Possible role of protein kinase C in regulation of flagellar motility and intracellular free Ca^{2+} concentration of fowl spermatozoa

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Summary

The presence and possible role of protein kinase C in the regulation of fowl sperm functions were investigated. Immunoblot analysis of sperm extract using antibody to protein kinase C revealed a cross-reacting protein of approximately 80 kDa. As the concentration of N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9) or 1-oleoyl-2-acetylglycerol (OAG), a protein kinase C activator, was increased, the motility of intact spermatozoa at 30°C was reduced. However, the inhibition of motility was reversed by reducing the concentrations of activators. Even in the presence of 1 mmol $CaCl_2$ l^{-1}, the addition of SC-9 and OAG inhibited the motility of intact spermatozoa. In contrast, the motility of demembranated spermatozoa was not inhibited by the addition of SC-9 or OAG at 30°C. On the other hand, the addition of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), a protein kinase C inhibitor, did not appreciably affect the motility of either intact or demembranated spermatozoa at 30°C. At 40°C, both intact and demembranated spermatozoa were almost immotile with or without any of the activators or inhibitor. Intracellular free Ca^{2+} concentrations, measured by means of a fluorescent Ca^{2+} indicator fura-2, gradually increased after the addition of SC-9 and OAG, but no changes were observed in H-7 treated spermatozoa. These results suggest that endogenous protein kinase C is present in the cytoplasmic matrix and/or the membrane, but not retained in the axoneme, and the activation of this enzyme may contribute to a decrease in the flagellar movement of fowl spermatozoa.

Introduction

In most biological systems where Ca^{2+} and cAMP are involved as second messengers, additional regulatory control is gained by protein kinase C exerting either positive forward effect, negative feedback control or both (Nishizuka, 1986). Protein kinase C, a Ca^{2+} and phospholipid dependent kinase, is a family of at least seven closely related subspecies. These subspecies differ in their distribution, regulation and enzymatic properties (Nishizuka, 1988).

Recently, protein kinase C has been identified in ejaculated human and epididymal bovine spermatozoa (Rotem et al., 1990a; Chaudhry and Casillas, 1992). An immunohistochemical approach to protein kinase C localization in human spermatozoa revealed that protein kinase C is localized in the equatorial segment in a distinct band and also in the principal piece of the tail (Rotem et al., 1990a,b). In bovine spermatozoa, immunoblot analysis of the partially purified enzyme with isozyme-specific monoclonal antibodies revealed the presence of α-, β- and γ-subspecies of protein kinase C. Furthermore, indirect immunofluorescence showed that the antibodies against α-, β- and γ-subspecies produced prominent staining of the postacrosomal region of the sperm head and a minor staining of the midpiece for the β-subspecies (Chaudhry and Casillas, 1992). In contrast, Roldan and Harrison (1988) suggested that potentially active protein kinase C is not present in ram spermatozoa.

It has been reported that phorbol esters and diacylglycerol analogs, the activators of protein kinase C, induced the acrosomal

reaction in human spermatozoa (De Jonge et al., 1991), enhanced the zona pellucida-induced acrosomal reaction in mouse spermatozoa (Lee et al., 1987; Endo et al., 1991) and stimulated human sperm motility (Rotem et al., 1990a,b). Since the head and the tail regions of the spermatozoa are physiologically separate compartments and the isozymes of protein kinase C are differentially located, it is tempting to speculate that different subspecies of protein kinase C may be involved in modulating the acrosome reaction and in regulating sperm motility (Chaudhry and Casillas, 1992).

It is well recognized that flagellar motility of spermatozoa may be controlled by a protein phosphorylation-dephosphorylation system (for reviews, see Tash and Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Majumder et al., 1990) Recently, we also suggested that cAMP-independent phosphorylation of a 43 kDa axonemal protein was likely to be a regulatory step in the maintenance of fowl sperm motility (Ashizawa et al., 1992b). Therefore, it is assumed that the activation of protein kinase C and subsequent protein phosphorylation are involved in the regulation of fowl sperm motility. However, the presence and role of protein kinase C in fowl spermatozoa have not been determined. In the present study, therefore, attempts were made to clarify the presence and role of protein kinase C in the regulation of flagellar motility and intracellular free Ca^{2+} concentration of fowl spermatozoa.

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 14 h light per 24 h.

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

1-(2-(5"-carboxyoxazol-2"-yl)-6-aminobenzofuran-5-oxy)-2-(2'-amino-5'-methylphenoxy)-ethane-N, N, N', N'-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) was purchased from Dojindo Laboratories, Inc. (Kumamoto). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9) were obtained from Seikagaku Co., Ltd. (Tokyo). Adenosine 5'-triphosphate (ATP), bovine serum albumin (BSA), 3, 3'-diaminobenzidine tetrahydrochloride (DAB), dithiothreitol, potassium glutamate, TES, Triton X-100 and Cremophor EL were purchased from Sigma Chemical Co. (St Louis, MO). Avidin-biotin peroxidase complex (ABC) was obtained from Vector Laboratories (Burlingame, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased

from Life Technologies, Inc. (Gaithersburg, MD). 1-oleoyl-2-acetylglycerol (OAG) and other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto).

Antisera

Immune rabbit serum against a synthetic 543-550 amino acid peptide of protein kinase $C_{consensus}$ (a polyclonal antibody) was purchased from Seikagaku Co., Ltd. (Tokyo). Biotinylated anti-rabbit IgG goat serum was obtained from MBL Co., Ltd. (Nagoya).

