Regulation of acrosome reaction of fowl spermatozoa: evidence for the involvement of protein kinase C and protein phosphatase-type 1 and/or -type 2A

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Abstract

The signal transduction pathways involved in the regulation of the acrosome reaction and motility of fowl spermatozoa were investigated. The motility and acrosomal integrity of fowl spermatozoa in TES/NaCl buffer, with or without homogenised inner perivitelline layers (IPVL), prepared from laid fowl eggs, was almost negligible at 40 °C. In the presence of 2 mmol CaCl₂/l at 40 °C, motility became vigorous and the acrosome reaction was stimulated when IPVL was added. In the absence of Ca²⁺, motility was stimulated by the addition of calyculin A and okadaic acid, both specific inhibitors of protein phosphatase-type 1 (PP1) and -type 2A (PP2A), but Okadaic acid, which is a weaker inhibitor of PP1, did not completely restore motility at 40 °C. However, the acrosome reaction was significantly and equally stimulated in a dose-dependent manner by both inhibitors in the range of 10–1000 nmol/l, when spermatozoa were incubated with IPVL but without Ca²⁺. These inhibitors did not stimulate the acrosome reaction in the absence of IPVL. The vigorous motility of spermatozoa, stimulated by the addition of Ca²⁺, was reduced gradually as the concentrations of SC-9, a selective activator of protein kinase C (PKC), were increased and a similar SC-9-induced inhibition was observed in the acrosome reaction in the presence of Ca²⁺ and IPVL. These results confirm that IPVL is necessary for the activation of the acrosome reaction in fowl spermatozoa and that Ca²⁺ plays an important role in the stimulation of motility and acrosomal exocytosis. Furthermore, it appears that the intracellular molecular mechanisms for the regulation of acrosome reaction of fowl spermatozoa are different from those for the restoration of motility, i.e., protein dephosphorylation involving PP1 and/or PP2A in the former, and PP1 alone in the latter case. In addition, the activation of PKC may contribute to a decrease in the flagellar movement and acrosome reaction of fowl spermatozoa.

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Introduction

In birds, at fertilisation, access of the sperm plasma membrane to the oolemma is blocked by a glycoprotein layer, the inner perivitelline layer (IPVL), which surrounds the oocyte at ovulation (Bellairs *et al.* 1963, Kido & Doi 1988). This layer may be considered to be analogous to the mammalian zona pellucida. In fact, it was shown that an IPVL glycoprotein of approximately 34 kDa has a high degree of homology to mammalian zona pellucida protein-3 (Waclawek *et al.* 1998), which acts as the primary sperm-binding protein (Bleil & Wassarman 1980), and triggers the acrosome reaction in mice (Bleil & Wassarman 1983). Recently, it has been demonstrated that oligosaccharides isolated from fowl-IPVL glycoproteins are capable of inducing the acrosome reaction in fowl spermatozoa. Preparations

containing only O-linked glycans were unable to induce the acrosome reaction whereas N-linked oligosaccharides released from the IPVL by PNGaseF treatment could induce the acrosome reaction. Addition of galactose to terminal N-acetylglucosamine residues suppressed the acrosome reaction-inducing capacity of the oligosaccharide preparation; however, this capacity could be restored by co-incubation with β -galactosidase. Therefore, it is suggested that the acrosome reaction-inducing factor is probably an N-linked oligosaccharide with terminal N-acetyl-glucosamine residues (Horrocks *et al.* 2000). Although some aspects of the inducer of the acrosome reaction in fowl spermatozoa have been identified, much remains to be learned about how the mechanism of the acrosome reaction is regulated within fowl spermatozoa. The addition of extracellular Ca²⁺ is found to be an absolute requirement for acrosomal

exocytosis at the avian body temperature of 40 °C (Robertson 1999).

