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

K. Ashizawa, S. Katayama, T. Kobayashi and Y. Tsuzuki

Laboratory of Animal Reproduction, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan

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 crossreacting protein of approximately 80 kDa. As the concentration of the protein kinase C activators *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9) or 1-oleoyl-2-acetyl glycerol (OAG) was increased, the motility of intact spermatozoa at 30°C was reduced. However, this inhibition of motility was reversed by reducing the concentrations of activators. Even in the presence of 1 mmol $\text{CaCl}_2 \text{ l}^{-1}$, 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. However, 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 in the presence or absence 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 or the membrane, but is not retained in the axoneme, and that 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 cyclic AMP (cAMP) are second messengers, additional regulatory control is gained by protein kinase C exerting either a positive forward effect or negative feedback control, or both (Nishizuka, 1986). The protein kinase C family, a Ca^{2+} -dependent and phospholipid-dependent kinase, consists 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 analogues, activators of protein kinase C, induce the acrosomal reaction in human spermatozoa (De Jonge *et al.*, 1991), enhance the zona-pellucida-induced acrosomal reaction in mouse spermatozoa (Lee *et al.*, 1987; Endo *et al.*, 1991) and stimulate 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 possible 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). 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). It is therefore 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, 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 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 4–6 males were diluted by a factor of approximately 10 in 150 mmol NaCl l^{-1} with 20 mmol *N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid l^{-1} (TES) 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) 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). ATP, 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 was obtained from Vector Laboratories (Burlingame, CA). Molecular weight standards for SDS-PAGE were purchased from Life Technologies, Inc. (Gaithersburg, MD). 1-Oleoyl-2-acetyl glycerol (OAG) and other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto).

Antisera

A rabbit serum raised against a synthetic peptide constituting amino acids 543–550 of protein kinase C_{consensus} (a polyclonal antibody) was purchased from Seikagaku Co., Ltd. Biotinylated anti-rabbit immunoglobulin G goat serum was obtained from MBL Co., Ltd (Nagoya).

Western immunoblot analysis of protein kinase C

Spermatozoa that had been washed as described above were mixed with equal volumes of concentrated ($\times 2$) Laemmli

(1970) sample buffer and were boiled for 5 min. Samples containing approximately 50 μg protein were loaded onto a 10% SDS-polyacrylamide slab gel, and subjected to electrophoresis. Western blotting was performed according to the protocol of Towbin *et al.* (1979), with some modifications. Briefly, the proteins were transferred electrophoretically to a polyvinylidene difluoride membrane sheet (AE-6660 Atto Co., Ltd, Tokyo). After transfer, nonspecific sites on the membranes were blocked by incubating them for 1 h at room temperature (20–25°C) in PBS containing 5% skimmed milk powder. The blots were then incubated overnight at 4°C with the 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 immunoglobulin G goat serum (1:200 dilution with 1% BSA in PBS) and then avidin-biotin peroxidase complex. After 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 preincubated aerobically in a shaking water bath at 30°C or 40°C for 10 min. After the preincubation, the dose-response and time course of motility of intact spermatozoa were measured at 30°C or 40°C after the addition of various concentrations of the protein kinase C activators SC-9 and OAG or the 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 the TES-NaCl buffer described above.

Demembranation and reactivation of spermatozoa were performed at 30°C and 40°C according to the method described by 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; type UR-157) 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 according to the method of Grynkiewicz *et al.* (1985), with some

Fig. 1. Immunoblot analysis of fowl sperm protein kinase C. Lane 1: incubation with antibody against a synthetic peptide constituting amino acids 543–550 of protein kinase C_{consensus}; lane 2: control.

modifications (Ashizawa *et al.*, 1992a). Fluorescence intensity was measured using a dual-wavelength spectrofluorimeter (Shimadzu, Model RF-5000, Kyoto), with the sample chamber kept at 30°C or 40°C by a circulating water jacket and the sperm suspension 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 (340:380 nm) 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 dissociation 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 the control lane (no antibody). In contrast, a protein of approximately 80 kDa was recognized by anti-protein kinase C antibody, even though the visible immunoreactive band was faint (Fig. 1).

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 ($\pm$ SEM) of five samples of spermatozoa. * $P < 0.01$ compared with value at $0 \text{ } \mu\text{mol l}^{-1}$.

Effects of protein kinase C activators and inhibitor on the motility of intact and demembrated 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 – $1000 \text{ } \mu\text{mol l}^{-1}$ (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).

Figure 2 shows the motility of spermatozoa 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: motility could be restored by diluting the sperm samples by a factor of approximately 8 in the activator-free assay medium.

However, inhibition of motility by the addition of SC-9 and OAG was not observed in demembrated and reactivated spermatozoa at 30°C: the motility of spermatozoa treated with

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 (by a factor of approximately 8) 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.

these activators was similar to that of spermatozoa subjected to control conditions and to H-7 treatment (Table 1).

Even the presence of Ca²⁺ 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 immediately after the addition of 1 mmol CaCl₂ 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 under control conditions or by the addition of H-7 at 30°C or 40°C.

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

Substances	Motility (%)	
	30°C	40°C
None (control)	64.0 ± 4.3	2.2 ± 1.0
SC-9 (20 μmol l ⁻¹)	69.5 ± 6.5	1.6 ± 1.0
OAG (150 μmol l ⁻¹)	54.4 ± 4.4	1.2 ± 0.7
H-7 (500 μmol l ⁻¹)	59.9 ± 1.6	3.4 ± 1.1

Each value represents the mean (± SEM) of five samples of spermatozoa. There were no significant differences between values within columns. SC-9: *N*-(6-phenylhexyl)-5-chloro-1-naphthalene sulfonamide; OAG: 1-oleoyl-2-acetylgllycerol; H-7: 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride.