Western immunoblot analysis of protein kinase C

Washed spermatozoa described above were mixed with equal volumes of concentrated (twofold) Laemmli (1970) sample buffer and were boiled for 5 min. Samples containing approximately 50 μg protein were loaded on to 10% SDS-polyacrylamide slab gel, and electrophoresed. Western blotting was performed basically according to the protocol of Towbin et al. (1979), but some modification. 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 protein kinase $C_{consensus}$ (1:100 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-rabbit IgG goat serum (1:200 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.

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 C activators (SC-9 and OAG) or inhibitor (H-7). The effects of the addition of $CaCl_2$ before the addition of activator or inhibitor were also examined at 30°C and 40°C to be compared with those of activator or 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). SC-9, OAG and H-7 were added to the reactivation medium. The suspension of intact or demembranated spermatozoa was placed on a microscope chamber (Sekisui Chemical Co., Ltd., Tokyo, UR-157 type) and the motility of spermatozoa was recorded by videomicroscopy at 30°C or

40°C on a thermostatically controlled warm plate (Katz and Overstreet, 1981).

Measurement of intracellular free Ca^{2+} concentrations of intact spermatozoa

Determination of the intracellular free Ca^{2+} concentrations was carried out with the fluorescent indicator fura-2, essentially according to Grynkiewicz et al. (1985), but with some modifications (Ashizawa et al., 1992a). Fluorescence intensity was measured with a dual-wavelength spectrofluorimeter (Shimadzu, Model RF-5000, Kyoto) with the sample chamber thermostated at 30°C or 40°C by a circulating water jacket and the sperm suspension was mixed continuously with a magnetic stirring bar. For the measurement of intracellular free Ca^{2+} concentrations, the excitation wavelength was set to 340 and 380 nm, respectively and the emission wavelength was set to 500 nm. The ratios (R:340/380nm) of fluorescence intensities were monitored continuously. Calculation of intracellular free Ca^{2+} was based upon the equation described by Grynkiewicz et al. (1985) where a dissocation constant (K_d) was 224 nmol l^{-1}.

Statistical analysis

Statistical comparisons were performed using Student's t-test.

Results

Immunoblot identification of protein kinase C in fowl spermatozoa

No appreciable immunoreactive protein was detected in control.

In contrast, approximately 80 kDa protein was recognized by anti-protein kinase C antibody, even though visible immunoreactive band was faint (Fig. 1).

Effects of protein kinase C activators and 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 protein kinase C activators such as SC-9 and OAG. In contrast, no inhibition of motility was observed following the addition of H-7, a protein kinase C inhibitor, within the range 0-1000 μmol l^{-1} (Fig. 2). At 40°C, the addition of activators or inhibitor did not appreciably affect the motility of intact spermatozoa which was almost negligible (Fig. 2).

Fig. 2 shows the motility in SC-9, OAG and H-7, at 5, 15 and 60 min respectively after the addition of drugs. The time-course of motility at 30°C and 40°C is shown in Fig. 3. Inhibition of motility by SC-9 and OAG was reversible at 30°C: the motility could be restored by the dilution (approximately 8 fold) in the activator free assay medium.

On the other hand, inhibition of motility by the addition of SC-9 and OAG was not observed in demembranated and reactivated spermatozoa at 30°C, and the motility was maintained as well as those of the control (no addition) and H-7 treatment (Table 1).

Even in the presence of Ca^{2+} before the addition of SC-9 and OAG could not prevent the inhibition of motility of intact spermatozoa at 30°C (Fig. 4a). At 40°C, the motility of intact spermatozoa was negligible, but the motility was restored instantly after the addition of

1 mmol $CaCl_2$ l^{-1}. However, the subsequent addition of SC-9 and OAG inhibited the motility again (Fig. 4b). In contrast, inhibition of motility was not observed by the addition of H-7 at 30°C and 40°C as well as those of control (no addition).

Effects of protein kinase C activators and inhibitor on the intracellular free Ca^{2+} concentrations of intact fowl spermatozoa

Intracellular free Ca^{2+} concentrations of intact spermatozoa increased rapidly after the addition of 1 mmol $CaCl_2$ l^{-1}, and then maintained a constant value at approximately 200 nmol l^{-1} at 30°C. Subsequently, the addition of SC-9 and OAG caused an increase of Ca^{2+} concentrations. In contrast, no changes of intracellular free Ca^{2+} concentrations of H-7 treated spermatozoa were observed as well as those of control (Fig. 5). Similar results were obtained at 40°C (data not shown).

Discussion

In the present study, immunoblot analysis of fowl sperm proteins showed that a protein of approximately 80 kDa was recognized by anti-protein kinase C antibody. This molecular weight corresponded to that detected by immunoblotting of various tissue extracts of rat using antibody to protein kinase C (Huang and Huang, 1986). They also suggested that the immunoreactive determinants for protein kinase C from the various sources are highly conserved (Huang and Huang, 1986). Furthermore, Asotra and Macklin (1993) reported that the sizes of individual protein kinase C isozyme-specific bands were ~80 kDa for

protein kinase C-α and -β. Therefore, it is possible that protein kinase C may be present in fowl spermatozoa, even though relatively low amounts of this enzyme.