With regard to the control of sperm motility, unlike mammalian spermatozoa, fowl spermatozoa display the unique phenomenon of reversible temperature-dependent immobilisation: in simple salt solutions, they become immotile at 40 °C, but motility is restored by decreasing the temperature (Munro 1938, Ashizawa & Nishiyama 1978, Ashizawa & Wishart 1987, Wishart & Ashizawa 1987, Ashizawa *et al.* 1989). It has been recognised that this flagellar movement of fowl spermatozoa may be regulated by a protein phosphorylation–dephosphorylation system (Ashizawa *et al.* 2000). Protein phosphorylation by protein kinase C (PKC) seems to be involved in reducing the motility of fowl spermatozoa (Ashizawa *et al.* 1994a).

Furthermore, it has been proposed that dephosphorylation by the activation of protein phosphatase-type 1 (PP1), present in the fowl sperm axoneme and/or accessory cytoskeletal components may be involved in the inhibition of fowl sperm motility at 40 °C, since, in addition to calyculin A and okadaic acid, inhibitors 1 and 2, specific inhibitors of PP1 (Cohen 1989), also stimulated the motility of demembranated spermatozoa at 40 °C (Ashizawa *et al.* 1994b). The regulatory serine/threonine protein phosphatases are classified into four main enzymes: type 1 (PP1), type 2A (PP2A), type 2B (PP2B), and type 2C (PP2C) (Cohen 1989). PP2B appears to be involved in the regulation of the acrosome reaction of fowl spermatozoa, but not their flagellar movement at body temperature, since the addition of specific inhibitors of PP2B significantly stimulated the acrosome reaction, but did not activate motility at 40 °C (Ashizawa *et al.* 2004). However, limited information is available on the involvement of PKC, PP1 or PP2A in the regulation of the acrosome reaction of fowl spermatozoa. Thus, in the following experiments, attempts were made to investigate the effects of PKC activator or PP1 and PP2A inhibitors on the acrosome reaction and motility of fowl spermatozoa.

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 a photoperiod of 14 h light:10 h darkness.

Semen was collected by the method of Bogdonoff & Shaffner (1954). Samples of semen pooled from four to six males were diluted approximately tenfold in 150 mmol NaCl/l with 20 mmol TES (*N*-Tris-[hydroxymethyl]-methyl-2-aminoethanesulfonic acid)/l 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 6×10^8 cells/ml.

Chemicals

Calyculin A and okadaic acid, specific inhibitors of PP1 and PP2A, were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), a broad-based inhibitor of serine/threonine protein kinase, and *N*-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9), a selective activator of PKC, were obtained from Seikagaku Co., Ltd. (Tokyo, Japan). All were dissolved in DMSO as a stock solution and stored at –30 °C until use. Adenosine 5'-triphosphate (ATP), bovine serum albumin, desiccated firefly tails, fluorescence isothiocyanate (FITC), conjugated peanut agglutinin (PNA), and TES were obtained from Sigma. Tween 20 was purchased from MP Biomedicals, Inc. (Aurora, OH, USA). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight standards were purchased from Amersham Biosciences UK, Ltd. (Buckinghamshire, UK). Other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

Antibodies

A rabbit polyclonal antibody raised against amino acids 1–309 representing the full-length PP2A catalytic subunit of human origin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase conjugated donkey anti-rabbit immunoglobulin was obtained from Amersham Biosciences UK, Ltd. (Buckinghamshire, UK).

Analysis of acrosome reaction and motility of spermatozoa

Inner perivitelline layers (IPVL) were separated from laid fowl eggs (Robertson *et al.* 1997) and were homogenised using a Teflon glass homogeniser on ice. The protein concentrations of IPVL homogenates were adjusted to 75 µg/ml with TES/NaCl buffer (pH 7.4), using bovine serum albumin as a standard. Fowl spermatozoa at concentrations adjusted to 1.2×10^7 cells/ml were incubated, with or without IPVL, for 30 min at 40 °C. The dose-response of the acrosome reaction was measured in the presence of various concentrations of calyculin A, okadaic acid, H-7 or SC-9 and the effects of the addition of CaCl₂ were also examined. The inhibition constant (*K_i*) values of calyculin A for PP1 and PP2A are around 2 nmol/l and 0.5–1 nmol/l, respectively (Ishihara *et al.* 1989) and the *K_i* values of okadaic acid

