Regulation of flagellar motility of fowl spermatozoa: Evidence for the involvement of intracellular free Ca^{2+} and calmodulin

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Summary

The possible role of intracellular free Ca^{2+} and calmodulin in regulating fowl sperm motility was investigated by using an intracellular Ca^{2+} chelator, 1, 2-bis (2-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) and calmodulin antagonists such as N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7), N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5) and trifluoperazine. Intact fowl spermatozoa maintained vigorous movement in a Ca^{2+}-free medium at 30°C. In contrast, the motility of spermatozoa loaded with BAPTA/AM was negligible at 30°C, but could be instantly restored by the addition of 2 mmol $CaCl_2$ l^{-1}. At this time, the intracellular free Ca^{2+} concentrations increased from 0 to about 100 nmol l^{-1}, measured by fluorescent Ca^{2+} indicator fura-2. At 40°C, neither control nor BAPTA/AM-treated spermatozoa were motile, but the motility of both spermatozoa was restored by the subsequent addition of 2 mmol $CaCl_2$ l^{-1}. Even in the presence of 2 mmol $CaCl_2$ l^{-1}, the addition of W-7 and trifluoperazine inhibited the motility of intact spermatozoa at 30°C and 40°C, and induced a concomitant decrease in the rate of oxygen consumption and the ATP concentrations, suggesting that energy depletion might be involved in the inhibition of motility. In contrast, the motility of demembranated spermatozoa was not inhibited by the addition of W-7 and trifluoperazine at 30°C. The addition of W-5, a weaker antagonist, did not appreciably affect the motility of either intact or demembranated spermatozoa. These results suggest that intracellular free Ca^{2+} is indispensable for the maintenance of fowl

sperm motility, and calmodulin which is in the cytoplasm and/or mitochondria, but not retained in the axoneme, is a prominent candidate as the signal transducer in Ca^{2+}–stimulated motility.

Introduction

It is well recognized that Ca^{2+} is a signal transducer in the regulation of sperm motility (for reviews, see Tash and Means, 1983; Lindemann and Kanous, 1989; Majumder et al., 1990). The effects of intracellular Ca^{2+} on sperm motility can be divided into two major classes: (i) modification of wave form and oscillation frequency (Gibbons and Gibbons, 1973; Brokaw et al., 1974; Brokaw, 1979; Lindemann et al., 1987; Suarez et al., 1993) and (ii) complete inhibition of motility at > 1 μmol Ca^{2+} l^{-1} (Gibbons and Gibbons, 1980; Mohri and Yanagimachi, 1980; Tash and Means, 1982).

Since the inhibition of mammalian sperm motility by elevated intracellular Ca^{2+} is paralleled by a diminution in the overall level of phosphorylation state of sperm proteins, some of which may be axonemal components (Tash and Means, 1982), it is logical to presume that the effects of Ca^{2+} on sperm motility may be in some part regulated by protein phosphorylation. The inhibitory effect of Ca^{2+} on protein phosphorylation could be explained by (i) a direct effect of Ca^{2+} on protein kinase activity, (ii) Ca^{2+}–stimulated phosphoprotein phosphatase activity or (iii) conformational changes within the axoneme which prevent enzyme–substrate interactions (Tash and Means, 1983).

On the other hand, fowl spermatozoa display a unique phenomenon: spermatozoa show a reversible inhibition of their motility

as the temperature is raised from 30°C to 40°C, the avian body temperature (Munro, 1938; Nevo and Schindler, 1968; Ashizawa and Nishiyama, 1978; Takeda, 1982; Ashizawa and Okauchi, 1984; Ashizawa and Wishart, 1987; Wishart and Ashizawa, 1987; Ashizawa et al., 1989a). One of the possible mechanisms of this phenomenon involves a loss of intracellular Ca^{2+} to the suspending medium at 40°C, with a subsequent resequestration of Ca^{2+} at lower temperature (Thomson and Wishart, 1989, 1991). In fact, motility is restored by decreasing the temperature or by the addition of Ca^{2+} at 40°C (Wishart and Ashizawa, 1987; Ashizawa et al., 1989a; Thomson and Wishart, 1989, 1991). Additionally, demembranated fowl sperm motility was also inhibited in Ca^{2+}-free medium (Ashizawa et al., 1992a) and reactivation was observed with the addition of 10^{-3} mol l^{-1} of Ca^{2+} (Ashizawa et al., 1989b). In such a high concentrations of Ca^{2+}, the motility of demembranated ram and hamster spermatozoa was inhibited (White and Voglmayr, 1986; Feng et al., 1988). Therefore, it seems likely that there is a species difference in sensitivity to Ca^{2+}, and Ca^{2+} is indispensable for the movement of fowl spermatozoa.

Calmodulin, the Ca^{2+} binding protein that modulates the activity of a number of key regulatory enzymes (Means et al., 1982), has been identified in a diverse range of species of spermatozoa, from sea urchins to humans (Brooks and Siegel, 1973; Jones et al., 1978, 1980; Garbers et al., 1980; Burgess, 1983; Moore and Dedman, 1984; Yamamoto, 1985; Camatini et al., 1986, 1991; Weinman et al., 1986; Fouquet et al., 1991; Kann et al., 1991). A function for calmodulin in the regulation of flagellar motility has been suggested by wave form changes in dog and sea urchin spermatozoa (Tash and Means, 1982; Brokaw and

Nagayama, 1985) or inhibitory action in human spermatozoa (Hong _et al._, 1985) resulting from anti–calmodulin drug treatment in spermatozoa.

Therefore, it may be assumed that Ca^{2+}–sensitive fowl sperm motility described above is regulated, for the most part, by interaction with the Ca^{2+}–calmodulin complex rather than by Ca^{2+} directly. However, the presence of calmodulin or its role in the regulation of motility has not been demonstrated in fowl spermatozoa. In the present work, attempts were made to clarify the role of calmodulin in the regulation of flagellar motility and metabolic activity of fowl spermatozoa by using calmodulin antagonists. We have shown that fowl spermatozoa are rendered immotile at 30°C and 40°C by the addition of calmodulin antagonists, even in the presence of Ca^{2+}.

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 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 1×10^9 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) and 1, 2-bis (2-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM) were purchased from Dojindo Laboratories, Inc., Kumamoto, Japan. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) and N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W-5) were obtained from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Trifluoperazine, adenosine 5'-triphosphate (ATP), dithiothreitol, potassium glutamate, TES, Triton X-100, Cremophor EL and desicated firefly tails were purchased from Sigma Chemical Co., St Louis, MO, USA. Other chemicals were of reagent grade from Nacalai Tesque, Inc., Kyoto, Japan.

Loading of spermatozoa with BAPTA/AM

Stock solutions of BAPTA/AM were made up to 50 mmol l^{-1} in dry dimethylsulphoxide (DMSO) and kept desiccated at 4°C in the dark until use. For the complete emulsification of BAPTA/AM, 1 μl of 10% Cremophor EL were added to 2 μl of BAPTA/AM, and then 1 ml of NaCl/TES buffer were added. This solution was sonicated for 2 min on ice with a ultrasonic processor with a microtip (Sonics and Materials, Inc., Danbury, CT, USA) at power of 50 W. This procedure subsequently gave optimal BAPTA/AM cellular loading and hydrolysis. 0.1 ml of sperm suspension was added to 1 ml BAPTA/AM solution and

shaken for 90 min at room temperature (20–25°C) in the dark. During this incubation, final concentrations of BAPTA/AM and spermatozoa were about 90 μmol l^{-1} and 0.9 x 10^8 ml^{-1}, respectively. Sperm preparations diluted with TES/NaCl buffer to the same concentrations described above and shaken for 90 min at room temperature were used as control.