Rotem et al. (1990a,b) demonstrated that the addition of protein kinase C activators such as a potent tumor-promoter phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and a membrane-permeable diacylglycerol analog OAG stimulated human sperm motility in a dose- and time-dependent manner. In contrast, the observations reported here have yielded apparently conflicting results: as the concentration of SC-9 or OAG was increased, the motility of intact fowl spermatozoa was reduced within 15 min of incubation at 30°C. We also observed the inhibition for motility by the addition of 100 μmol TPA l^{-1}, although 60 min of incubation was needed for the complete inhibition of motility (data not shown). From these results, it seems that protein kinase C may have an opposite effect on the regulation of fowl sperm motility than that of mammalian spermatozoa. However, such difference is not surprising, since it seems likely that the regulation of sperm motility might be basically different between such species as judged from several lines of evidence. That is: (i) demembranated fowl spermatozoa can be motile with the addition of even at millimolar concentrations of Ca^{2+} (Ashizawa et al., 1989b), whereas in such a high concentrations of Ca^{2+}, demembranated mammalian sperm motility is inhibited (White and Voglmayr, 1986; Feng et al., 1988) and (ii) cAMP is indispensable for the initiation and stimulation of flagellar motility of mammalian spermatozoa (for reviews, see Tash and Means, 1983; Lindemann and Kanous, 1989), but is not for fowl spermatozoa, especially at 40°C (Ashizawa et al., 1989b, 1992b).

On the other hand, the addition of H-7, a protein kinase C inhibitor, did not appreciably affect the intact fowl sperm motility at 30°C. These results suggest that fowl sperm motility may not be simply stimulated or inhibited by the changes of protein kinase C activity. Perhaps, in a basic regulatory system of fowl sperm motility, protein kinase C-dependent phosphoprotein(s) which are responsible for the inhibition of motility may exist: when this protein(s) are phosphorylated more than a certain threshold by activating protein kinase C, fowl sperm motility is inhibited. In contrast, when this protein(s) are dephosphorylated or phosphorylated less than a threshold, i.e., by the addition of H-7 or no treatment, spermatozoa can maintain their motility. In this experiments, however, the target and precise mechanisms of action of protein kinase C remained to be elucidated. Further study is needed to examine which protein(s) are altered during the inhibition of fowl sperm motility by the activation of protein kinase C.

Generally, both vigour of motility and metabolic activity of mammalian spermatozoa increase with rising temperature, and maximum metabolic activity of spermatozoa occurs between 40 and 47°C (Beck and Salisbury, 1943; Freund et al., 1959; Salisbury and Lodge, 1962). Unlike mammalian spermatozoa, fowl spermatozoa display a unique phenomenon: in most synthetic diluents, they become immotile at the body temperature of 40-41°C, but motility is restored by decreasing the temperature (Munro, 1938; Ashizawa and Nishiyama, 1978; Ashizawa and Okauchi, 1984; Ashizawa and Wishart, 1987, 1992; Wishart and Ashizawa, 1987; Thomson and Wishart, 1989, 1991). However, the detailed mechanisms on molecular levels have remained unsolved. In

the present work, as mentioned above, the motility of intact spermatozoa was inhibited by the addition of protein kinase C activators such as SC-9 and OAG, and was restored by reducing the concentrations of activators. Nevertheless, it is possible that the activation of protein kinase C might not be directly involved in the temperature-dependent reversible immobilization of fowl spermatozoa, since the addition of SC-9 and OAG did not inhibit the motility of demembranated spermatozoa. In contrast, the temperature-dependent immobilization was also observed in demembranated spermatozoa as with intact spermatozoa, suggesting that the axoneme itself might be directly involved in this immobilization (Ashizawa et al., 1989b).

In most types of cell, immunocytochemical analysis using monoclonal antibodies against protein kinase C indicates that intracellular localization of this enzyme varies with cell types, but this enzyme is recovered mainly from the soluble fraction, and is apparently translocated to membrane in a Ca^{2+}-dependent fashion when cells are stimulated (Nishizuka, 1986). Our results also indicate that most of protein kinase C in fowl spermatozoa might be located in the cytoplasmic matrix and/or the membrane, but not retained in the axoneme, since as described above, inhibition for motility of intact spermatozoa by the addition of protein kinase C activators was not observed in demembranated spermatozoa.

The effect of Ca^{2+} on fowl sperm motility is less straighfoward. Depending on the conditions, Ca^{2+} may stimulate (Ashizawa et al., 1989a; Thomson and Wishart, 1989, 1991; Ashizawa et al., 1992a) or inhibit (Ashizawa et al., 1994). In the present work, the motility of intact spermatozoa was negligible at 40°C, but was restored after the

addition of Ca^{2+}. However, the subsequent addition of SC-9 and OAG inhibited the motility again. At this time, intracellular free Ca^{2+} concentrations of spermatozoa gradually increased after the addition of SC-9 and OAG, but no changes were observed in H-7 treated spermatozoa, suggesting that an activation of protein kinase C caused an increase of intracellular free Ca^{2+} concentrations in fowl spermatozoa. However, this phenomenon would not be a primary factor in the inhibition of sperm motility, since demembranated fowl spermatozoa could be still motile even at millimolar concentrations of Ca^{2+} (Ashizawa et al., 1989b). Several lines of evidence suggest that protein kinase C modulates ion conductance by phosphorylating membrane proteins such as channels, pumps and ion exchange protein (Nishizuka, 1986). It is proposed that protein kinase C may take part in the enhancement of Ca^{2+} entry by the stimulation of Ca^{2+} channels, since microinjection of TPA or protein kinase C itself into bag cell neurones from the mollusc Aplysia enhances the voltage-sensitive Ca^{2+} current (De Riemer et al., 1985). In contrast, protein kinase C may also stimulate the removal of intracellular Ca^{2+} by activation of the Ca^{2+}-transport ATPase and the Na^{+}-Ca^{2+} exchange protein (Nishizuka, 1988), since the cytosolic Ca^{2+} concentration is frequently decreased by the addition of TPA in various cell types (Tsien et al., 1982; Moolenaar et al., 1984; Drummond, 1985; Sagi-Eisenberg et al., 1985). In fowl spermatozoa, the former possibility is suggested, although the precise mechanisms of such phenomenon remain to be determined.