for PP1 and PP2A are around 60–500 nmol/l and 0.5–1 nmol/l, respectively (Ishihara *et al.* 1989). The K_i values of H-7 for PKC, cAMP-dependent protein kinase (PKA), protein kinase G (PKG) and myosin light chain kinase (MLCK) are 6.0 μ mol/l, 3.0 μ mol/l, 5.8 μ mol/l and 97 μ mol/l, respectively (Kawamoto & Hidaka 1984). The K_m value of PKC, activated by SC-9 for substrate myosin light chain is 5.8 μ mol/l (Ito *et al.* 1986). Ordinarily, 10- to 100-fold higher concentrations are required for the whole cells.

Acrosome-reacted spermatozoa were identified using FITC-conjugated PNA, which binds to acrosome-reacted, but not acrosome-intact spermatozoa, using a fluorescence microscope at $\times 1000$, as described by Horrocks *et al.* (2000).

For motility determination, the suspension of spermatozoa was placed into a microscope slide chamber (Sekisui Chemical Co., Ltd., UR-157 type, Tokyo, Japan) on a thermostatically controlled warm plate, and the motility of spermatozoa was recorded by video microscopy (magnification on the 12-inch black and white monitor was approximately $\times 600$) at 40 °C (Katz & Overstreet 1981).

The percentages of acrosome-reacted and motile spermatozoa were derived by assessing a total of approximately 100 spermatozoa, distributed uniformly among three or more fields.

Analysis of ATP concentrations of intact spermatozoa

ATP content of spermatozoa in the absence of IPVL was assayed in boiled sperm extracts by firefly bioluminescence (Wishart 1982). Numbers of spermatozoa were estimated by the method of Wishart & Ross (1985), using a double-beam spectrophotometer (Shimadzu, Model UV-150-02, Kyoto, Japan). The concentration of ATP was expressed in terms of nmol ATP/ 10^9 spermatozoa.

Western immunoblot analysis

Spermatozoa that had been washed as described above and adjusted to the concentrations of 4×10^8 cells/ml were mixed with equal volumes of concentrated ($\times 2$) Laemmli (1970) sample buffer and were boiled for 5 min. Samples containing approximately 15 μ g protein were loaded onto 12.5% SDS-polyacrylamide slab gel and subjected to electrophoresis. For positive control, human breast carcinoma (T-47D) whole cell lysate (approximately 25 μ g protein) was loaded onto the same gel. Western blotting was performed according to the protocol of Towbin *et al.* (1979), with some modifications. Briefly, proteins were transferred electrophoretically to a polyvinylidene difluoride membrane sheet (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After transfer, non-specific sites on the membranes were blocked by incubating

them for 1.5 h at room temperature (20–25 °C) in 0.1% Tween 20 in Tris buffered saline (TTBS) containing 5% skimmed milk powder. The blots were then incubated overnight at 4 °C with the antibody to PP2A (1:200 dilution with TTBS). For negative control, the blots were incubated in TTBS alone. The blots were further incubated for 1 h at room temperature (20–25 °C) with donkey anti-rabbit immunoglobulin conjugated with horseradish peroxidase (1:2000 dilution with TTBS). After each incubation, the membranes were rinsed extensively in TTBS. Finally, blots were developed with the Amersham ECL detection kit (Amersham Biosciences UK) for 5 min. Immunocomplexes were detected with Amersham photoimager system (Typhoon 9410) exposures for around 5 min.

Statistical analysis

Percentages of acrosome reaction and motility were transformed using arc sine transformation. All data were subjected to statistical analysis by Duncan's multiple-range tests (Duncan 1955).