Measurement of motility of intact and demembranated spermatozoa

In the first series of experiments, the sperm preparations with or without BAPTA/AM treatment were incubated aerobically in a shaking water bath at 30°C or 40°C. Five minutes after the start of incubation, 2 mmol $CaCl_2$ l^{-1} was added. During incubation of spermatozoa, the suspension of spermatozoa was placed in a microscope chamber (Sekisui Chemical Co., Ltd., Tokyo, Japan, UR–157 type, 0.07 mm in depth) and the motility of spermatozoa was recorded by videomicroscopy (magnification on the 12–inch black and white monitor was approximately x600) at 30°C or 40°C on a thermostatically controlled warm plate (Katz and Overstreet, 1981). Measurements were made on totally 200–300 spermatozoa, distributed uniformly among the 3 or more fields, to determine percentage motility.

In the second series of experiments, to examine the effects of calmodulin antagonists on the motility of intact and demembranated spermatozoa, the dose–response and time–course of motility were measured at 30°C after addition of various concentrations of W–5, W–7 and trifluoperazine. The effects of the addition of $CaCl_2$ before the addition of calmodulin antagonists were also examined at 30°C and 40°C to be compared with those of calmodulin antagonists 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 according to the method described previously (Ashizawa et al., 1989b). The extraction medium used consisted of 0.1% Triton X-100, 0.2 mol 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}, 0.2 mol 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).

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, Japan) 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}.

Measurement of oxygen consumption and ATP concentrations of intact spermatozoa

Oxygen consumption of spermatozoa was determined polarographically with a Clark electrode by the method of Kielley (1963), using a YSI model 53 biological oxygen monitor (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA). The rate of oxygen consumption was expressed in terms of μl O_2 consumption per 10^8 spermatozoa h^{-1}. 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).

ATP concentration in spermatozoa was assayed by firefly bioluminescence in a boiled extract (Wishart, 1982).

Statistical analysis

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

Results

Effects of BAPTA/AM on the motility and intracellular free Ca^{2+} concentrations of intact fowl spermatozoa

Intact fowl spermatozoa which were not treated with BAPTA/AM (control) maintained vigorous movement at 30°C in the presence or absence of $CaCl_2$ in the suspending medium. On the contrary, the motility of spermatozoa loaded with BAPTA/AM, an intracellular calcium chelator, was negligible at 30°C, but was instantly restored by the addition of 2 mmol $CaCl_2$ l^{-1} (Fig. 1a). At 40°C, neither control nor BAPTA/AM-treated spermatozoa were motile, but the motility of both

preparations was restored by the subsequent addition of $CaCl_2$ (Fig. 1b).

The intracellular free Ca^{2+} concentration of intact spermatozoa before the addition of $CaCl_2$ was approximately 20 nmol l^{-1} at 30°C and 40°C. After the addition of $CaCl_2$, the Ca^{2+} concentration increased rapidly and plateaued at approximately 200 nmol l^{-1}. In contrast, the intracellular free Ca^{2+} concentration of BAPTA/AM-treated spermatozoa before the addition of $CaCl_2$ was almost 0 nmol l^{-1} at 30°C and 40°C. However, the addition of 2 mmol $CaCl_2$ l^{-1} caused an influx of Ca^{2+} from the medium and the Ca^{2+} concentration increased gradually at 30°C. Similar results were obtained at 40°C (Fig. 2a,b).

Effects of calmodulin antagonists 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 calmodulin antagonists, W-7 and trifluoperazine. In contrast, no inhibition of motility was observed following the addition of W-5, a weaker antagonist used as a control drug for W-7, within the range 0-500 μmol l^{-1} (Fig. 3).

Fig. 3 shows the inhibition of motility at 20 min after the addition of calmodulin antagonists. The time-course of motility inhibition by calmodulin antagonists at 30°C is shown in Fig. 4. Inhibition of motility by trifluoperazine was irreversible, but was reversible in W-7-treatment: the motility could be restored by the dilution (20 fold) in W-7 free assay medium to decrease the concentrations of W-7 after 20 min of exposure.

On the other hand, inhibition for motility by the addition of W-7

and trifluoperazine was not observed in demembranated and reactivated spermatozoa at 30°C (Fig. 5), and the motility maintained at least 30 min as well as those of control and W-5-treated spermatozoa (Fig. 6).

Even the presence of Ca^{2+} before the addition of W-7 and trifluoperazine could not prevent the inhibition of motility of intact spermatozoa at 30°C (Fig. 7a). At 40°C, the motility of intact spermatozoa was negligible, but the motility was restored instantly after the addition of 2 mmol $CaCl_2$ l^{-1}. However, the subsequent addition of W-7 and trifluoperazine inhibited the motility again (Fig. 7b). During this period, the intracellular free Ca^{2+} concentration maintained a constant value at approximately 200 nmol l^{-1} (data not shown). Furthermore, the motility of BAPTA/AM-treated spermatozoa also could not be restored by the addition of both W-7 and $CaCl_2$ at 30°C and 40°C, but was instantly restored by the addition of $CaCl_2$ alone (Figs. 7c,d).

Effects of BAPTA/AM and calmodulin antagonists on the oxygen consumption and ATP concentrations of intact fowl spermatozoa

The oxygen consumption of spermatozoa which were treated with BAPTA/AM at 30°C and 40°C were 3.4 ± 0.1 and 4.2 ± 0.3 (μl O_2 10^{-8} spermatozoa h^{-1}, the mean$\pm$SEM of five samples), respectively. At both temperatures, there were significant differences ($P<0.01$) compared with spermatozoa which were not treated with BAPTA/AM (control) (5.4 ± 0.2 at 30°C and 6.2 ± 0.4 at 40°C).

Furthermore, the rate of oxygen consumption of intact spermatozoa increased slightly after the addition of 2 mmol $CaCl_2$ l^{-1} at

30°C and 40°C, but decreased significantly ($P<0.01$) following the addition of W-7 and trifluoperazine. In contrast, there was no significant decrease after the addition of W-5 as well as those in control (Figs. 8a,b).

The ATP concentrations of BAPTA/AM-treated spermatozoa decreased slightly at 30°C (38.2±3.5 nmol 10^{-9} spermatozoa, the mean± SEM of five samples) compared with those of no treated spermatozoa (control) (42.6±4.7), but there was no significant difference between them. However, at 40°C, there was significant difference ($P<0.01$) between BAPTA/AM-treated spermatozoa and control (31.7±2.9 in BAPTA/AM treatment and 55.1±6.0 in control).

In addition, the ATP concentrations of intact spermatozoa decreased slightly at 30°C and 40°C after the addition of $CaCl_2$. The subsequent addition of W-7 and trifluoperazine in spite of the presence of Ca^{2+} caused the significant decrease ($P<0.01$) of ATP concentrations compared with those of no addition (control) and W-5 treatment (Figs. 9a,b).

Discussion

Unlike mammalian spermatozoa, fowl spermatozoa display a reversible, temperature-dependent immobilization (see Introduction). This phenomenon might provide a unique model for basic studies on the cellular mechanisms involved in the regulation of flagellar movement. It is particularly useful for studying the stimulatory effect of Ca^{2+}, since at 40°C fowl sperm motility is negligible until the addition of millimolar Ca^{2+} to the suspending medium whereupon 'maximal' motility

is achieved (Wishart and Ashizawa, 1987; Ashizawa et al., 1989a; Thomson and Wishart, 1989, 1991). However, the detailed mechanisms on molecular levels have remained unresolved.

In the present work, we have shown that the motility of intact fowl spermatozoa loaded with an intracellular Ca^{2+} chelator, BAPTA/AM, was negligible at 30°C, even though spermatozoa are naturally motile at this temperature. However, motility was instantly restored by the addition of excess Ca^{2+} (Fig. 1a). At this time, the intracellular free Ca^{2+} concentrations increased from 0 to about 100 nmol l^{-1} (Fig. 2a). Thus it would appear that intracellular free Ca^{2+} is indispensable for the maintenance of flagellar motility of fowl spermatozoa.

