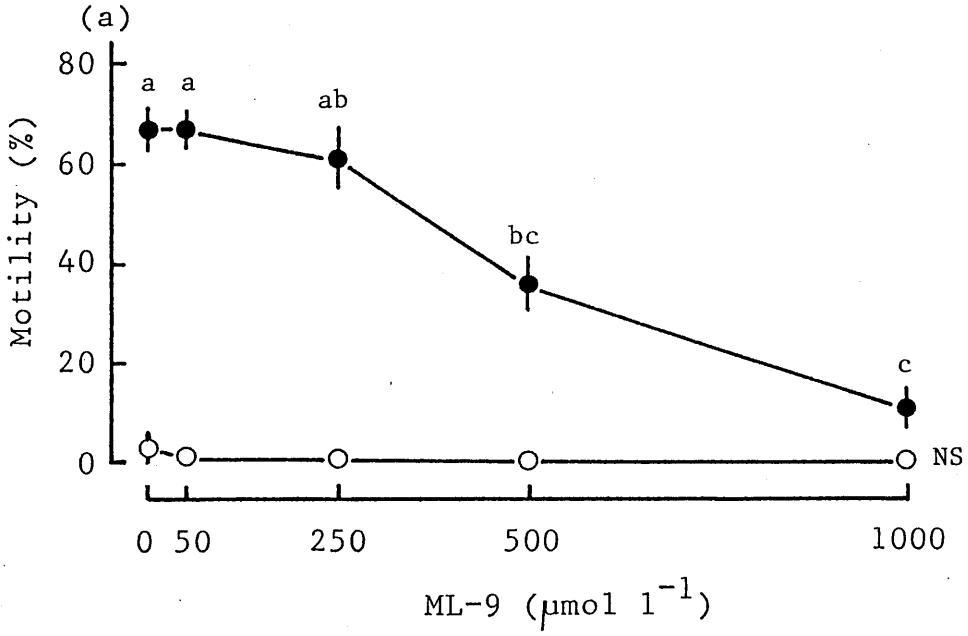

Fig. 1a

Ashizawa et al

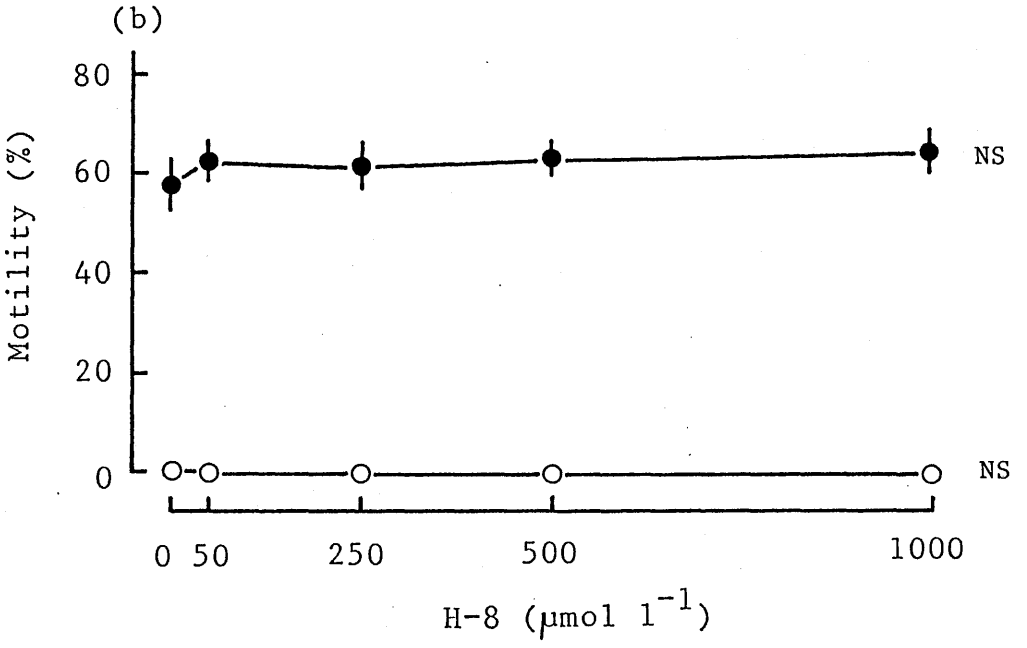

Fig. 1b