Results

Effects of calyculin A, okadaic acid, H-7 and SC-9 on the motility and acrosome reaction of fowl spermatozoa

The motility of spermatozoa in TES/NaCl buffer alone (control) with or without IPVL at 40 °C was almost negligible (Figs. 1a–3a). When calyculin A was added, motility became vigorous (Fig. 1a), and the acrosome reaction was stimulated in the presence, but not in the absence of IPVL (Fig. 1b), in a dose-dependent manner, respectively. Similar dose-dependent stimulation of acrosome reaction was observed when okadaic acid was added to spermatozoa in the presence of IPVL (Fig. 2b). Okadaic acid also stimulated the motility of spermatozoa, although calyculin A appeared to be more potent, stimulating motility at a tenfold lower concentration than okadaic acid (Figs. 1a, 2a). The addition of 2 mmol CaCl_2 /l alone also stimulated motility, whether or not IPVL was present (Fig. 3a), and the acrosome reaction in the presence, but not in the absence of IPVL (Fig. 3b). The presence of both Ca^{2+} and inhibitor with IPVL resulted in a slightly higher stimulation of the acrosome reaction than the addition of Ca^{2+} or inhibitor alone, but this was not significant (Fig. 3b).

In the absence of added Ca^{2+} , neither H-7, a broad-based inhibitor of serine/threonine protein kinase, nor SC-9, a selective activator of PKC, restored motility at 40 °C or stimulated the acrosome reaction in the presence of IPVL (data not shown). Contrary to the absence of Ca^{2+} , when 2 mmol CaCl_2 /l was added, no inhibition or stimulation was observed by the addition of H-7 compared with the control (no addition of H-7): the presence of H-7 permitted the restoration of motility at

Figure 1 Effect of calyculin A on (a) the motility and (b) acrosome reaction of fowl spermatozoa incubated with or without IPVL in the absence of Ca^{2+} at 40 °C. Each value represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.05$) from each other.

40 °C (Fig. 4a) and, in the presence of IPVL, induced the acrosome reaction (Fig. 4b) in the range of 1–100 micromol/l. However, the vigorous motility of spermatozoa, stimulated by the addition of Ca^{2+} was reduced gradually as the concentrations of SC-9 were increased (Fig. 5a) and a similar SC-9-induced inhibition was observed in the acrosome reaction in the presence of Ca^{2+} and IPVL (Fig. 5b).

Effects of calyculin A, okadaic acid, H-7 and SC-9 on the ATP concentrations of fowl spermatozoa

The ATP concentrations of spermatozoa following exposure to 100 nmol okadaic acid/l, 100 μ mol H-7/l or 10 μ mol SC-9/l alone at 40 °C had almost the same values compared to those of untreated spermatozoa (control) (around 40–45 nmol per 10^9 spermatozoa). Additionally, the ATP concentrations of spermatozoa decreased significantly in the presence of 2 mmol CaCl_2 /l or 100 nmol calyculin A/l alone, presumably due to the restoration of motility (around 30 nmol per 10^9 spermatozoa). Thus, the addition of inhibitors or activator is not simply affecting membrane damage or inhibiting energy production in these spermatozoa, but

may be acting on some part of the regulatory cascade of motility or the acrosome reaction.

Immunoblot identification of PP2A in fowl spermatozoa

No appreciable immunoreactive protein was detected in the negative control lane (no antibody). In contrast, a protein of approximately 36 kDa was recognised by the anti-PP2A antibody, which corresponds to the molecular weight of the catalytic subunit of PP2A, even though the visible immunoreactive band was faint, compared with the positive control (Fig. 6).

Discussion

The acrosome reaction, which is an exocytotic secretory response, is required for sperm penetration and fusion with the egg investments and plasma membrane (Rotem *et al.* 1992), and is regulated by the activation of compartmentalised intracellular signalling pathways (Urner & Sakkas 2003). The molecular mechanisms and the signal transduction pathways mediating the processes of acrosome reaction are partially defined: changes in protein phosphorylation and

Figure 2 Effect of okadaic acid on (a) the motility and (b) acrosome reaction of fowl spermatozoa incubated with or without IPVL in the absence of Ca^{2+} at 40 °C. Each value represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.05$) from each other.