Calmodulin is a highly conserved, ubiquitous protein involved in mediating the influence of Ca^{2+} on such cellular processes as signal transduction, cell motility and membrane fusion through its ability to activate key intracellular enzymes (Means et al., 1982). The present results suggest that calmodulin may regulate fowl sperm motility at a point downstream from Ca^{2+} fluxes, since the addition of calmodulin antagonists such as W-7 and trifluoperazine inhibited the motility of intact spermatozoa at 30°C and 40°C even in the presence of Ca^{2+} (Figs. 7a,b) and the motility of BAPTA/AM-treated spermatozoa also could not be restored by the addition of both W-7 and Ca^{2+}, but was instantly restored by the addition of Ca^{2+} alone (Figs. 7c, d). Furthermore, our results indicate that most of calmodulin in fowl spermatozoa might be located in the cytoplasmic matrix, the mitochondria and/or the membrane, but not retained in the axoneme, since inhibition of motility of intact spermatozoa by the addition of W-7 and trifluoperazine was not observed in demembranated spermatozoa

(Figs. 5 and 6). These results were different from those reported previously: calmodulin of sea urchin spermatozoa remains tightly bound to the axoneme after the extraction with Triton X-100 and millimolar Ca^{2+} that is required to produce 'potentially symmetric' flagella, although a portion of calmodulin is readily solubilized during extraction, and may be either membrane associated or simply soluble in the cytoplasmic matrix (Brokaw and Nagayama, 1985).

With regard to the action of calmodulin antagonists such as W-7 and trifluoperazine, an alternative explanation should be noted: these antagonists could be interfering, not only with calmodulin, but with membrane-bound, Ca^{2+}-dependent protein kinase C. The activity of this kinase system is strictly independent of calmodulin and yet is susceptible to nonspecific suppression by calmodulin antagonists (Schatzman et al., 1981, 1983). However, inhibition of both intact and demembranated fowl sperm motility at 30°C was not observed by the addition of H-7, the most potent and specific protein kinase C inhibitor (Ashizawa et al., unpublished data). Therefore, it is logical to presume that the inhibition of fowl sperm motility by the addition of W-7 and trifluoperazine might be due to the inhibition of Ca^{2+}/calmodulin-regulated enzyme activities.

In this experiments, however, the target and precise mechanisms of action of calmodulin in the regulation of sperm motility remain to be elucidated. One possibility is that the inhibition of motility is probably due to the energy depletion on sperm motility, since our results have, for the first time, shown that the oxygen consumption and ATP concentrations of fowl spermatozoa decreased markedly after the addition of W-7 and trifluoperazine (Figs. 8 and 9). It is still

unclear whether the energy depletion by the addition of W-7 and trifluoperazine is caused by either a calmodulin-regulated or some other, unidentified process. It has been suggested that calmodulin may be involved in the control of axonemal function by regulating a number of key enzymes, including dynein ATPase, myosin light chain kinase, cyclic nucleotide phosphodiesterases and adenylate cyclase (Tash and Means, 1983). A possible explanations for the influence of calmodulin on sperm motility is that calmodulin in spermatozoa might stimulate the activity of adenylate cyclase responsible for generating the cAMP which is known to be essential for the stimulation of mammalian sperm motility (Gross et al., 1987). However, unlike mammalian spermatozoa, demembranated fowl sperm motility could not be restored by the addition of cAMP at 40°C (Ashizawa et al., 1989b, 1992b).

On the other hand, attempts to demonstrate a direct association of calmodulin with dynein have yielded conflicting results (Blum et al., 1980; Gitelman and Witman, 1980). The high molar ratios of calmodulin to dynein required to alter dynein ATPase activity (Hisanaga and Pratt, 1984) suggest either that conditions of axoneme or dynein preparation pivotal to preserving such an interaction have not been optimized or that calmodulin regulates motility through an indirect interaction with dynein via another component (Tash, 1989). Evidence for an intermediate calmodulin-regulated component has been obtained by the identification of calmodulin binding proteins in mammalian spermatozoa (Moore and Dedman, 1984; Aitken et al., 1988; Tash et al., 1988; Leclerc et al., 1989; Wasco et al., 1989; Manjunath et al., 1993).

Calmodulin appears to mediate many of the effects of Ca^{2+} on

fowl sperm motility. However, it should be noted that not all effects of Ca^{2+} on fowl sperm flagellar movement might be mediated by calmodulin, since demembranated sperm motility was inhibited in Ca^{2+}-free medium and was restored by the subsequent addition of Ca^{2+} alone at 30°C (Ashizawa et al., 1992a), even though most of calmodulin might be removed from axoneme by the demembranation.

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Legends for figures

Fig. 1. The time course of motility of intact fowl spermatozoa with (●) or without BAPTA/AM (■) at 30°C (a) and 40°C (b). At arrow, 2 mmol $CaCl_2$ l^{-1} was added (○,□). Each point represents the mean (±SEM) of five samples of spermatozoa. *$P<0.01$ compared with value of no addition of Ca^{2+} at each period.

Fig. 2. Changes of intracellular free Ca^{2+} concentrations in fowl spermatozoa with or without BAPTA/AM at 30°C (a) and 40°C (b) measured by fura-2 fluorescence. 2 mmol $CaCl_2$ l^{-1} was added at arrow.

Fig. 3. Motility of intact fowl spermatozoa after addition of various concentrations of calmodulin antagonists, W-7 (●), W-5 (○) and trifluoperazine (■) at 30°C. Spermatozoa were assayed for motility 20 min after the addition of calmodulin antagonists at each concentration. Each point represents the mean (±SEM) of five samples of spermatozoa. *$P<0.01$ compared with value at 0 μmol l^{-1}.

Fig. 4. The time course of motility of intact fowl spermatozoa at 30°C after addition of 500 μmol W-7 l^{-1} (●), 500 μmol W-5 l^{-1} (○) and 100 μmol trifluoperazine l^{-1} (■); (□) no addition of antagonist (control). At arrow, sperm samples were diluted (20 fold) in antagonist free assay medium to decrease extracellular antagonist concentrations. Each point represents the mean (±SEM) of five samples of spermatozoa. *$P<0.01$ compared with value at 0 min.

Fig. 5. Motility of demembranated fowl spermatozoa after addition of various concentrations of calmodulin antagonists, W–7 (●), W–5 (○) and trifluoperazine (■) at 30°C. Spermatozoa were assayed for motility 20 min after the addition of calmodulin antagonists at each concentration. Each point represents the mean (± SEM) of five samples of spermatozoa. *$P<0.01$ compared with value at 0 μmol l^{-1}.

Fig. 6. The time course of motility of demembranated fowl spermatozoa at 30°C after addition of 100 μmol W–7 l^{-1} (●), 100 μmol W–5 l^{-1} (○) and 50 μmol trifluoperazine l^{-1} (■); (□) no addition of antagonist (control). Each point represents the mean (± SEM) of five samples of spermatozoa. *$P<0.01$ compared with value at 0 min.

Fig. 7. Motility of intact fowl spermatozoa after addition (↓) of 2 mmol $CaCl_2$ l^{-1} (○), 500 μmol W–7 l^{-1} (●), 500 μmol W–5 l^{-1} (■) and 100 μmol trifluoperazine l^{-1} (□) at 30°C (a), and 2 mmol $CaCl_2$ l^{-1} (○), 100 μmol W–7 l^{-1} (●), 100 μmol W–5 l^{-1} (■) and 50 μmol trifluoperazine l^{-1} (□) at 40°C (b). Motility of BAPTA/AM–treated intact fowl spermatozoa before (●) and after addition (↓) of 2 mmol $CaCl_2$ l^{-1} (□) and 2 mmol $CaCl_2$ l^{-1} + 500 μmol W–7 l^{-1} (○) at 30°C (c), and 2 mmol $CaCl_2$ l^{-1} (□) and 2 mmol $CaCl_2$ l^{-1} + 100 μmol W–7 l^{-1} (○) at 40°C (d). Each point represents the mean (± SEM) of five samples of spermatozoa. *$P<0.01$ compared with value of no addition of calmodulin antagonist (control) at each period.