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Table 1. The effects of SC-9, OAG and H-7 on the motility of demembranated fowl spermatozoa at 30°C and 40°C

Substances(conc.)	Motility (%)	
	30° C	40° C
None (control)	64.0± 4.3[a]	2.2± 1.0[a]
SC-9 (20μ mol l^{-1})	69.5± 6.5[a]	1.6± 1.0[a]
OAG (150μ mol l^{-1})	54.4± 4.4[a]	1.2± 0.7[a]
H-7 (500μ mol l^{-1})	59.9± 1.6[a]	3.4± 1.1[a]

Each value represents the mean (±SEM) of five samples of spermatozoa. Within columns, values with different superscripts differ significantly ($P<0.01$) from each other.

Legends for figures

Fig. 1. Immunoblot analysis of fowl sperm protein kinase C. Lane 1: incubation with antibody against a synthetic 543–550 amino acid peptide of protein kinase $C_{consensus}$; lane 2: control (see Materials and Methods).

Fig. 2. Motility of intact fowl spermatozoa after addition of various concentrations of (a) SC-9, (b) OAG and (c) H-7 at 30°C (●) and 40°C (○). Each point represents the mean (±SEM) of five samples of spermatozoa. *P<0.01 compared with value at 0 μmol l^{-1}.

Fig. 3. The time course of motility of intact fowl spermatozoa at 30°C (●) and 40°C (○) after addition of (a) 10 μmol SC-9 l^{-1}, (b) 100 μmol OAG l^{-1} and (c) 500 μmol H-7 l^{-1}. At arrow, sperm samples were diluted (approximately 8 fold) in drug free assay medium to decrease extracellular concentrations of drug. Each point represents the mean (±SEM) of five samples of spermatozoa. *P<0.01 compared with value at 0 min.

Fig. 4. Motility of intact fowl spermatozoa after addition (↓) of 1 mmol $CaCl_2$ l^{-1} (○), 20 μmol SC-9 l^{-1} (●), 150 μmol OAG l^{-1} (□) and 500 μmol H-7 l^{-1} (■) at (a) 30°C and (b) 40°C. Each point represents the mean (±SEM) of five samples of spermatozoa. *P<0.01 compared with value of no addition of drug (control) at each period.

Fig. 5. Changes of intracellular free Ca^{2+} concentrations in fowl spermatozoa at 30°C measured by fura-2 fluorescence after addition (↓) of 1 mmol $CaCl_2$ l^{-1}, 20 μmol SC-9 l^{-1}, 150 μmol OAG l^{-1} and 500 μmol H-7 l^{-1}.

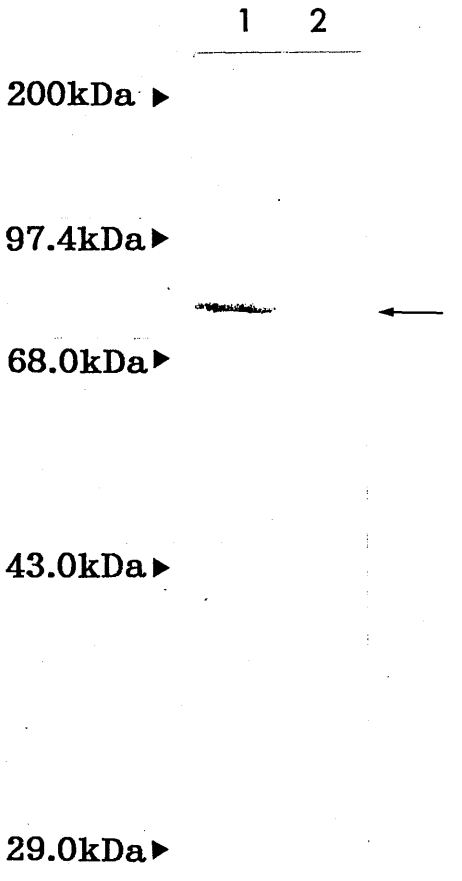

Fig. 1
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(a)

