Effects of calpain and Rho-kinase inhibitors on the acrosome reaction and motility of fowl spermatozoa *in vitro*

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Abstract

At the avian body temperature of 40°C, intact fowl spermatozoa require Ca^{2+} for the initiation of motility and a combination of both Ca^{2+} and homogenized inner perivitelline layer (IPVL) together to induce the acrosome reaction. Within the range of 1–100 $\mu\text{mol/l}$, neither PD 150606 (a Ca^{2+} -dependent calpain inhibitor) nor Y-27632 (an inhibitor of Ca^{2+} -dependent Rho-kinase) were able to inhibit the acrosome reaction induced by the presence of Ca^{2+} and IPVL. However, PD 150606, although not Y-27632, was able to inhibit sperm motility initiated by Ca^{2+} , as well as motility initiated by calyculin A – a specific inhibitor of protein phosphatases, which also initiates sperm motility at 40°C. The addition of PD 150606 did not reduce the ATP concentrations of intact spermatozoa, nor the motility of demembranated spermatozoa. Immunoblot analysis of sperm extract using a polyclonal antibody against calpain 12 revealed a cross-reacting protein of approximately 80 kDa. These results suggest that Rho-kinase is not involved in the regulation of the acrosome reaction or of motility in fowl spermatozoa. In contrast, calpain appears to be involved in the regulation of flagellar movement, but not in that of the acrosome reaction. Furthermore, it seems that endogenous calpain is present in the cytoplasmic matrix and/or the plasma membrane, but not retained in the axoneme and/or accessory cytoskeletal components.

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Introduction

It is well known that Ca^{2+} is a signal transducer in the regulation of the acrosome reaction and motility of spermatozoa (for review see Tash & Means 1983, Lindemann & Kanous 1989, Fraser 1995, Benoff 1998, Baldi *et al.* 2000, Guraya 2000, Topfer-Petersen *et al.* 2000). In mammalian spermatozoa, an increase in the intracellular Ca^{2+} occurs during the acrosome reaction and this increase of Ca^{2+} is required to trigger the molecular events that culminate in the acrosome reaction (for review see Breitbart & Naor 1999). On the other hand, the effects of intracellular Ca^{2+} on sperm motility can be divided into two major classes: modification of wave form and oscillation frequency (Gibbons & Gibbons 1973, Brokaw *et al.* 1974, Brokaw 1979, Lindemann *et al.* 1987, Suarez *et al.* 1993, Aoki *et al.* 1999); and complete inhibition of motility at high concentrations of Ca^{2+} (Tash & Means 1982).

In fowl spermatozoa, the addition of extracellular Ca^{2+} is found to be an absolute requirement for acrosomal exocytosis at 40°C, the normal body temperature of these birds. When incubated with preparations of the homogenized inner perivitelline layers (IPVLs), which may be considered to be analogous to the mammalian zona pellucida

(Waclawek *et al.* 1998), fowl spermatozoa were unable to undergo the acrosome reaction. However, the addition of Ca^{2+} to the salt solution resulted in a significant increase in acrosomal exocytosis (Robertson 1999). Additionally, unlike mammalian spermatozoa, fowl sperm motility is reversibly inhibited as the temperature is raised from 30°C to 40°C. Motility is restored by decreasing the temperature or by the addition of Ca^{2+} at 40°C (Munro 1938, Ashizawa & Nishiyama 1978, Ashizawa & Wishart 1987, 1992, Ashizawa *et al.* 1989a, 1994a, Wishart & Ashizawa 1987). Therefore, Ca^{2+} seems to play a key role for the stimulation of the acrosome reaction and the motility of fowl spermatozoa at the avian body temperature.