Fig. 8. Oxygen consumption (μl O_2 10^{-8} spermatozoa h^{-1}) of intact fowl spermatozoa after addition of 2 mmol $CaCl_2$ l^{-1}, 500 μmol W–7 l^{-1}, 500 μmol W–5 l^{-1} and 100 μmol trifluoperazine l^{-1} at 30°C (a), and 2 mmol $CaCl_2$ l^{-1}, 100

μmol W–7 l^{-1}, 100 μmol W–5 l^{-1} and 50 μmol trifluoperazine l^{-1} at 40°C (b). Each value represents the mean (±SEM) of five samples of spermatozoa. *P<0.01 compared with value of no addition of calmodulin antagonist (control).

Fig. 9. ATP concentrations in intact fowl spermatozoa after addition (↓) of 2 mmol $CaCl_2$ l^{-1} (○), 500 μmol W–7 l^{-1} (●), 500 μmol W–5 l^{-1} (■) and 100 μmol trifluoperazine l^{-1} (□) at 30°C (a), and 2 mmol $CaCl_2$ l^{-1} (○), 100 μmol W–7 l^{-1} (●), 100 μmol W–5 l^{-1} (■) and 50 μmol trifluoperazine l^{-1} (□) at 40°C (b). Each point represents the mean (±SEM) of five samples of spermatozoa. *P<0.01 compared with value of no addition of calmodulin antagonist (control) at each period.

(a)

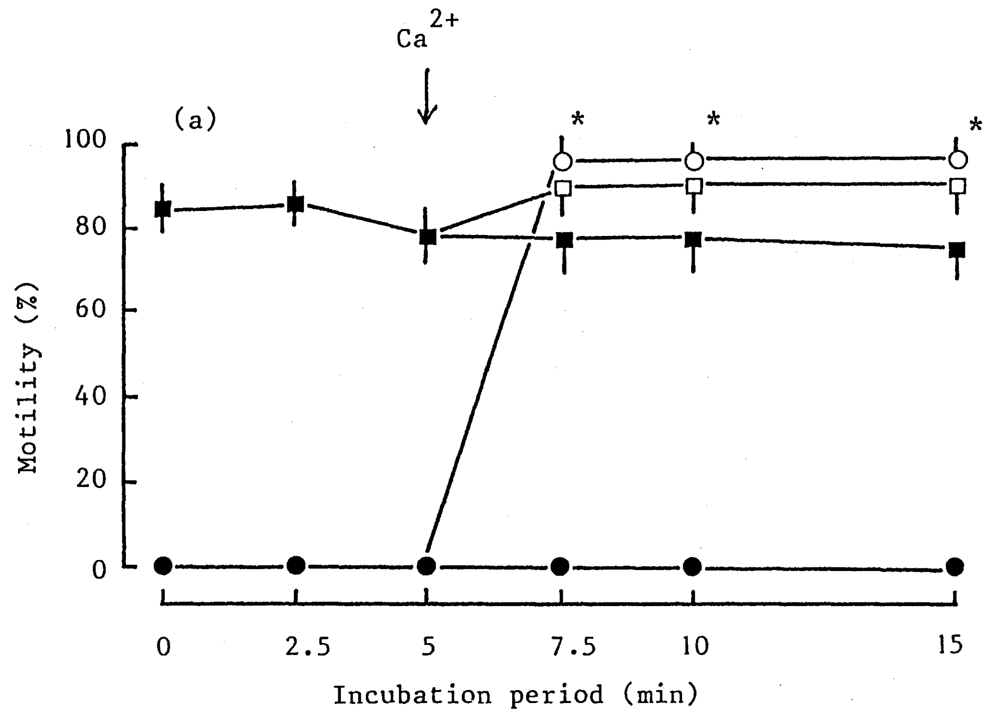

Fig. 1a

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(b)

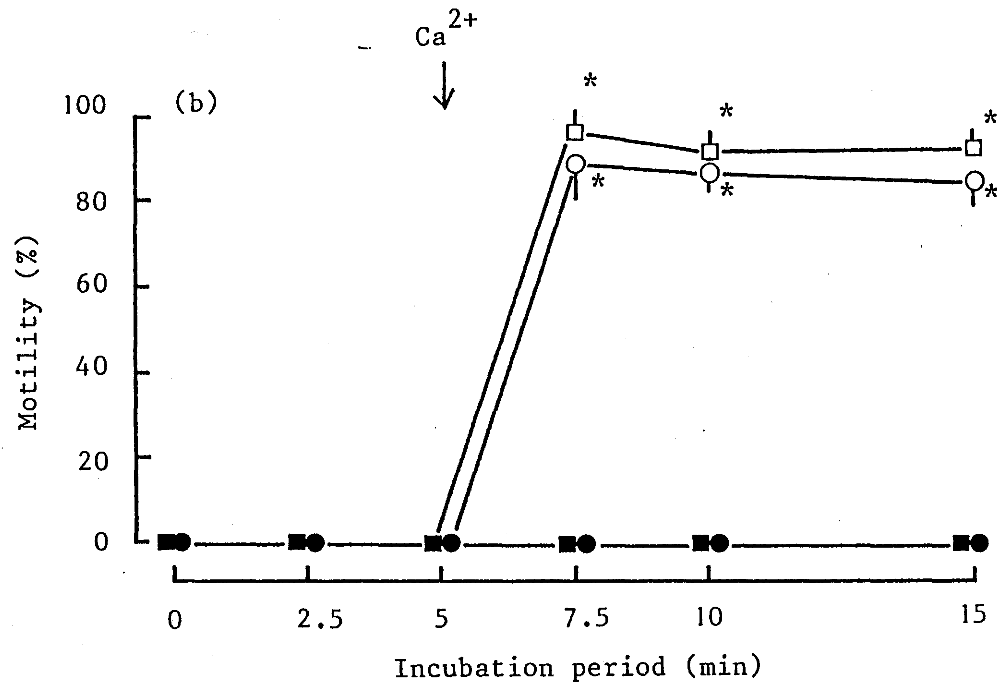

Fig. 1b

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(a)