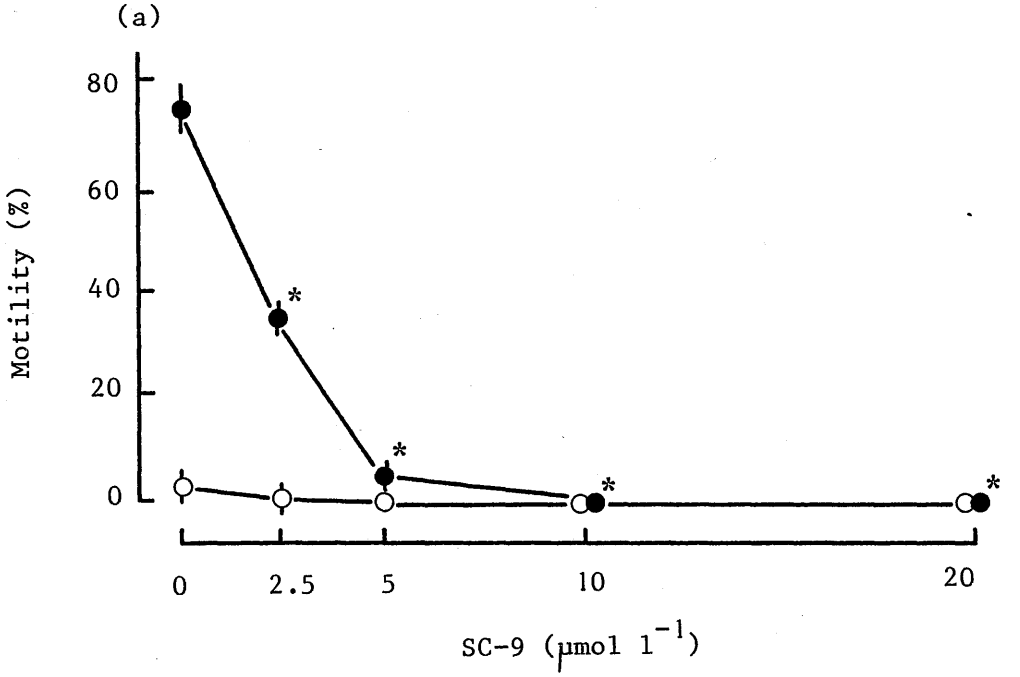

Fig. 2a
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(b)

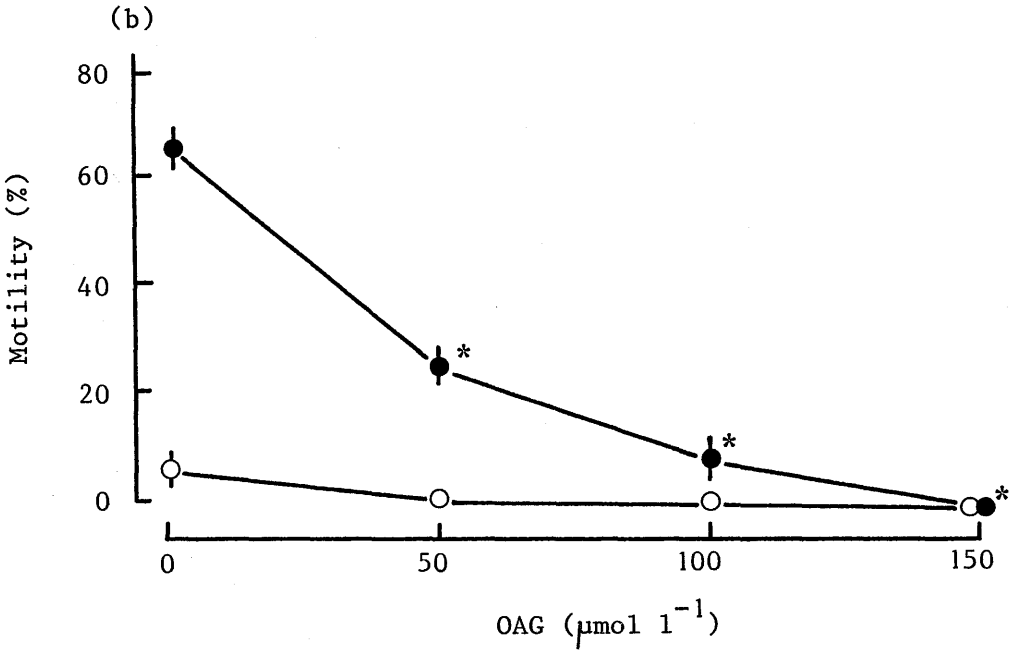

Fig. 2b

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(C)

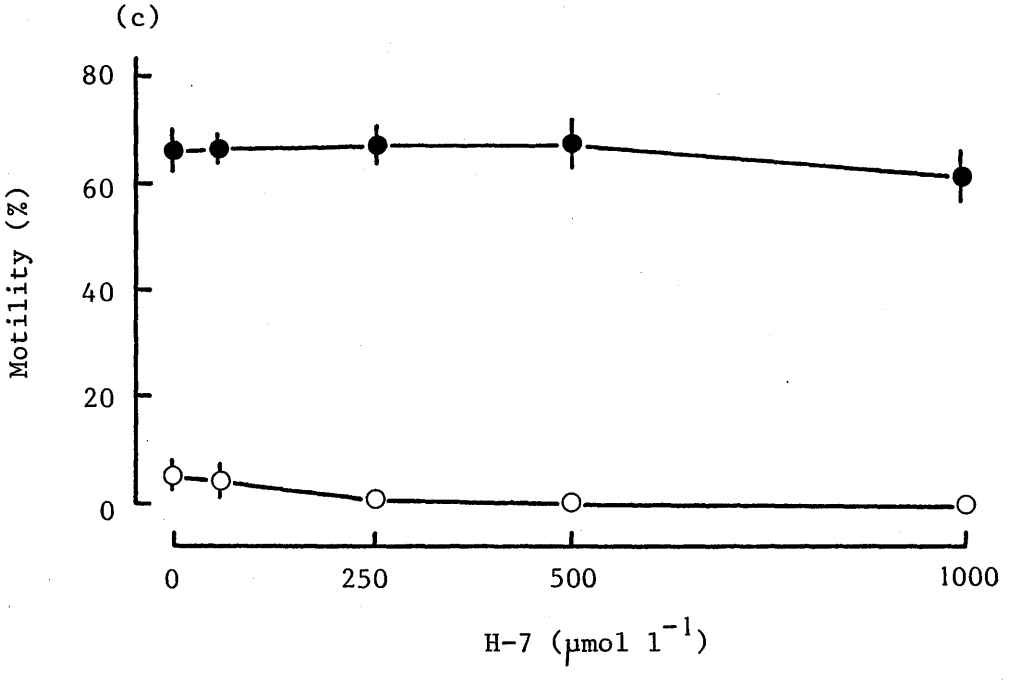

Fig. 2c
Ashizawa et al.

