Stimulation of motility and respiration of intact fowl spermatozoa by calyculin A, a specific inhibitor of protein phosphatase-1 and -2A, via a Ca^{2+} -dependent mechanism

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Intact fowl spermatozoa became almost immotile at 40°C. In contrast, the presence of 10–1000 nmol calyculin A l^{-1} , a specific inhibitor of protein phosphatase-1 (PP1) and -2A (PP2A), permitted activation of sperm motility in a dose-dependent manner. Calyculin A also stimulated the rate of oxygen consumption by spermatozoa, and induced a concomitant decrease in ATP concentrations, suggesting a coupling of ATP hydrolysis to the rate of oxidative phosphorylation. However, the motility and oxygen consumption of spermatozoa loaded with an intracellular Ca^{2+} chelator, 1,2-bis (2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, tetraacetoxymethyl ester (BAPTA/AM), were not stimulated by calyculin A alone, but only after the subsequent addition of 2 mmol CaCl_2 l^{-1} . These results suggest that inhibition of the activities of endogenous PP1 and PP2A may stimulate the motility and metabolic activity of fowl spermatozoa at 40°C via a mechanism that requires intracellular free Ca^{2+} .

Introduction

Unlike mammalian spermatozoa, fowl spermatozoa show definitive temperature-dependent changes in their motility. In most synthetic diluents, they become immotile at the avian body temperature of 40–42°C, and motility is restored by decreasing the temperature (Munro, 1938; Ashizawa and Nishiyama, 1978; Takeda, 1982; Ashizawa and Okauchi, 1984; Wishart and Ashizawa, 1987; Ashizawa *et al.*, 1989a, b; Thomson and Wishart, 1989, 1991; Ashizawa *et al.*, 1992a). Munro (1938) suggested that avian spermatozoa may be immotile during most of their sojourn in the female reproductive tract. This view is supported by the observation that in the uterovaginal sperm storage tubules, where avian spermatozoa can remain for several weeks before fertilization, spermatozoa appear to be immotile (Bakst, 1987). This immotility may be useful for the prolonged survival of spermatozoa in the tubules (Ashizawa and Nishiyama, 1978). In addition, sperm motility does not seem to be required in domestic hens for the movement of spermatozoa within the oviduct from the shell gland (uterus) to the magnum, as dead spermatozoa or carbon powder inserted in the shell gland are transported along the reproductive tract (Mimura, 1939). However, it has been shown that only motile spermatozoa can traverse from the posterior to the anterior vagina (Takeda, 1974), and it has been suggested that factors within seminal plasma, deposited with the spermatozoa, may enhance intravaginal sperm transport (Terada *et al.*, 1984). Two factors that stimulate sperm motility at 40°C, Ca^{2+} and an unidentified regulator of low M_r , have

been identified in seminal plasma (Ashizawa and Wishart, 1987). Furthermore, Ashizawa and Wishart (1992) demonstrated that the fluid that collects in the ovarian pocket at the time of ovulation contains factors similar to those found in seminal plasma, and suggested that the sperm motility-stimulating factors may be implicated in promoting fertilization in domestic hens even at 40°C.

In addition to such extracellular stimulating signals, reversible phosphorylation of intracellular proteins on serine, threonine and tyrosine residues by protein kinases and phosphatases is widely accepted as a principal mechanism by which eukaryotic cells respond to extracellular signals. Many protein kinases and phosphatases have multiple substrates *in vivo*, enabling a diversity of responses to these extracellular stimuli (for a review, see Cohen, 1989; Holmes and Boland, 1993; Hubbard and Cohen, 1993). With regard to sperm motility, protein phosphorylation by cAMP-dependent protein kinase seems to play a primary role in the second messenger regulatory mechanisms of flagellar axoneme-based movement in a variety of species except fowl (for a review, see Tash and Means, 1983; Brokaw, 1987; Lindemann and Kanous, 1989; Majumder *et al.*, 1990; Mohri, 1993; Morisawa, 1994).