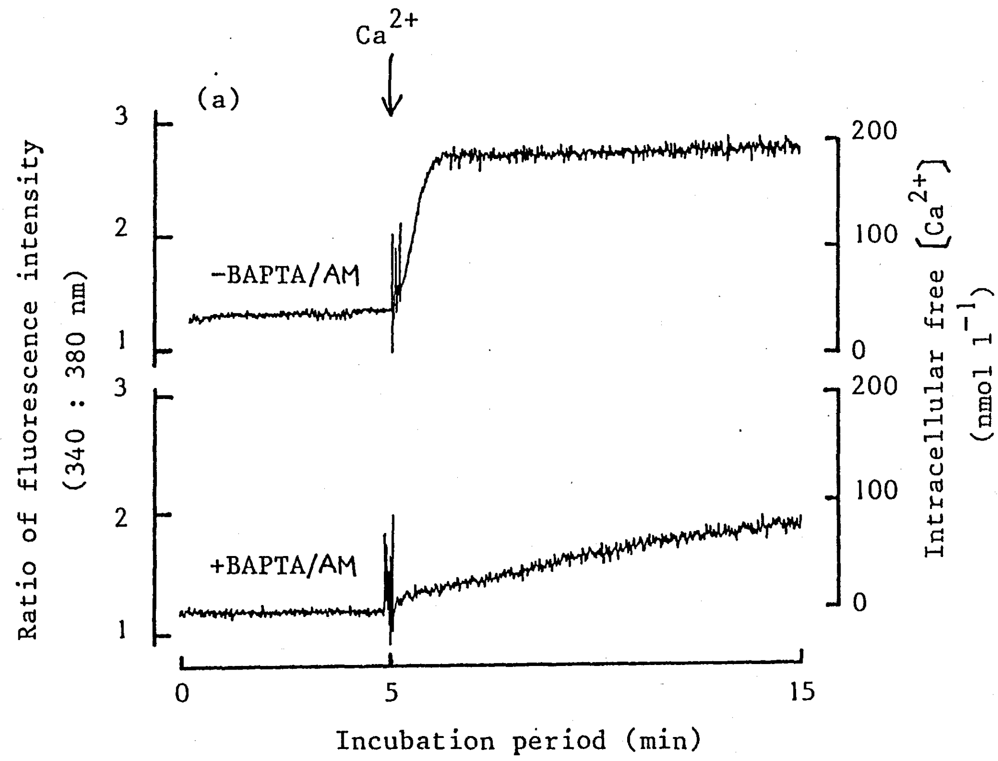

Fig 2a
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(b)

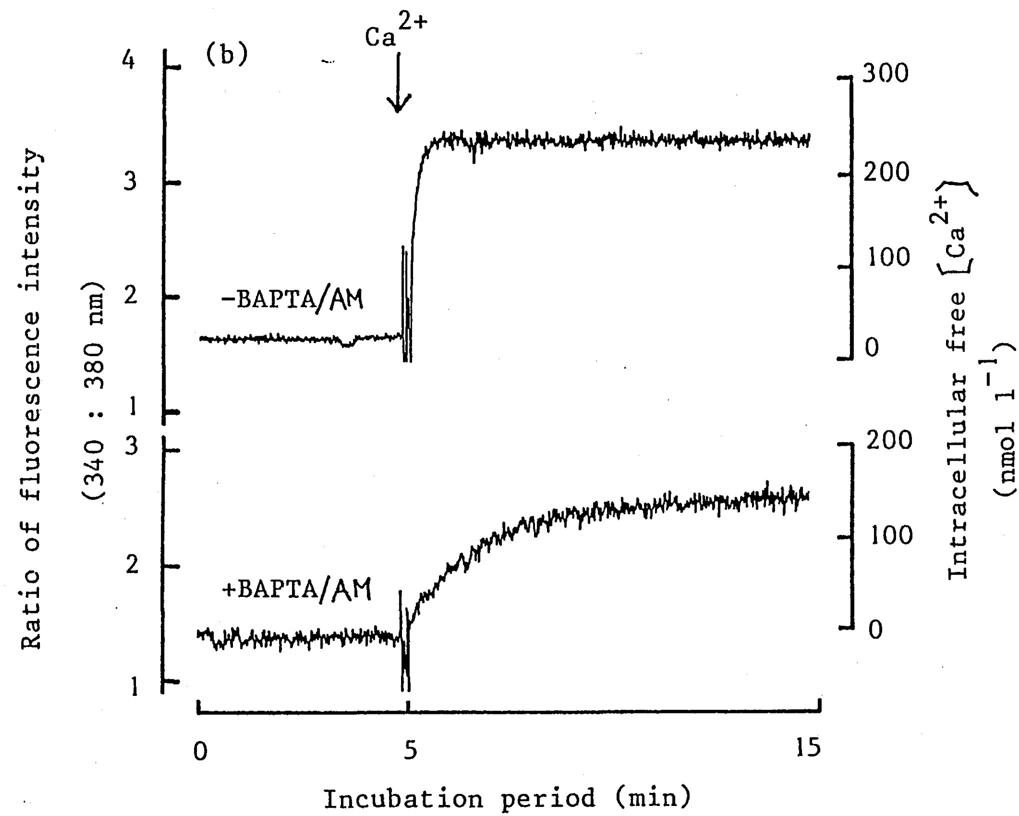

Fig. 2b

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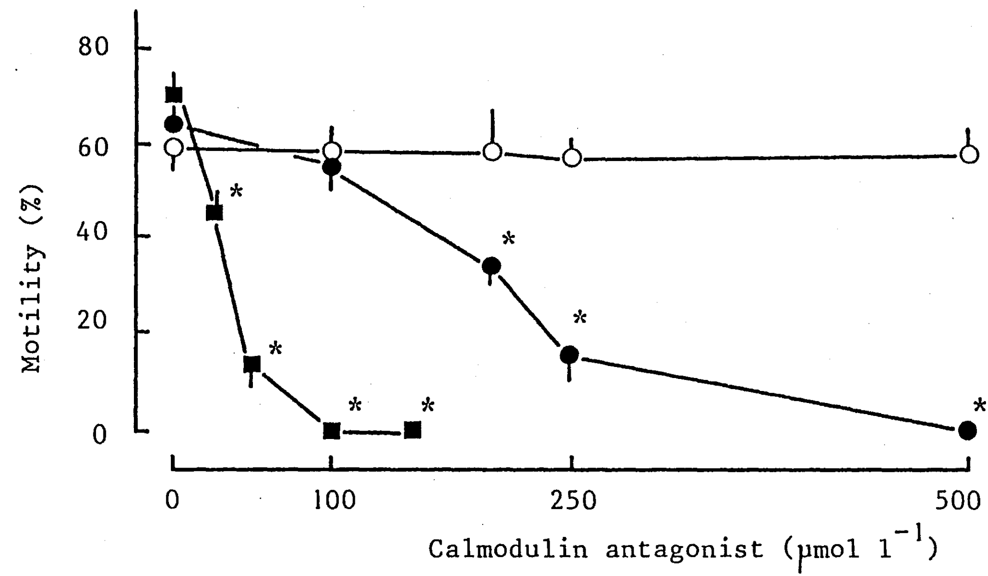

Fig. 3

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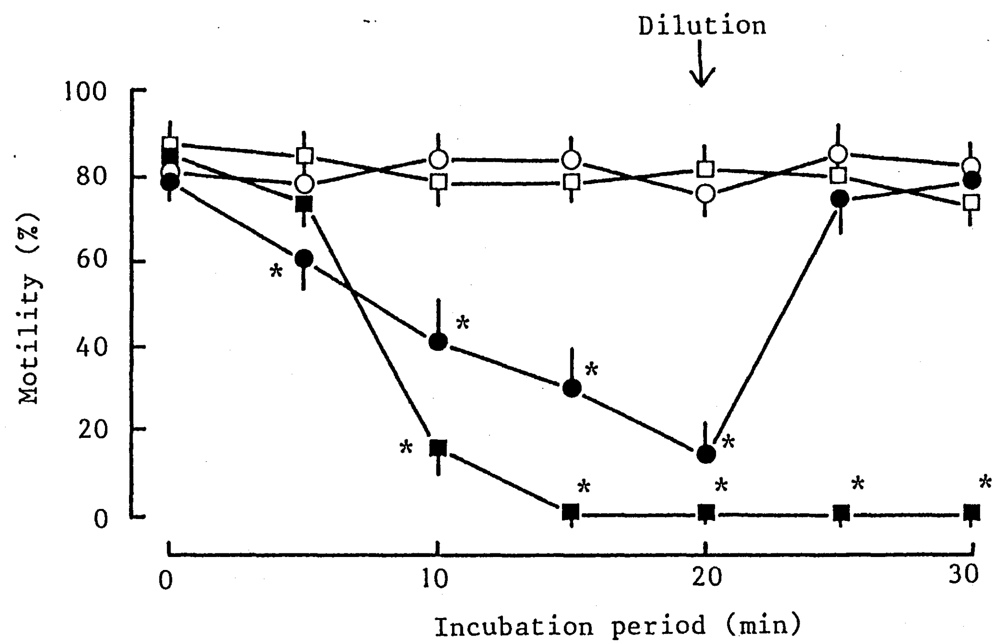


Fig. 4
Ashizawa et al.