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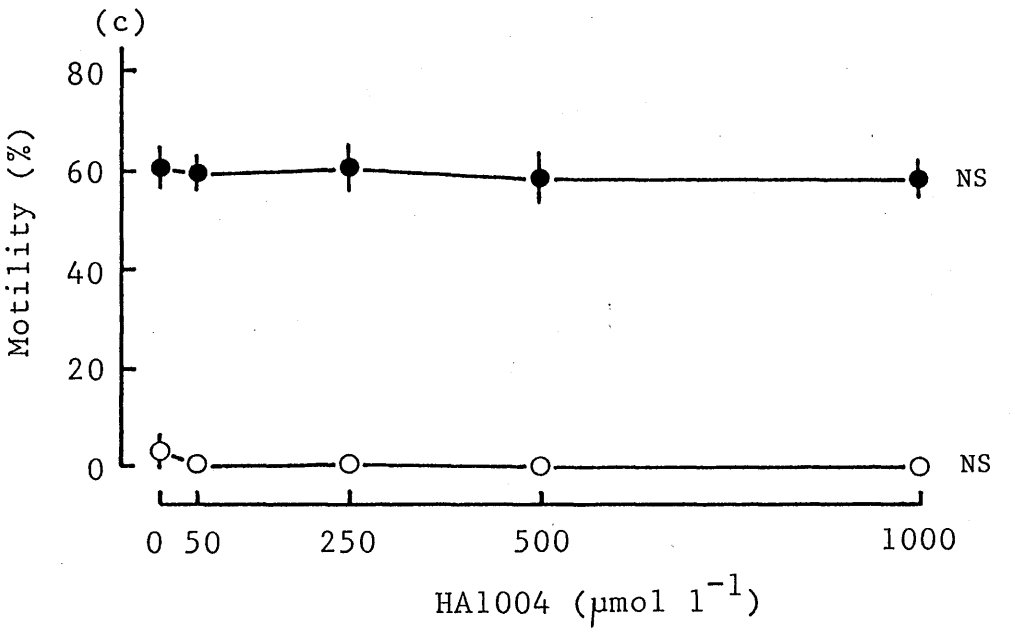

Fig. 1c

Ashizawa et al.