However, a new generation of protein phosphatase inhibitors, such as okadaic acid and calyculin A, isolated from various microorganisms, have proved extremely useful for understanding the role of protein dephosphorylation by phosphatases (Cohen *et al.*, 1990). For example, it has been demonstrated that the contraction of chemically skinned smooth muscle fibres is enhanced by the addition of okadaic acid or calyculin A (Ozaki *et al.*, 1987a, b; Takai *et al.*, 1987; Suzuki and Itoh, 1993), although calyculin A is effective at

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lower concentrations than is okadaic acid (Hartshorne *et al.*, 1989). Since calyculin A has a potency similar to that of okadaic acid as an inhibitor of protein phosphatase-2A (PP2A), but is 10–100 times more effective as an inhibitor of protein phosphatase-1 (PP1) (Ishihara *et al.*, 1989a), it is suggested that PP1 is a major myosin phosphatase *in vivo* (Cohen, 1989).

The treatment of human spermatozoa with calyculin A allows them to undergo the acrosome reaction sooner, suggesting that dephosphorylation by protein phosphatases, probably PP1, may be involved in the regulation of capacitation (Furuya *et al.*, 1993). Furthermore, it has been proposed that PP1 may be a dominant regulator for temperature-dependent flagellar movement of fowl spermatozoa since, in addition to calyculin A and okadaic acid, inhibitors 1 and 2, which are specific inhibitor proteins of PP1 (Cohen, 1989), also stimulate the motility of demembrated spermatozoa at 40°C (Ashizawa *et al.*, 1994a). However, limited information is available concerning the effects of PP1 and PP2A on the metabolic activity of spermatozoa, or that of other cells such as smooth muscle.

In the following experiment, therefore, attempts were made to investigate the effects of calyculin A, a specific inhibitor of PP1 and PP2A, on the motility, metabolic activity and Ca^{2+} flux of intact 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 a photoperiod of 14 h light:10 h dark.

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-aminoethanesulfonic 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

Calyculin A was purchased from Wako Pure Chemical Industries, Ltd (Osaka) 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) was obtained from Dojindo Laboratories Inc. (Kumamoto). Adenosine 5'-triphosphate (ATP), Cremophor EL, TES, Triton X-100 and desiccated firefly tails were purchased from Sigma Chemical Co. (St Louis, MO). Other chemicals were of reagent grade from Nacalai Tesque Inc. (Kyoto).

Analysis of sperm motility

Sperm samples were preincubated aerobically in a shaking water bath at 40°C for 10 min. The dose–response relationship of motility of intact spermatozoa in TES–NaCl buffer was then measured at 40°C after the addition of various concentrations of calyculin A. The suspension of spermatozoa was placed in a microscope chamber (Sekisui Chemical Co., Tokyo, UR-157 type, 0.07 mm in depth) 25 min after the start of incubation, and the motility of spermatozoa was recorded by videomicroscopy (magnification on the 12-inch black and white monitor was approximately $\times 600$) at 40°C on a thermostatically controlled warm plate (Katz and Overstreet, 1981). Measurements to determine the percentage motility were made on 200–300 spermatozoa, distributed uniformly among the three or more fields.

For BAPTA/AM-treated experiments, intact spermatozoa were loaded with BAPTA/AM by the methods described by Ashizawa *et al.* (1994b). Briefly, sperm suspension (0.1 ml) 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 $\mu\text{mol l}^{-1}$ and $0.9 \times 10^8 \text{ ml}^{-1}$, 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 controls. After the 10 min preincubation at 40°C, the sperm preparations with or without BAPTA/AM treatment were incubated aerobically in a shaking water bath at 40°C. At 3 and 15 min after the start of incubation, 500 nmol calyculin A l^{-1} and 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ were added, respectively. During incubation of spermatozoa, the motility was assayed as described above.

Analysis of oxygen consumption and ATP concentrations

The 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., Yellow Springs, OH). The rate of oxygen consumption was expressed in terms of $\mu\text{l O}_2$ consumption 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).

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

Analysis of intracellular free Ca^{2+} concentrations

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.*, 1992b). Fluorescence intensity was measured with a dual-wavelength spectrofluorometer (Shimadzu, Model RF-5000, Kyoto) with the sample chamber regulated 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, and the emission wavelength was set to 500 nm. The

ratio (340:380 nm) of fluorescence intensity was monitored continuously. The calculation of intracellular free Ca^{2+} was based upon the equation described by Grynkiewicz *et al.* (1985), where a dissociation constant (K_d) was 224 nmol l^{-1} .