(a)

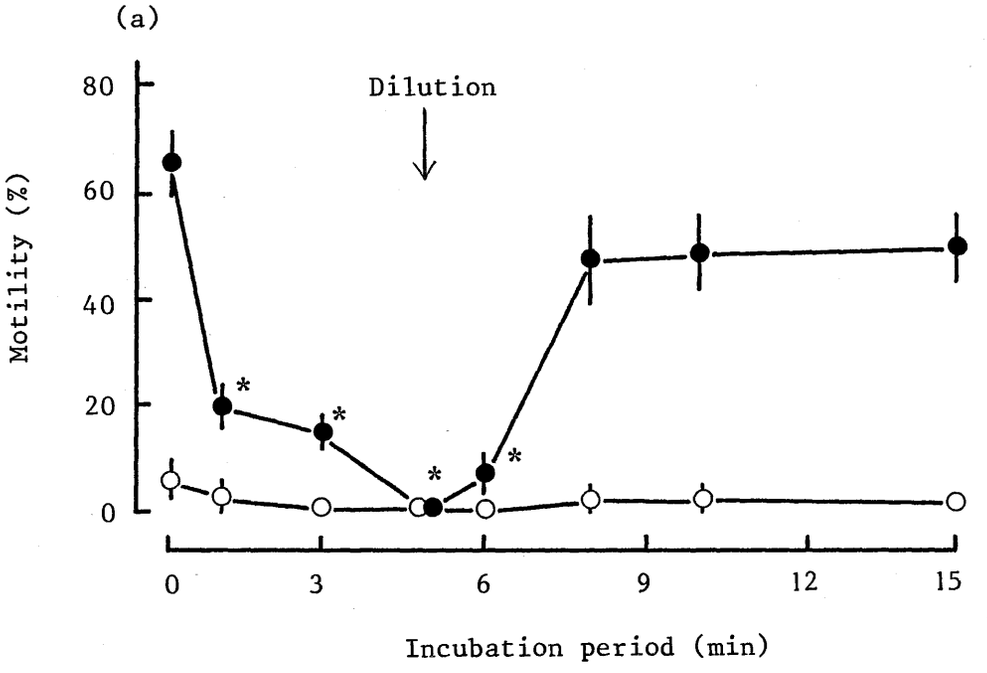

Fig. 3a
Ashizawa et al.

(b)

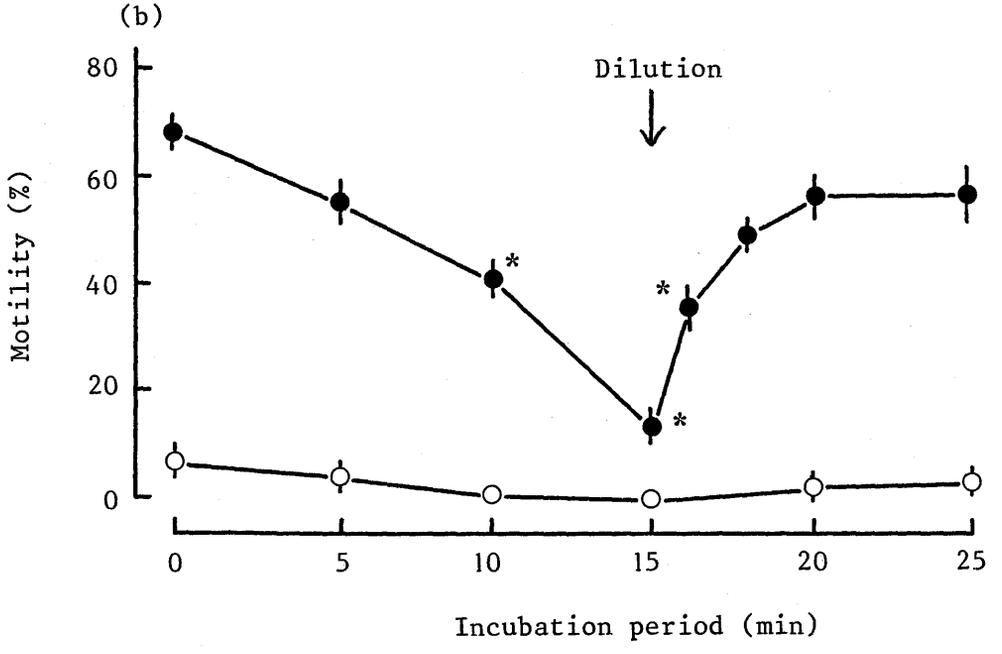

Fig. 3b
Ashizawa et al.

(C)