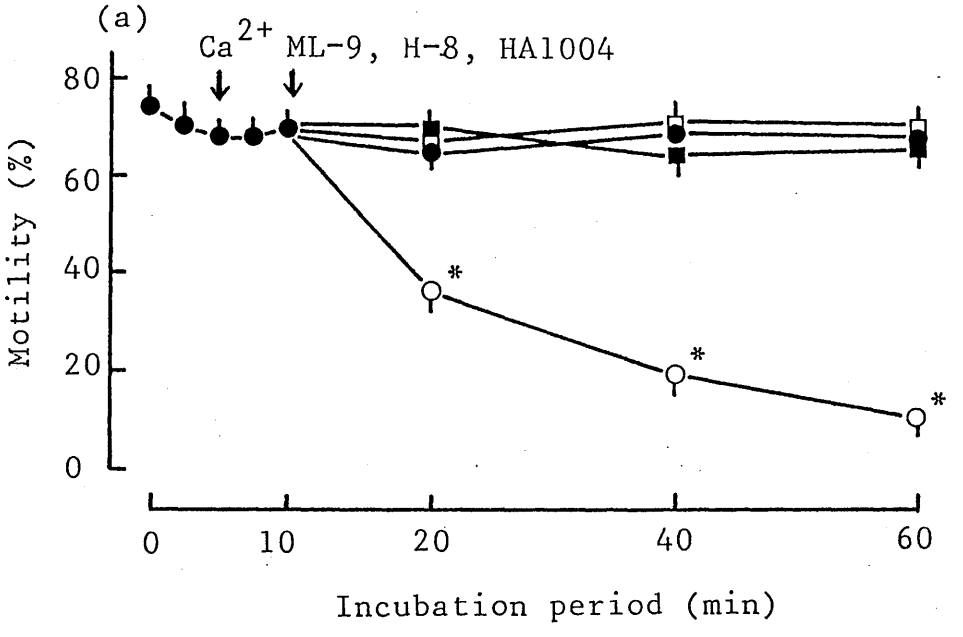

Fig. 2a

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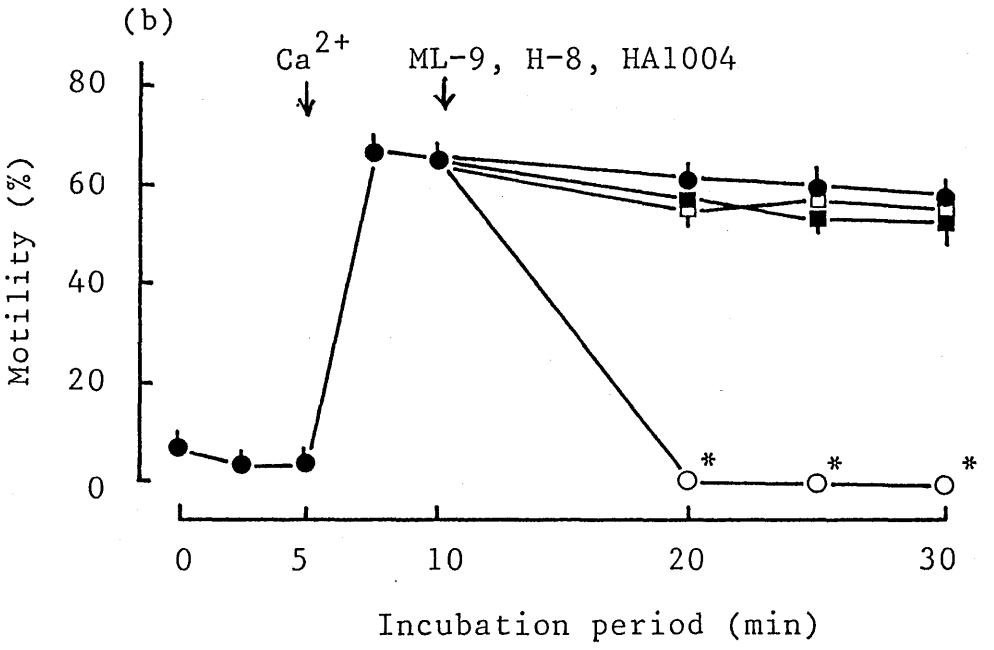

Fig. 2b

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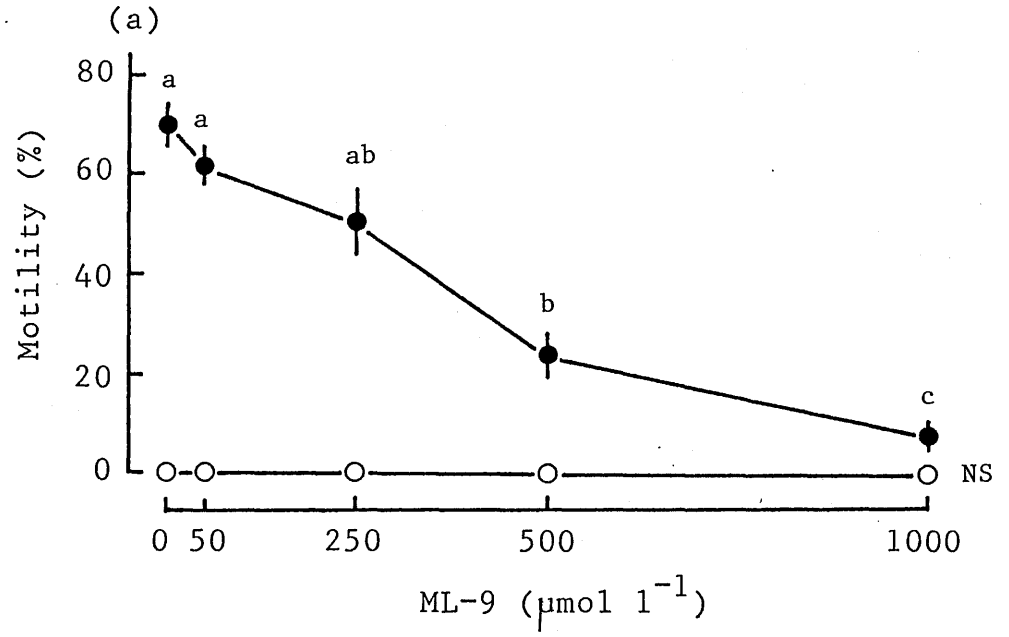