Fig. 4. Motility of intact fowl spermatozoa after addition (at arrows) of (○) 1 mmol CaCl₂ 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 when no drug was added (control) at each period.

Effects of protein kinase C activators and inhibitor on the intracellular free Ca²⁺ concentrations of intact fowl spermatozoa

Intracellular free Ca²⁺ concentrations of intact spermatozoa increased rapidly after the addition of 1 mmol CaCl₂ l⁻¹; if no drug was added, it 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 in Ca²⁺

Fig. 5. Changes in intracellular free concentrations of Ca^{2+} in fowl spermatozoa at 30°C measured by fura-2 fluorescence after addition (at arrows) of $1\text{ mmol CaCl}_2\text{ l}^{-1}$, $20\text{ }\mu\text{mol SC-9 l}^{-1}$, $150\text{ }\mu\text{mol OAG l}^{-1}$ and $500\text{ }\mu\text{mol H-7 l}^{-1}$. TES-NaCl: incubation buffer (control).

concentrations. In contrast, no change in the intracellular free Ca^{2+} concentration was observed in spermatozoa treated with H-7 (Fig. 5). Similar results were obtained at 40°C (data not shown).

Discussion

In the study reported here, immunoblot analysis of fowl sperm proteins showed that a protein of approximately 80 kDa was recognized by an antibody to part of protein kinase C. This molecular mass corresponds to that detected by immunoblotting various tissue extracts of the rat using an antibody to protein kinase C (Huang and Huang, 1986). These authors also suggested that the immunoreactive determinants for protein kinase C from 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 are approximately 80 kDa for the α and β subspecies of protein kinase C. It is therefore possible that protein kinase C may be present in fowl spermatozoa, even though it occurs in relatively low amounts.

Rotem *et al.* (1990a, b) demonstrated that the addition of protein kinase C activators, such as the potent tumour promoter phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) and the membrane-permeable diacylglycerol analogue OAG, stimulated human sperm motility in a dose-dependent 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 of motility by the addition of $100\text{ }\mu\text{mol TPA l}^{-1}$, although an incubation period of 60 min was needed for complete inhibition (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 on that of mammalian spermatozoa. However, such a difference is not surprising, given several lines of evidence pointing towards differential regulation of sperm motility in fowl and mammals. First, demembranated fowl spermatozoa can be motile even in the presence of millimolar concentrations of Ca^{2+} (Ashizawa *et al.*, 1989b), whereas in such high concentrations of Ca^{2+} , the motility of demembranated mammalian spermatozoa is inhibited (White and Voglmayr, 1986; Feng *et al.*, 1988). Second, 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).

The addition of H-7, a protein kinase C inhibitor, did not appreciably affect the motility of intact fowl spermatozoa at 30°C . These results suggest that fowl sperm motility may not be simply stimulated or inhibited by changes in protein kinase C activity. Perhaps a basic regulatory system of fowl sperm motility involves a phosphoprotein(s) that is dependent on protein kinase C and that is responsible for the inhibition of motility: when this protein(s) is phosphorylated more than a certain threshold amount by activating protein kinase C (e.g. by SC-9 or OAG), fowl sperm motility is inhibited. In contrast, when this protein(s) is dephosphorylated or phosphorylated less than a threshold amount (e.g. by the addition of H-7 or under control conditions) spermatozoa can retain their motility. However, in this study the target and precise mechanisms of action of protein kinase C remain to be elucidated. Further study is needed to examine which protein(s) is altered during the inhibition of fowl sperm motility by the activation of protein kinase C.

In general, both motility and metabolic activity of mammalian spermatozoa increase with increasing 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, detailed mechanisms of this phenomenon at the molecular level remain unsolved. In the study reported here, the motility of intact spermatozoa was inhibited by adding protein kinase C activators such as SC-9 and OAG

and was restored by reducing the concentrations of these 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. However, temperature-dependent immobilization was observed in demembranated spermatozoa as with intact spermatozoa, suggesting that the axoneme itself might be directly involved in this immobilization (Ashizawa *et al.*, 1989b).

Immunocytochemical analysis using monoclonal antibodies against protein kinase C indicates that intracellular localization of this enzyme varies with cell type; however, it is known that this enzyme is recovered mainly from the soluble fraction, and is apparently translocated to the 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 or the membrane, but is not retained in the axoneme, since inhibition of 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 straightforward. 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) motility. In the present study, 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 in intracellular free Ca^{2+} concentrations in fowl spermatozoa. However, this phenomenon is not a primary factor in the inhibition of sperm motility, since demembranated fowl spermatozoa could still be 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 proteins (Nishizuka, 1986). It is proposed that protein kinase C may have a role in upregulating Ca^{2+} entry by stimulating Ca^{2+} channels, as microinjection of TPA or protein kinase C itself into bag cell neurones from *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 activating the Ca^{2+} -ATPase and the Na^{+} - Ca^{2+} -ATPase (Nishizuka, 1988), as 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, it is suggested that protein kinase C increases intracellular free Ca^{2+} concentrations, although the precise mechanisms of such a process remain to be determined.

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