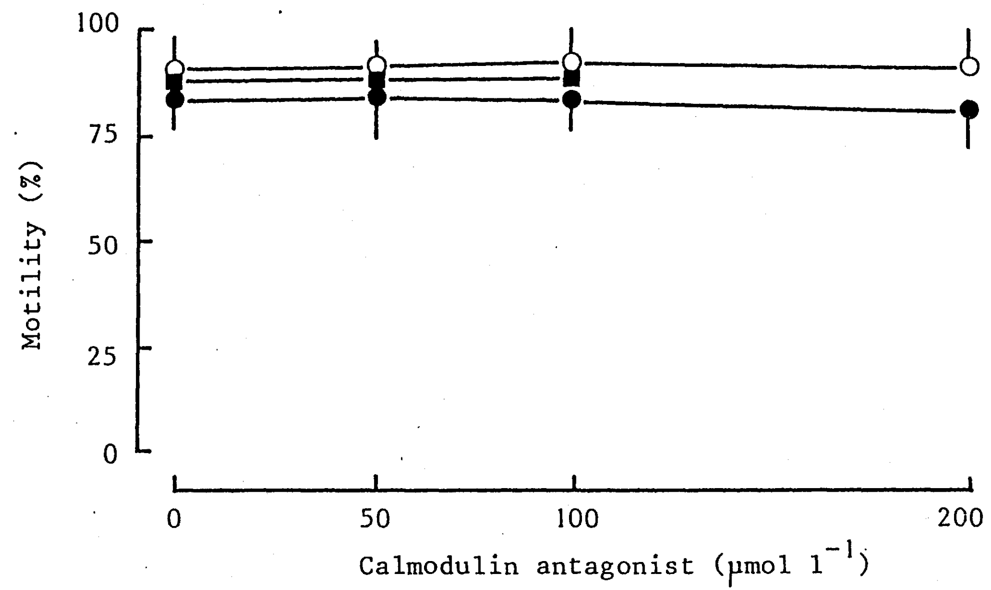


Fig. 5
Ashizawa et al.

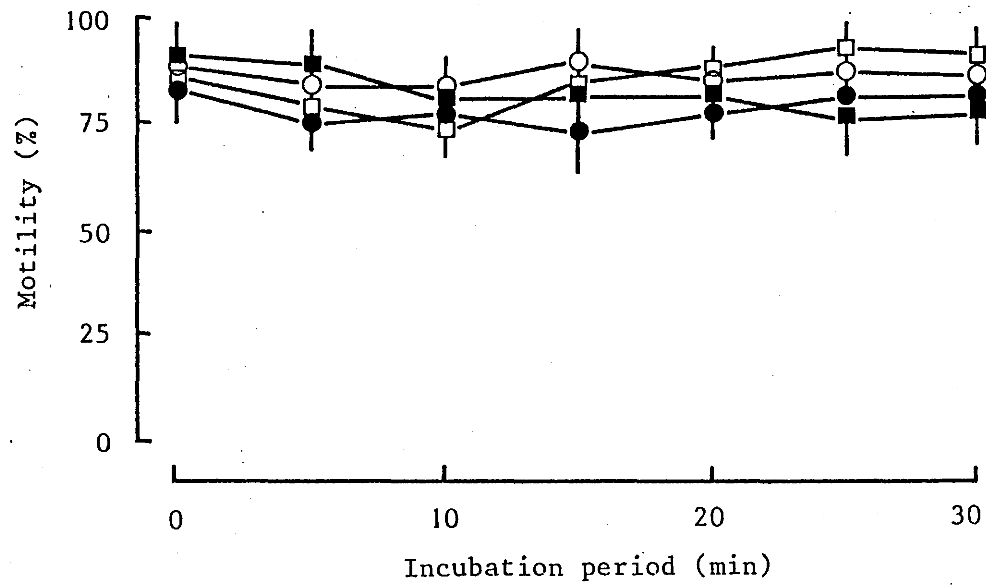


Fig. 6
Ashizawa et al.

(a)

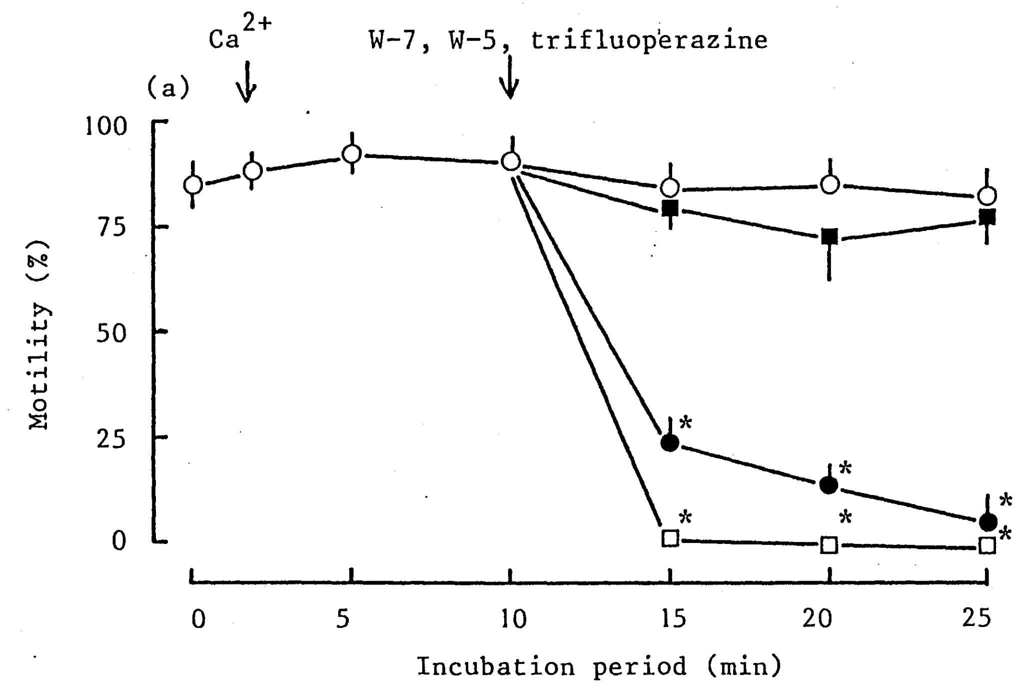


Fig 7a
Ashizawa et al.

(b)

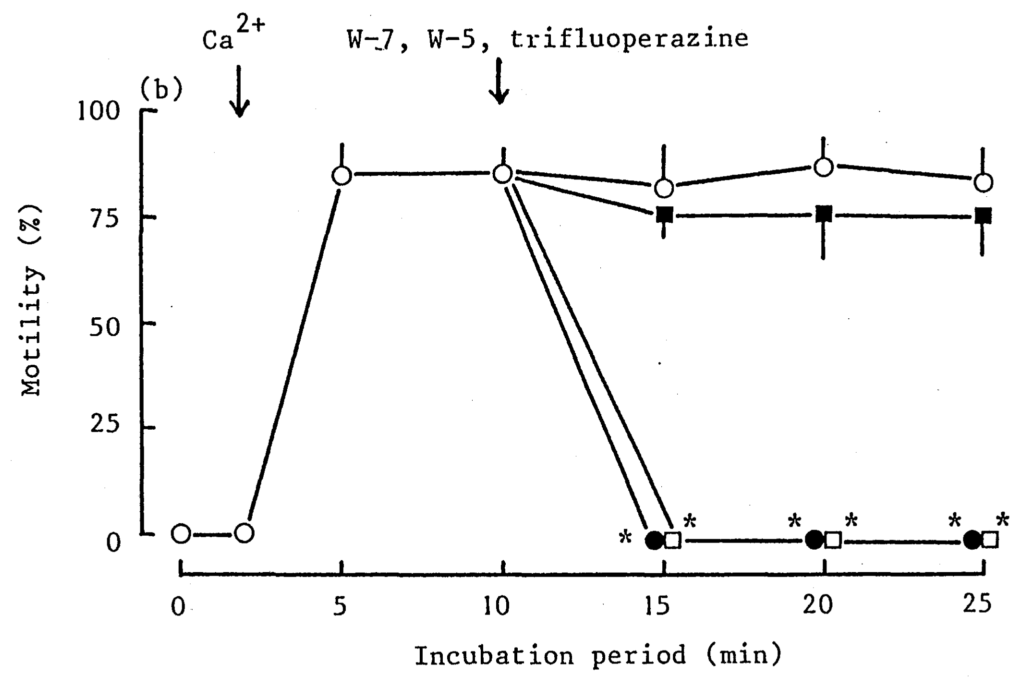


Fig. 7b
Ashizawa et al.

