Protein phosphatase-type 2B is involved in the regulation of the acrosome reaction but not in the temperature-dependent flagellar movement of fowl spermatozoa

K Ashizawa, G J Wishart¹, A R A H Ranasinghe, S Katayama and Y Tsuzuki

Laboratory of Animal Reproduction, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan and ¹Department of Molecular and Life Sciences, University of Abertay Dundee, Dundee DD1 1HG, UK

Correspondence should be addressed to K Ashizawa; Email: ashizawa@cc.miyazaki-u.ac.jp

Abstract

The motility and acrosomal integrity of fowl spermatozoa in TES/NaCl buffer, with or without homogenized inner perivitelline layers (IPVL) prepared from laid fowl eggs, was almost negligible at 40 °C. However, motility became vigorous even at 40 °C when 2 mmol CaCl₂/l was added, and the acrosome reaction was also stimulated in the presence, but not in the absence, of IPVL. The presence of deltamethrin or fenvalerate, specific inhibitors of protein phosphatase-type 2B (PP2B), did not permit the restoration of motility at 40 °C but, in the presence of IPVL, these compounds stimulated the acrosome reaction in a dose-dependent manner in the range of 1–1000 nmol/l. These results suggest 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 the acrosome reaction of fowl spermatozoa are different from those for the restoration of motility, i.e. protein dephosphorylation by PP2B in the former but not in the latter case.

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Introduction

The acrosome of spermatozoa is a Golgi-derived organelle that forms a cap over the anterior part of the nucleus. The acrosome reaction, which is an exocytotic secretory response, is required for sperm penetration and fusion with the egg plasma membrane (Rotem *et al.* 1992) and involves the following: fusion of the outer acrosomal membrane with the overlying plasma membrane; vesiculation and disappearance of the fused membranes; and release of enzymes and other components contained within the acrosomal matrix (Oura & Toshimori 1990).

Like other exocytotic events, the acrosome reaction can be stimulated by a variety of signalling pathways, including a Ca²⁺-dependent process. In mammalian spermatozoa, several Ca²⁺-dependent processes have been shown to occur, including activation of phospholipases C and A₂, protein kinase C and cAMP-dependent protein kinase pathways. This means that protein phosphorylation seems to play a primary role in the second messenger regulatory mechanisms of the acrosome reaction (for review see Benoff 1998, Breitbart & Naor 1999, Baldi *et al.* 2000, Guraya 2000, Topfer-Petersen *et al.* 2000, Urner & Sakkas 2003).

If phosphorylation is required for the activation of 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). With regard to fowl sperm motility, it has been proposed that inhibition of sperm motility at body temperature (40 °C), known as the reversible temperature-dependent immobilization, may be due to the activation of PP1 (Ashizawa *et al.* 1994a, 1997). However, limited information is available on the involvement of protein phosphatases in the regulation of acrosome reaction in almost all species from invertebrates to vertebrates. We report here that 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.

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 10-fold 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

Deltamethrin and fenvalerate, specific inhibitors of PP2B, were purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). ATP, bovine serum albumin (BSA), desiccated firefly tails, fluorescein isothiocyanate (FITC)-conjugated peanut agglutinin (PNA) and TES were obtained from Sigma Chemical Co. (St Louis, MO, USA). Bicinchoinic acid protein assay reagent was from Pierce Chemical Co. (Rockford, IL, USA). Other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

Analysis of acrosome reaction and motility of spermatozoa

The homogenized inner perivitelline layers (IPVL) were prepared from laid fowl eggs, using a Teflon–glass homogenizer on ice. The protein concentrations of IPVL were adjusted to 75 µg/ml with TES/NaCl buffer (pH 7.4), using BSA as a standard. With or without IPVL, fowl spermatozoa were incubated for 30 min at 40 °C. Sperm concentrations were adjusted to 1.2×10^7 cells/ml. The dose–response of the acrosome reaction and motility was measured in the presence of various concentrations of deltamethrin or fenvalerate and the effects of the addition of CaCl_2 were also examined. Acrosome-reacted spermatozoa were identified using a fluorescent microscope and FITC-conjugated PNA which binds to acrosome-reacted, but not acrosome-intact, spermatozoa. The protocols for the preparation of IPVL and the assessment of acrosome reaction were essentially those described by Robertson *et al.* (1997) and Horrocks *et al.* (2000) respectively.

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

The percentages of acrosome reaction and motility were made on a total of approximately 100 spermatozoa distributed uniformly among three or more fields.