Fig. 3a
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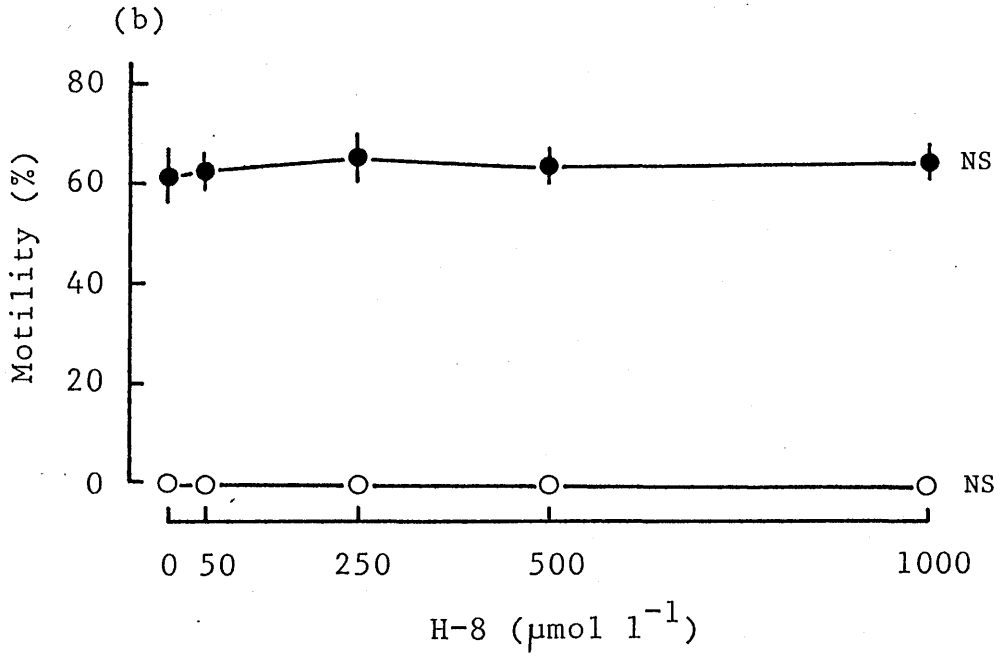
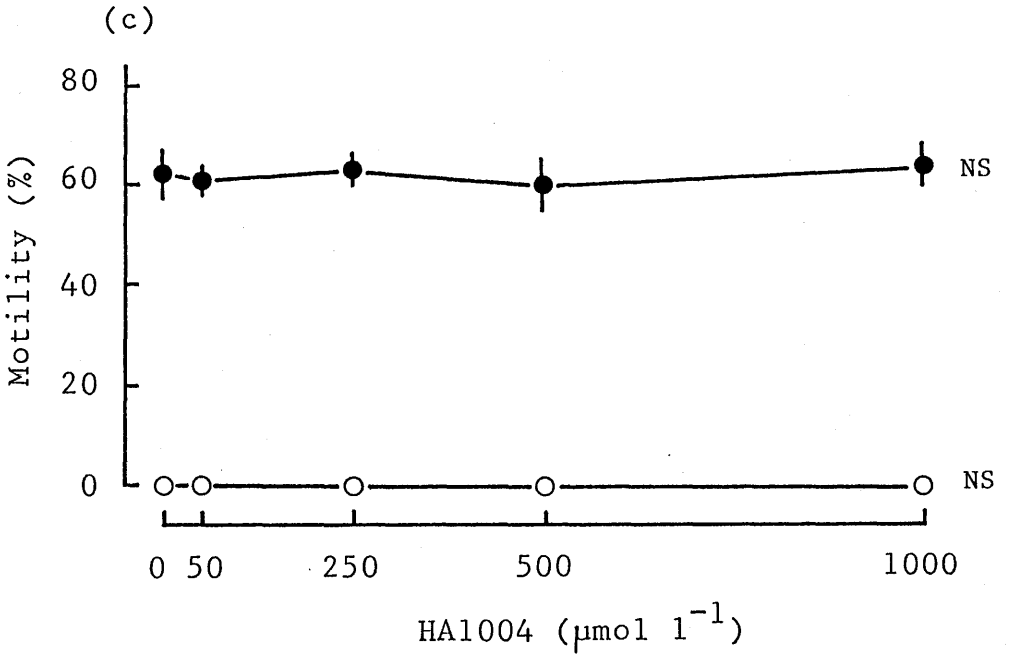


Fig. 3b
Ashizawa et al.



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Ashizawa et al.