(C)

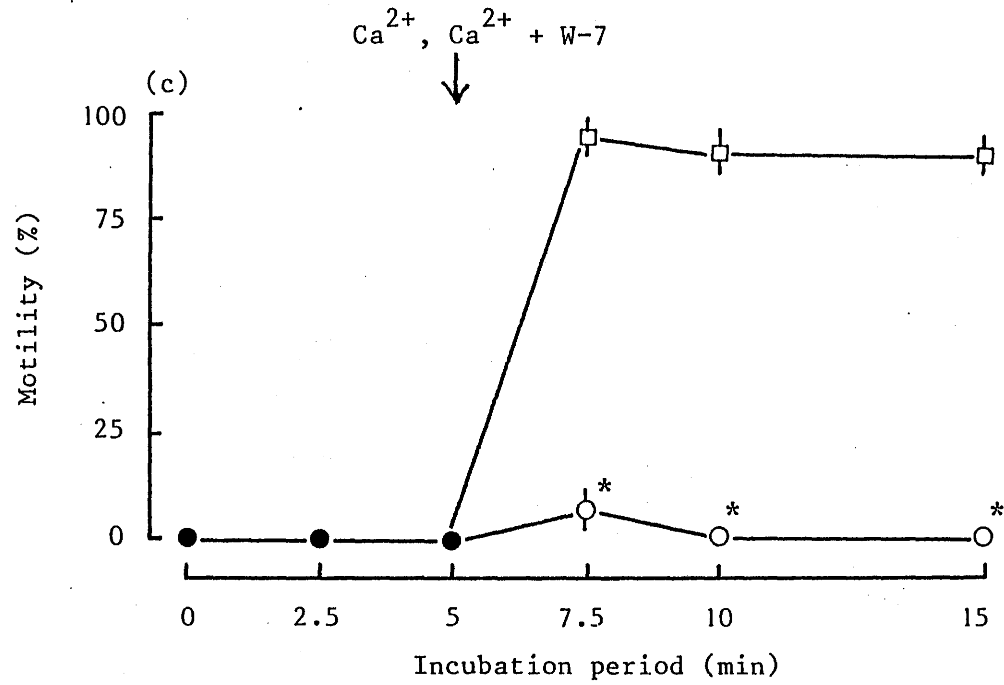
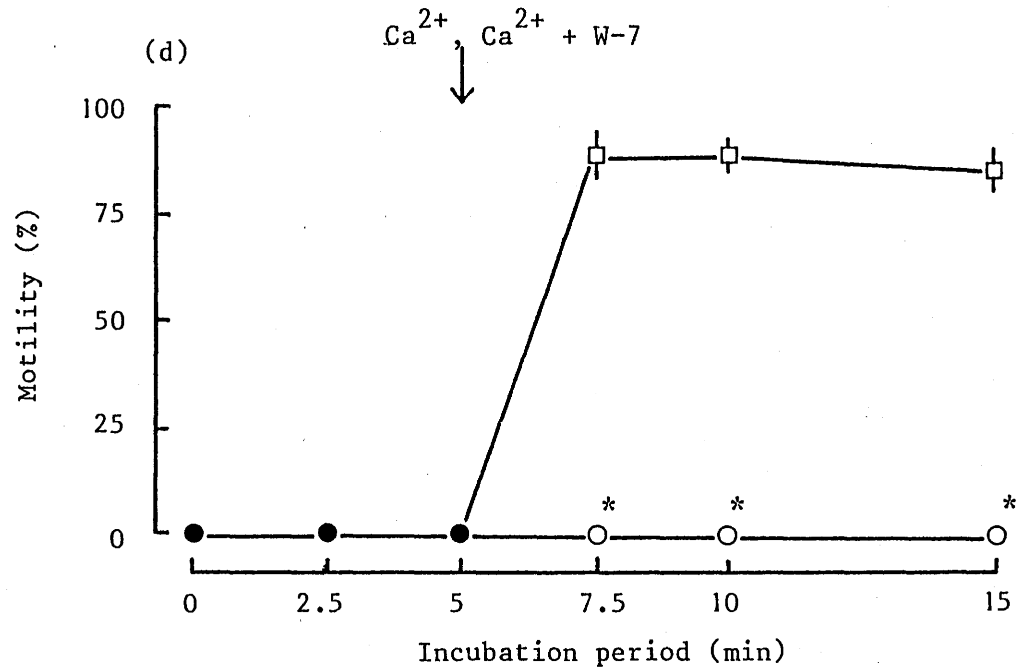


Fig. 7c
Ashizawa et al.

(d)



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Ashizawa et al.

(a)

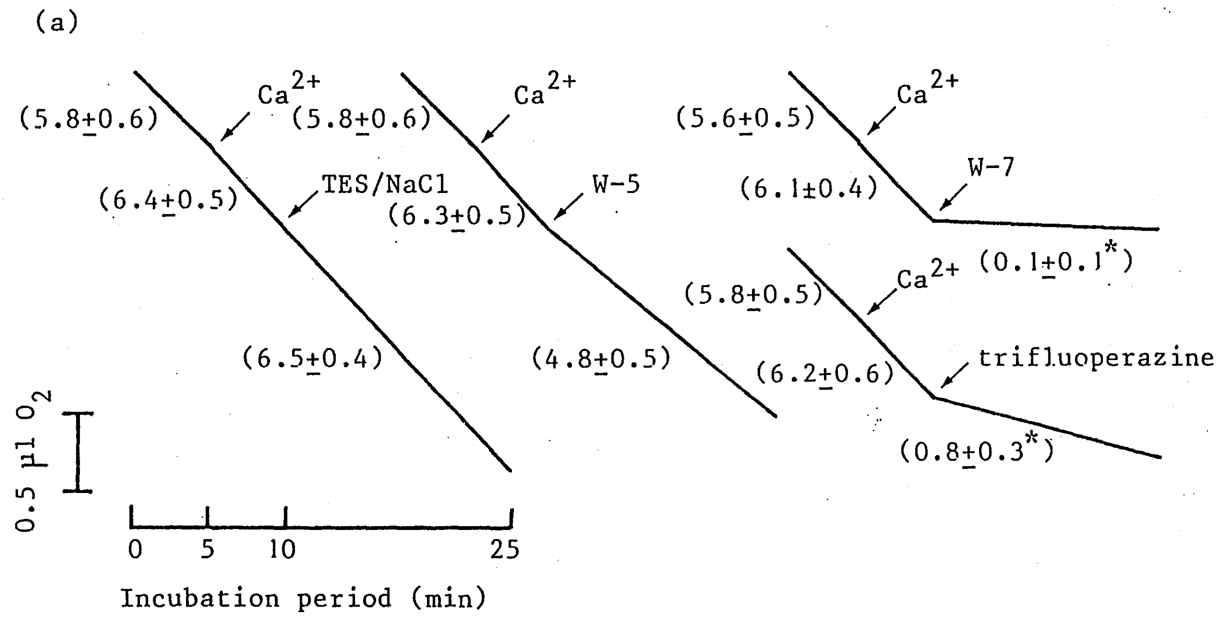

Fig. 8a
Ashizawa et al.