Statistical analysis

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

Results

Effects of calyculin A on the motility and metabolic activity of intact fowl spermatozoa

The motility of intact spermatozoa was markedly affected by the addition of calyculin A at 40°C . As the concentration of calyculin A was increased, the motility was stimulated gradually and maximum motility was obtained at a concentration of 250 nmol l^{-1} (Fig. 1a).

As with the stimulation of motility, the rate of oxygen consumption also increased with increasing concentrations of calyculin A at 40°C . Conversely, the ATP concentrations decreased gradually in a dose-dependent manner, suggesting that spermatozoa used endogenous ATP due to the initiation of motility (Fig. 1b).

Effects of calyculin A on the motility and metabolic activity of BAPTA/AM-treated fowl spermatozoa

At 40°C , neither control nor BAPTA/AM-treated spermatozoa were motile. The motility of control spermatozoa was restored by the addition of calyculin A alone. However, the motility of spermatozoa loaded with BAPTA/AM, an intracellular Ca^{2+} chelator, could not be restored even in the presence of calyculin A until the subsequent addition of $2 \text{ mmol CaCl}_2 \text{ l}^{-1}$ 15 min after the start of incubation (Fig. 2a).

The concentration of ATP in control spermatozoa decreased significantly ($P < 0.01$) after the addition of calyculin A. In contrast, there was no significant decrease in BAPTA/AM-treated spermatozoa, but the concentration of ATP decreased slightly after the addition of CaCl_2 (Fig. 2b).

The rates of oxygen consumption of spermatozoa treated or untreated with BAPTA/AM at 40°C were significantly different ($P < 0.05$); 3.6 ± 0.3 and $5.0 \pm 0.3 \mu\text{l O}_2 \cdot 10^{-8} \text{ spermatozoa h}^{-1}$, respectively. Furthermore, the rate of oxygen consumption of control preparations increased significantly ($P < 0.01$) after the addition of calyculin A. In contrast, the oxygen consumption of BAPTA/AM-treated preparations could not be stimulated by the addition of calyculin A alone. However, the subsequent addition of $2 \text{ mmol CaCl}_2 \text{ l}^{-1}$ stimulated the oxygen consumption significantly ($P < 0.01$) compared with the value before the addition of calyculin A or CaCl_2 (Fig. 2c).

Effects of calyculin A on the intracellular free Ca^{2+} concentration of fowl spermatozoa

Intracellular free Ca^{2+} concentrations, measured by means of a fluorescent Ca^{2+} indicator Fura-2, increased rapidly after the

Fig. 1. (a) Motility and (b) oxygen consumption (○) and ATP concentration (●) of intact fowl spermatozoa after addition of various concentrations of calyculin A at 40°C . Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value at 0 nmol l^{-1} (control).

addition of $2 \text{ mmol CaCl}_2 \text{ l}^{-1}$ at 40°C . Subsequently, the addition of calyculin A maintained almost the same Ca^{2+} concentration in the spermatozoa as that of the control (no addition of calyculin A) (Fig. 3a).

The intracellular free Ca^{2+} concentration of BAPTA/AM-treated spermatozoa before the addition of CaCl_2 was almost 0 nmol l^{-1} , even in the presence of calyculin A. However, the addition of $2 \text{ mmol CaCl}_2 \text{ l}^{-1}$ caused an influx of Ca^{2+} from the medium and the Ca^{2+} concentrations increased gradually (Fig. 3b).

Discussion

Generally, little information is available on the effects of PP1 and PP2A on the metabolic activity of cells. In the present study, the presence of calyculin A, a specific inhibitor of PP1 and PP2A, stimulated the rate of oxygen consumption and the motility of intact fowl spermatozoa in a dose-dependent manner at 40°C . These results suggest that inhibition of endogenous PP1 and PP2A activities stimulate the motility and respiration of fowl spermatozoa. Furthermore, it is assumed that the stimulation of respiration by calyculin A probably follows from the increase of motility, since calyculin A also stimulated the motility of demembrated spermatozoa at 40°C (Ashizawa *et al.*, 1994a). Thus, the stimulatory effect of