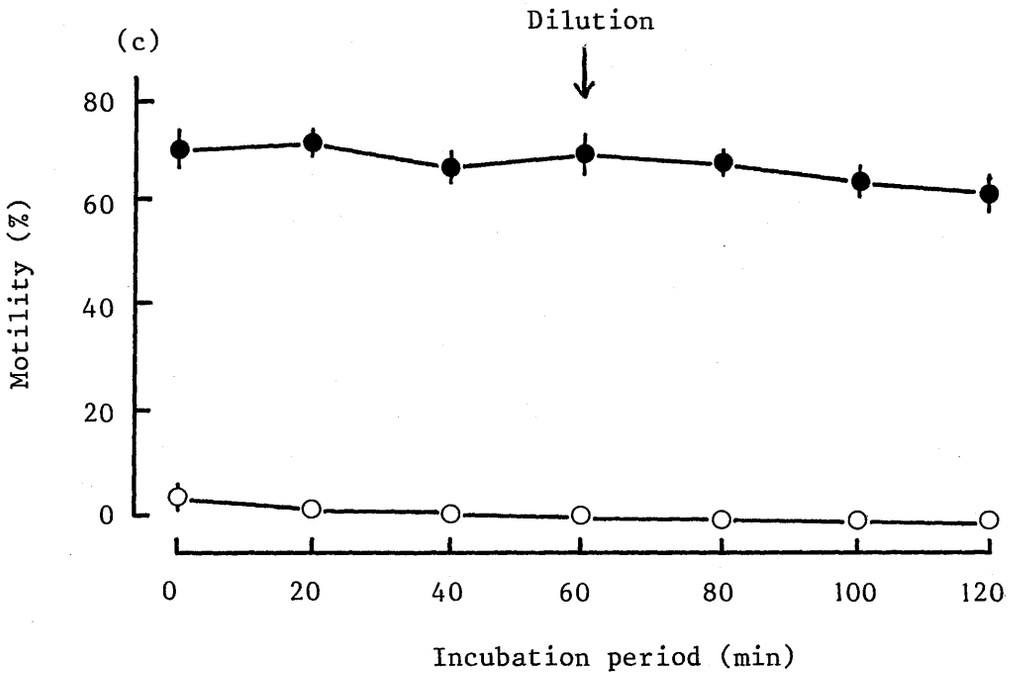

Fig. 3C
Ashizawa et al.

(a)

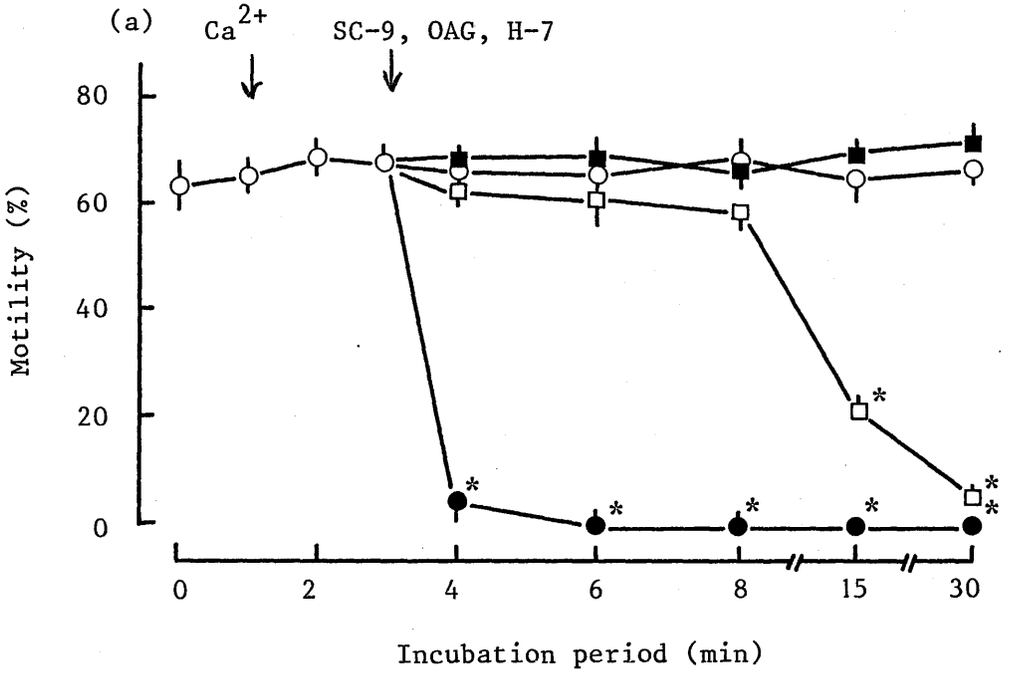

Fig. 4a
Ashizawa et al.

(b)

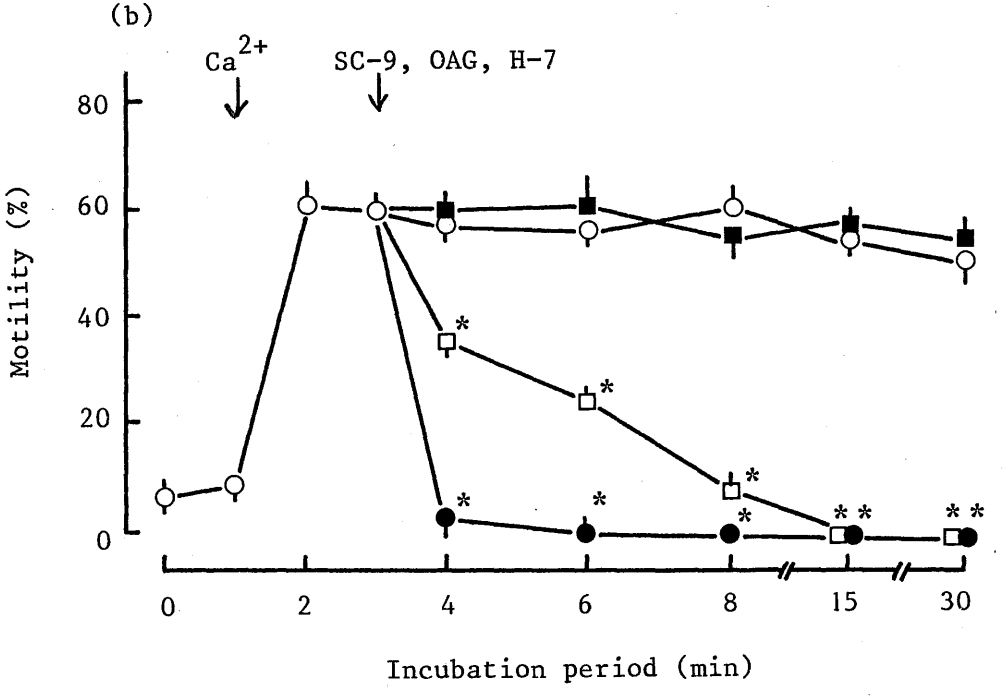

Fig. 4b
Ashizawa et al.