Analysis of ATP concentrations of spermatozoa

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

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

The motility of spermatozoa in TES/NaCl buffer (control) with or without IPVL at 40 °C was almost negligible (Figs 1a–3a). However, motility became vigorous at 40 °C when 2 mmol CaCl_2 /l was added (Fig. 3a), and the acrosome reaction was stimulated in the presence but not in the absence of IPVL (Fig. 3b). Deltamethrin or fenvalerate, specific inhibitors of PP2B, did not restore motility at 40 °C (Figs 1a and 2a) but did, in the presence of IPVL, induce the acrosome reaction in a dose-dependent manner in the range of 1–1000 nmol/l (Figs 1b and 2b). Deltamethrin stimulated the acrosome reaction at a 10-fold lower concentration than fenvalerate. This might be due to the different potency of these inhibitors, since deltamethrin is approximately 10- to 100-fold more effective than fenvalerate as an inhibitor of PP2B (Enan & Matsumura 1992). The presence of both Ca^{2+} and inhibitor with IPVL resulted in slightly higher stimulation of the acrosome reaction than the addition of Ca^{2+} or inhibitor alone, but this was not significant (Fig. 3b).

The ATP concentrations of spermatozoa following exposure to deltamethrin or fenvalerate at 40 °C were almost the same values compared with those of untreated spermatozoa (control). Additionally, the ATP concentrations of spermatozoa decreased significantly in the presence of Ca^{2+} alone or Ca^{2+} with inhibitor, presumably due to the restoration of motility (Fig. 4).

Discussion

At the time of ovulation, the avian oocyte is surrounded by the IPVL (Bain & Hall 1969), which may be considered to be analogous to the mammalian zona pellucida. In fact, it was recently shown that an IPVL glycoprotein of approximately 34 kDa has a high degree of homology to murine zona pellucida protein-3 (Waclawek *et al.* 1998), which acts as the primary sperm binding protein (Bleil & Wassarman 1980) and triggers the acrosome reaction (Bleil & Wassarman 1983). In addition, extracellular Ca^{2+}

Figure 1 Effects of deltamethrin on (a) the motility and (b) the 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.

is known to be required for the induction of the acrosome reaction in most species (Fraser 1995). The present results confirm that, in fowl spermatozoa, the IPVL is necessary for the activation of the acrosome reaction and that Ca^{2+} also plays an important role in the stimulation of acrosomal exocytosis (Robertson 1999).

At the avian body temperature of 40 °C, when suspended in a simple medium of buffered NaCl, fowl spermatozoa become immotile (Munro 1938, Ashizawa & Nishiyama 1978, Ashizawa *et al.* 1989). Motility is restored by decreasing the temperature or by the addition of Ca^{2+} or body fluids such as seminal plasma or the fluid of the female ovarian pocket taken at the time of ovulation (Ashizawa & Wishart 1987, 1992, Wishart & Ashizawa 1987, Ashizawa *et al.* 1994b). The present results show that the presence of IPVL has no effect on this phenomenon and that Ca^{2+} , but not PP2B inhibitors, stimulate the

Figure 2 Effects of fenvalerate on (a) the motility and (b) the 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.

motility of spermatozoa at 40 °C. However, both Ca^{2+} and PP2B inhibitors were able to induce the acrosome reaction in fowl spermatozoa in the presence of IPVL. Therefore, it seems that Ca^{2+} is necessary for the stimulation of both motility and the acrosome reaction, but that PP2B might be involved in the regulation of the acrosome reaction. During these incubations, spermatozoa maintained almost the same concentrations of intracellular ATP as those of the control (no addition of PP2B inhibitors), in spite of the inhibition of motility. Thus, it appears that the addition of PP2B inhibitors does not simply affect membrane damage or inhibit energy production in these spermatozoa, but may be acting on some part of the regulatory cascade in the acrosome reaction: the inhibitory dephosphorylation action of PP2B might occur in the cascade between sperm-IPVL receptor binding and the

Figure 3 Effects of Ca²⁺ (2 mmol/l), fenvalerate (100 nmol/l) and deltamethrin (10 nmol/l) on (a) the motility and (b) the 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.

acrosome reaction. However, the precise working point is still unclear.

In conclusion, the intracellular molecular mechanisms for the regulation of the acrosome reaction of fowl spermatozoa are different from those for the temperature-dependent immobilization and restoration of motility, i.e. protein dephosphorylation by PP2B in the former but not in the latter case. We are pursuing investigation of the possible involvement of the other classes of serine/threonine protein phosphatase, such as PP1 and PP2A, in the regulation of acrosome reaction.

Figure 4 Effects of Ca²⁺ (2 mmol/l), fenvalerate (100 nmol/l) and deltamethrin (10 nmol/l) on the ATP concentrations of fowl spermatozoa incubated 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.

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