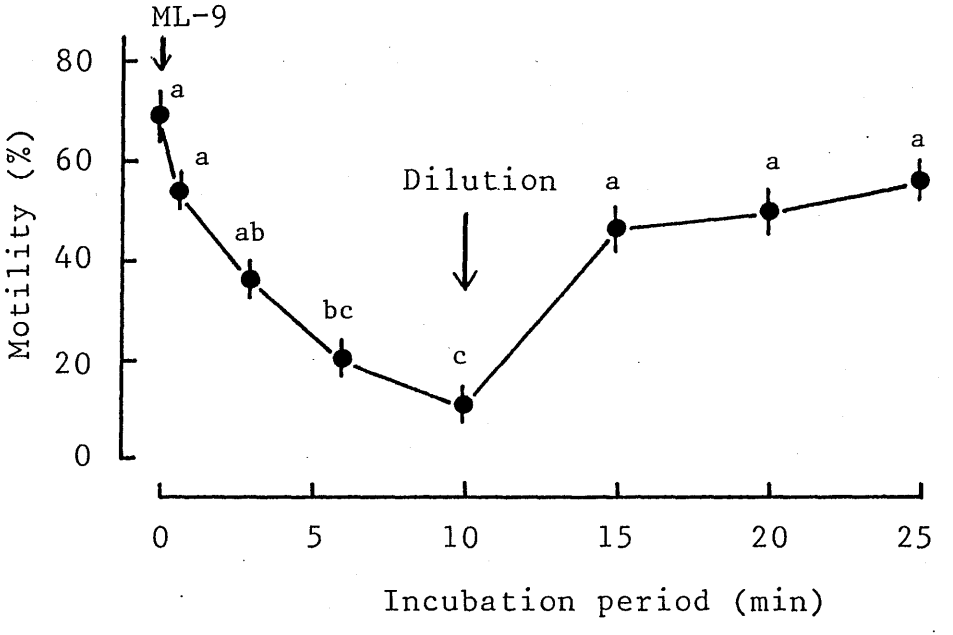

Fig. 4
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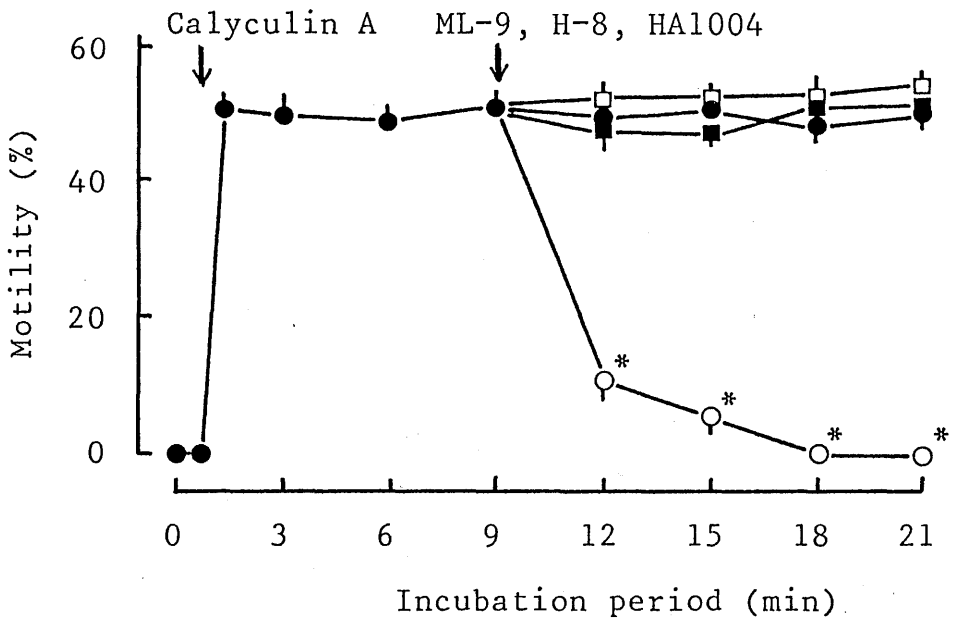

Fig. 5

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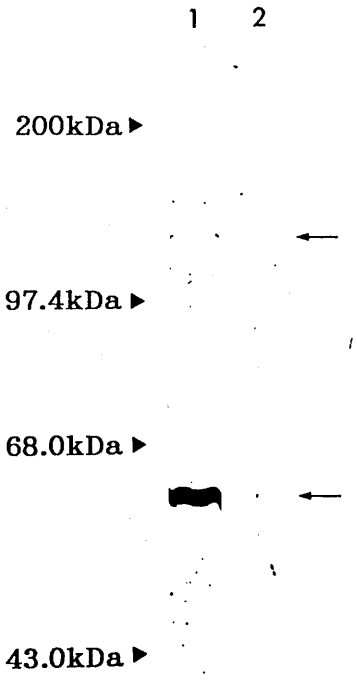

Fig. 6.
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Fig. 7.
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