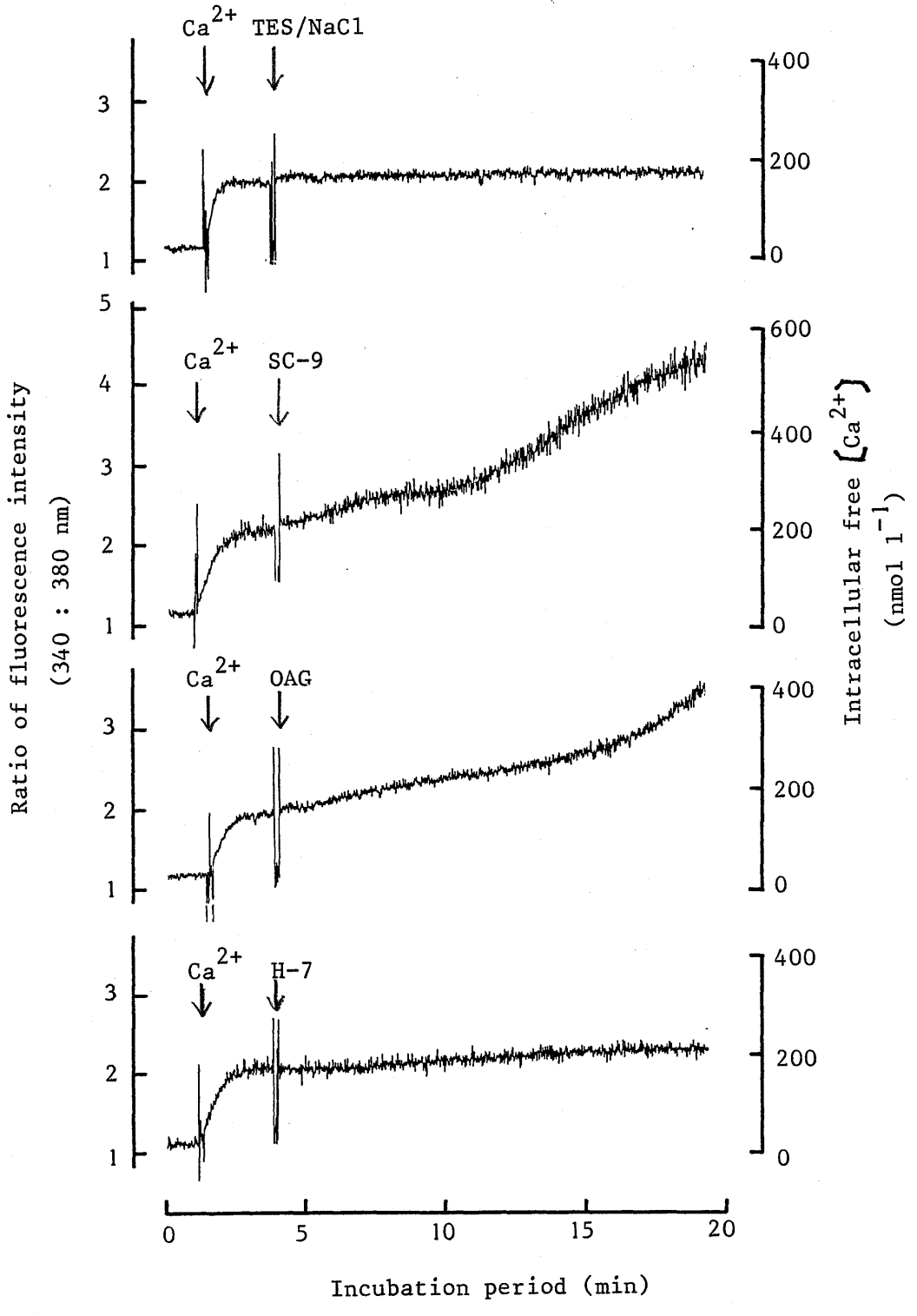

Fig. 5

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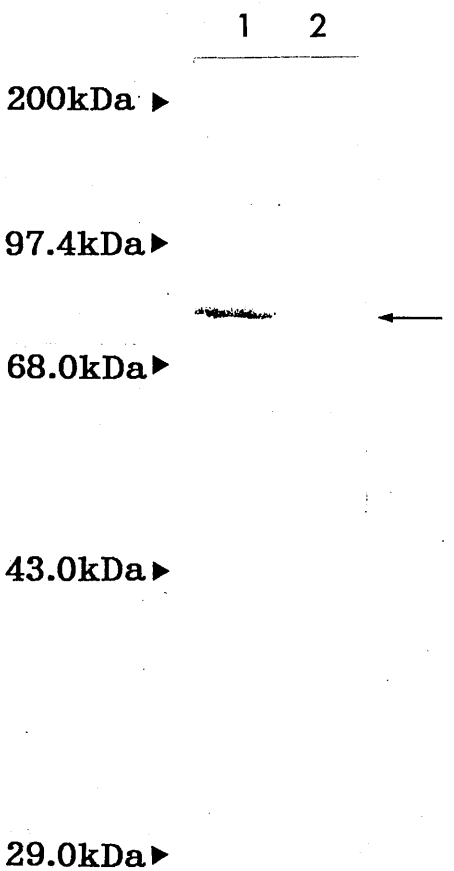

Fig. 1
Ashizawa et al.