Fig. 2. (a) Motility and (b) ATP concentration of intact (○) and intracellular Ca^{2+} chelator BAPTA/AM-treated (●) fowl spermatozoa after addition (at arrows) of 500 nmol calyculin A l^{-1} and 2 mmol CaCl_2 l^{-1} at 40°C. Each point represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value at 0 min. (c) Oxygen consumption ($\mu\text{l O}_2$ 10^{-8} spermatozoa h^{-1}) of intact and BAPTA/AM-treated fowl spermatozoa at 40°C after addition of 500 nmol calyculin A l^{-1} and 2 mmol CaCl_2 l^{-1} . Each value represents the mean ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value before the addition of calyculin A.

calyculin A on motility may be primarily the result of direct action on the axoneme, probably with coupling of ATP hydrolysis by the various types of ATPases (including dynein ATPase) to the rate of oxidative phosphorylation and respiration.

The motility and respiration of fowl spermatozoa are strongly influenced by their intracellular free Ca^{2+} concentrations (Thomson and Wishart, 1989, 1991; Ashizawa *et al.*, 1992b, 1994b). The present study shows that calyculin A stimulated the motility and rate of oxygen consumption of control spermatozoa, but not spermatozoa loaded with the intracellular Ca^{2+} chelator, BAPTA/AM. However, the subsequent addition of 2 mmol CaCl_2 l^{-1} stimulated both parameters significantly ($P < 0.01$). Thus, it appears that calyculin A stimulates sperm motility via a Ca^{2+} -dependent mechanism.

At the axonemal level, demembranated fowl spermatozoa, stimulated by calyculin A at 40°C, lost their motility after the addition of 1 mmol Ca^{2+} l^{-1} to the reactivation medium. When the Ca^{2+} concentration was varied from 10 nmol to 100 $\mu\text{mol l}^{-1}$ by the subsequent addition of various concentrations of EGTA, motility was restored in inverse proportion to the Ca^{2+} concentration (Ashizawa *et al.*, 1994a). Initially, it might seem likely that these results are inconsistent with those of intact spermatozoa described above. However, in the present work, the intracellular free Ca^{2+} concentration of intact fowl spermatozoa (measured by means of a fluorescent Ca^{2+} indicator, Fura-2) was approximately 50 nmol l^{-1} after the addition of calyculin A in BAPTA/AM-untreated spermatozoa, and maximally 200 nmol l^{-1} after the addition of both calyculin A and Ca^{2+} in BAPTA/AM-treated spermatozoa (Fig. 3b). These values were similar to those from preparations in which demembranated spermatozoa showed higher motility (Ashizawa *et al.*, 1994a). Therefore, it is suggested that Ca^{2+} at nanomolar concentrations is necessary for the stimulation of motility induced by calyculin A in both intact and demembranated fowl spermatozoa, but such a stimulatory effect may not occur at zero or millimolar Ca^{2+} concentration.

It has been proposed that calyculin A is involved in the activation of the voltage-dependent Ca^{2+} channels in intact smooth muscle cells, although this effect appears to be independent of the calyculin A-induced contraction (Ishihara *et al.*, 1989b). Klumpp *et al.* (1990) suggest that the voltage-dependent Ca^{2+} channels on the ciliary membrane of the protozoan *Paramecium* are inactivated by a dephosphorylation event, and that okadaic acid blocks this dephosphorylation, inducing Ca^{2+} influx and the reversal of ciliary beat and initiating backward swimming. If there is such action in calyculin A or okadaic acid, intracellular free Ca^{2+} concentration should increase after the addition of such inhibitors. However, intracellular free Ca^{2+} concentration in fowl spermatozoa did not increase even in the presence of calyculin A, compared with controls (Fig. 3a). A possible explanation for these results is that there is either an alternative mechanism in the regulation of Ca^{2+} channels of fowl spermatozoa or that both a Ca^{2+} ATPase-dependent pump system and Ca^{2+} channels are activated by calyculin A, resulting in a balance of influx and efflux of Ca^{2+} . Further investigations of Ca^{2+} mobilization are required.

Fig. 3. Changes of intracellular free Ca^{2+} concentrations in intact fowl spermatozoa (a) with or without calyculin A at 40°C , and (b) with or without BAPTA/AM at 40°C , measured by means of a fluorescent Ca^{2+} indicator Fura-2. Arrows indicate the addition of 2 mmol $\text{CaCl}_2 \text{ l}^{-1}$ and 500 nmol calyculin A l^{-1} .

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