(b)

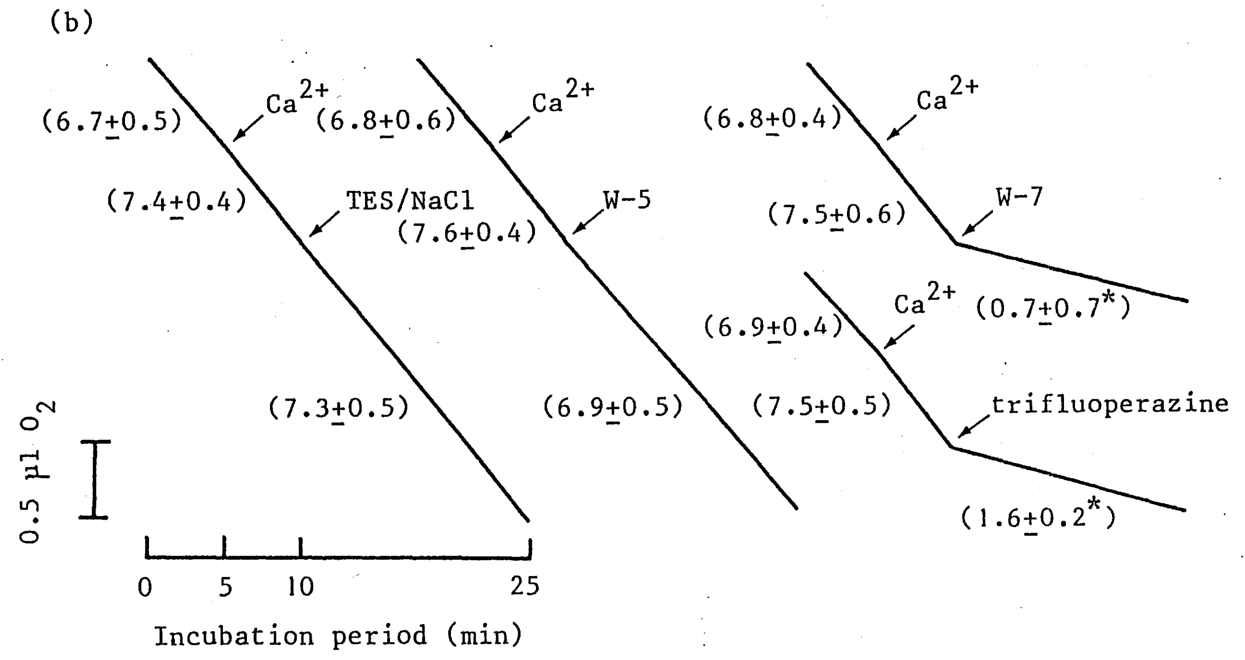

Fig. 8b

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(a)

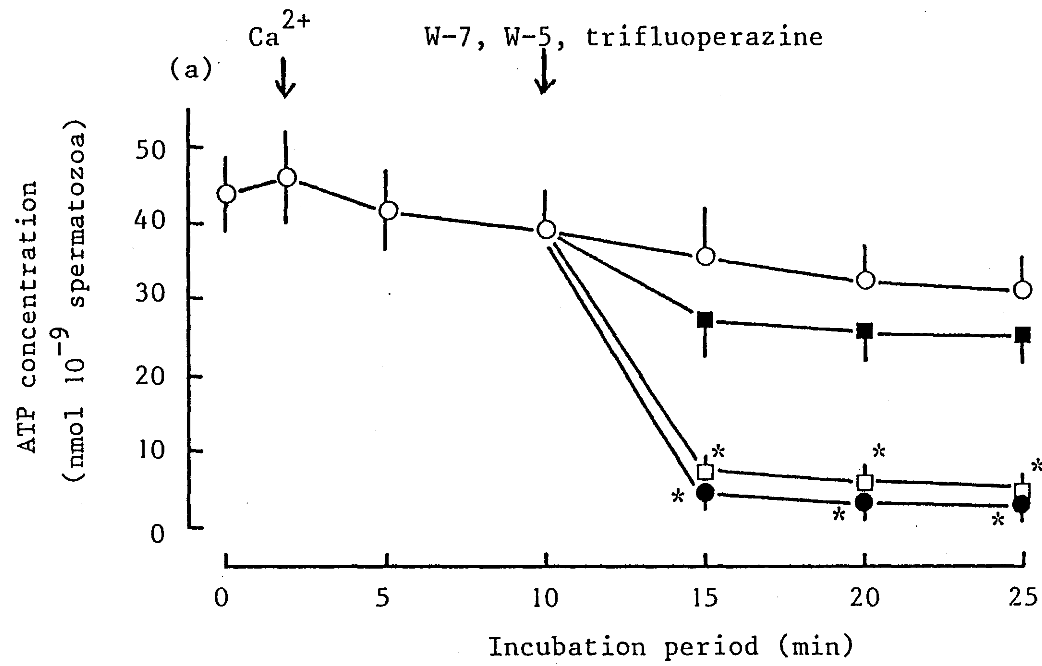

Fig. 9a
Ashizawa et al.

(b)

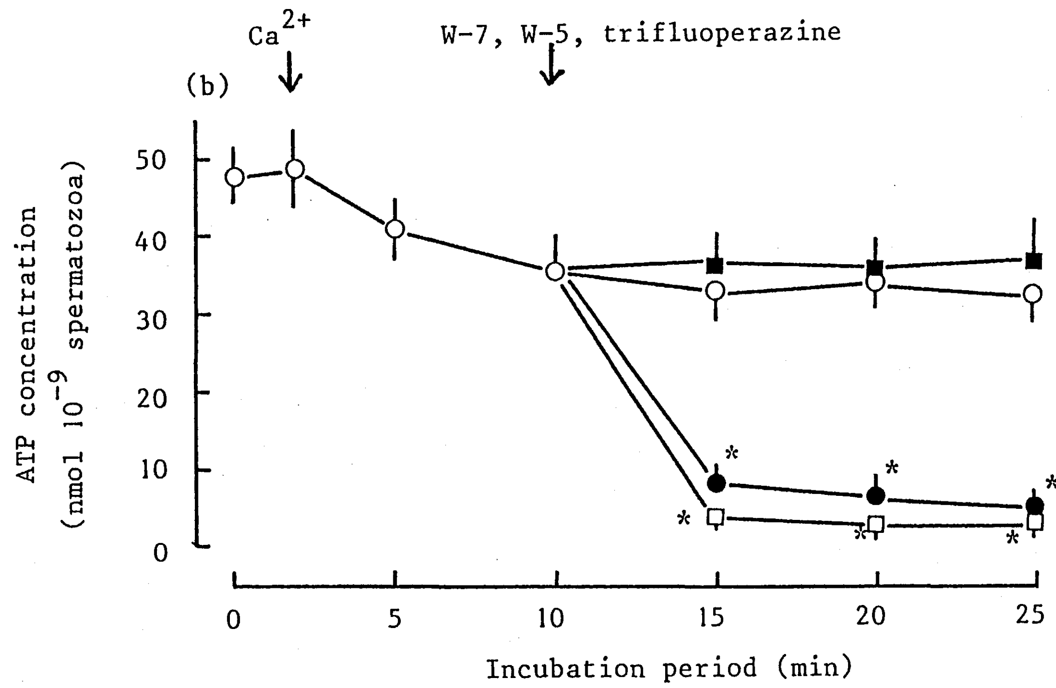

Fig. 9b

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