Figure 3 Effect of Ca²⁺ (2 mmol/l), calyculin A (100 nmol/l) and okadaic acid (100 nmol/l) on (a) the motility and (b) acrosome reaction of fowl spermatozoa incubated with or without IPVL at 40 °C. Each value represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.05$) from each other.

dephosphorylation of sperm proteins, including Ca²⁺-dependent processes, seem to play a primary role in the second messenger regulatory mechanisms (for a review, see Benoff 1998, Breitbart & Naor 1999, Baldi *et al.* 2000, Guraya 2000, Topfer-Petersen *et al.* 2000, Urner & Sakkas 2003). There is evidence for the involvement of cAMP-dependent protein kinase (PKA) in the mammalian acrosome reaction: adenylate cyclase activity and cAMP generation increased during this process in human spermatozoa (De Jonge *et al.* 1991); adenylate cyclase stimulators such as forskolin, and the cAMP analogue dibutyryl cAMP, induced the acrosome reaction in a dose-dependent manner in human and ram spermatozoa (Anderson *et al.* 1992, Garde & Roldan 2000, Harrison & Meizel 2000); and PKA inhibitors have been reported to suppress human sperm acrosome reaction (Harrison & Meizel 2000).

PKC activators, such as phorbol esters and synthetic diacylglycerol (DAG) induce the mammalian sperm acrosome reaction, and the localisation of PKC in the acrosomal region of the sperm head also indicates the involvement of PKC in this process (Breitbart & Naor 1999). In addition, induction of the human sperm

acrosome reaction by solubilised zona pellucida was partially reduced by pre-treatment with inhibitors of PKA, PKC and protein kinase G (PKG) and their combined inhibitory effect was additive (Bielfeld *et al.* 1994). These results suggest a concomitant role for PKA, PKC and PKG in human ZP-induced acrosome reaction (Bielfeld *et al.* 1994). Protein tyrosine kinase also appears to be involved in the regulation of the acrosome reaction of mammalian spermatozoa (for a review, see Urner & Sakkas 2003).

From our previous work, PKC appeared to be present in fowl spermatozoa, since immunoblot analysis of fowl sperm proteins showed that a protein of approximately 80 kDa was recognised by an antibody to PKC (Ashizawa *et al.* 1994a). Furthermore, the present study demonstrates that the activation of PKC may contribute to a decrease in the acrosome reaction of fowl spermatozoa, since the addition of SC-9, a selective activator of PKC, inhibited the acrosome reaction response to IPVL and Ca²⁺ in a dose-dependent manner. A similar inhibition by SC-9 of motility stimulation by Ca²⁺ was consistent with previous work (Ashizawa *et al.* 1994a). From these results, it seems that PKC has an opposite effect on the

Figure 4 Effect of H-7 on (a) the motility and (b) acrosome reaction of fowl spermatozoa incubated with or without IPVL in the presence of 2 mmol CaCl₂/l at 40 °C. H-7 was added at the start of incubation and spermatozoa were incubated for a total of 30 min. After 15 min of incubation, CaCl₂ was added to the inhibitor-treated spermatozoa. Each value represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.05$) from each other.

Figure 5 Effect of SC-9 on (a) the motility and (b) acrosome reaction of fowl spermatozoa incubated with or without IPVL in the presence of 2 mmol CaCl_2/l at 40 °C. SC-9 was added at the start of incubation and spermatozoa were incubated for a total of 30 min. After 15 min of incubation, CaCl_2 was added to the activator-treated spermatozoa. Each value represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly ($P < 0.05$) from each other.

regulation of fowl sperm motility and acrosome reaction than that of mammalian spermatozoa. However, such a difference is not surprising, given that several lines of evidence point towards differential regulation of sperm motility in fowl and mammals. First, demembrated fowl spermatozoa can be motile even in the presence of millimolar concentrations of Ca^{2+} (Ashizawa *et al.* 1989), whereas such high concentrations of Ca^{2+} inhibit the motility of demembrated mammalian spermatozoa (White & Voglmayer 1986, Feng *et al.* 1988). Secondly, cAMP is indispensable for the initiation and stimulation of flagellar motility of mammalian spermatozoa (for a review, see Tash & Means 1983, Lindemann & Kanous 1989), but is not necessary for fowl spermatozoa, especially at 40 °C (Ashizawa *et al.* 1992).

In the study reported here, the addition of H-7, a broad-based inhibitor of serine/threonine protein kinase (especially of PKC, PKA and PKG), did not appreciably affect the motility or acrosome reaction of fowl spermatozoa. These results suggest that fowl sperm motility and the acrosome reaction may not be simply stimulated or inhibited by changes in the activity of these kinases, especially PKC. Perhaps their inhibition involves a PKC-dependent phosphoprotein(s) that is active only when phosphorylated more than a certain threshold amount (e.g. in response to SC-9). 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 maintain, but do not increase their motility or acrosome reaction status. However, in this study, the target and precise mechanisms of action of PKC remain to be elucidated. Further study is needed to examine which protein(s) is altered during the inhibition of fowl sperm motility or acrosome reaction by the activation of PKC.

If phosphorylation by protein kinases is involved in the acrosome reaction, then dephosphorylation of proteins by specific regulatory phosphatases should

also affect the acrosome reaction. Such regulatory serine/threonine protein phosphatases are classified into four main enzymes; type 1 (PP1), type 2A (PP2A), type 2B (PP2B) and type 2C (PP2C) (Cohen 1989). Another phosphatase family is protein tyrosine phosphatase (PTPs), which removes phosphate groups from phosphorylated tyrosine residues of proteins (for a review, see Montalibet *et al.* 2005). Both mouse and human spermatozoa contain highly active tyrosine phosphatases and inhibition of tyrosine phosphatases with sodium pervanadate, bis(*N,N*-dimethylhydroxamido) hydroxovanadate, ethyl-3,4-dephostatin and phenylarsine oxide prevents the acrosome reaction, suggesting that PTPs play a role in mammalian sperm exocytosis (Tomes *et al.* 2004). With regard to fowl spermatozoa, it is suggested that PP2B appears to be involved in the regulation of the acrosome reaction, since the addition of specific inhibitors of PP2B, such as

Figure 6 Immunoblot analysis of fowl sperm protein phosphatase type-2A. Lane 1: fowl sperm lysate incubated without a PP2A polyclonal antibody (negative control); lane 2: human breast carcinoma (T-47D) whole cell lysate incubated with a PP2A polyclonal antibody (positive control); lane 3: fowl sperm lysate incubated with a rabbit polyclonal antibody raised against amino acids 1-309, representing full-length PP2A catalytic subunit of human origin.

deltamethrin or fenvalerate, significantly stimulated the acrosome reaction (Ashizawa *et al.* 2004).

Okadaic acid is a very potent inhibitor of PP1 and PP2A (Cohen *et al.* 1990). Calyculin A has a potency similar to that of okadaic acid as an inhibitor of PP2A, but is 10- to 100-fold more effective as an inhibitor of PP1 (Ishihara *et al.* 1989). The present study showed that the motility of fowl spermatozoa at 40 °C was stimulated by calyculin A and was effective at 10- to 100-fold lower concentrations of calyculin A than those of okadaic acid, confirming earlier results (Ashizawa *et al.* 1994b). In contrast, the acrosome reaction in the presence of IPVL was stimulated in the same dose-dependent manner by both 10–1000 nmol/l okadaic acid and calyculin A. These results suggest that PP1 and/or PP2A are involved in the regulation of acrosome reaction but that only PP1 is involved in the regulation of motility of fowl spermatozoa. It seems that both phosphatases are present in fowl spermatozoa, since immunoblotting of sperm extract using an antibody to PP1 α revealed a major cross-reacting protein of 36–37 kDa which corresponds to the molecular weight of the catalytic subunit of PP1 α (Ashizawa *et al.* 1994b), and in this study, a protein of approximately 36 kDa was recognised by anti-PP2A antibody which corresponds to the molecular weight of the catalytic subunit of PP2A.

Therefore, in conclusion, different or different combinations of protein phosphatases are involved in the regulation of the acrosome reaction of fowl spermatozoa than those involved in the regulation of fowl sperm motility, i.e., protein dephosphorylation by PP2B and PP1 and/or PP2A in the former, and PP1 alone in the latter case. In addition, the activation of PKC may contribute to a decrease in the flagellar movement and acrosome reaction of fowl spermatozoa.

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