Ca^{2+} -dependent cysteine protease (calpain), an enzyme responsible for degradation of axonal and muscle cytoskeletal elements, has been isolated and characterized from fowl tissues such as skeletal muscle (Kawashima *et al.* 1984, Wolfe *et al.* 1989, Johari *et al.* 1993, Birkhold & Sams 1994, Sorimachi *et al.* 1995), brain, sciatic nerve and gastrocnemius muscle (el-Fawal *et al.* 1990). In most mammalian tissues and cells, there are more than two forms of calpain – namely calpain 1 and calpain 2 – which have identical substrate specificities, but require low and

high Ca^{2+} concentrations respectively, for their activation (Murachi 1989, Croall & DeMartino 1991). The small GTPase Rho also plays pivotal roles in the Ca^{2+} sensitization of smooth muscle contraction (Hirata *et al.* 1992, Gong *et al.* 1996, Otto *et al.* 1996) and a recent study demonstrates that Rho-kinase (Rho-associated kinase), one of target proteins of Rho, modulates smooth muscle contraction in a Ca^{2+} -dependent manner (Kureishi *et al.* 1997). Rho and Rho-kinase activities have also been identified in fowl smooth muscle (Feng *et al.* 1999, Anabuki *et al.* 2000, Stevenson *et al.* 2004).

Although there is no previous evidence for the existence of these proteins in fowl spermatozoa, both calpain (Schollmeyer 1986, Rojas *et al.* 1999, Rojas & Moretti-Rojas 2000, Yudin *et al.* 2000, Aoyama *et al.* 2001) and Rho (Castellano *et al.* 1997) have been found in mammalian and sea urchin spermatozoa respectively: calpain appears to be involved in the regulation of the acrosome reaction (Schollmeyer 1986, Yudin *et al.* 2000, Aoyama *et al.* 2001) and the cell fusion process that takes place during penetration of the oocyte (Rojas *et al.* 1999, Rojas & Moretti-Rojas 2000). The presence of Rho in the acrosomal region, the middle piece of the head and in the flagellum of sea urchin spermatozoa has been demonstrated by both immunofluorescence and immunogold staining and, based on this cellular localization, it has been assumed that Rho may participate in regulating motility and the actin polymerization that accompanies the acrosome reaction (Castellano *et al.* 1997). If so, Rho-kinase should also participate in the regulatory mechanisms of the acrosome reaction and motility of spermatozoa.

However, no information is available concerning the effects of calpain and Rho-kinase on the regulation of the acrosome reaction and motility of avian spermatozoa, although potent and selective inhibitors of calpain and Rho-kinase, namely PD 150606 (Wang *et al.* 1996) and Y-27632 (Uehata *et al.* 1997) respectively, have been developed. In the following experiment, therefore, attempts were made to investigate the effects of PD 150606 and Y-27632 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 and 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

Y-27632 ((*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide), a cell-permeable selective inhibitor of Rho-kinase, a generous gift from Yoshitomi Pharmaceutical Industries, Ltd (Osaka, Japan), was dissolved in distilled water as a stock solution (100 mmol/l) and stored at –30 °C until use. PD 150606 (3-(4-iodophenyl)-2-mercapto-(*Z*)-2-propenoic acid), a cell-permeable selective calpain inhibitor, obtained from Calbiochem-Novabiochem Co. (La Jolla, CA, USA), was dissolved in DMSO as a stock solution (10 mmol/l) and stored at –30 °C until use. Calyculin A, a specific inhibitor of protein phosphatase-type 1 (PP1) and -type 2A (PP2A), was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). 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 from Pierce Chemical Co. (Rockford, IL, USA). SDS-PAGE molecular weight standards were purchased from Amersham. Other chemicals were of reagent grade from Nacalai Tesque, Inc. (Kyoto, Japan).

Antibodies

A rabbit polyclonal antibody raised against a synthetic peptide based on the amino-terminal end of domain-I in the large subunit of calpain 12 (molecular weight, 80 kDa) was purchased from Triple Point Biologics, Inc. (Forest Grove, OR, USA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulins donkey serum was obtained from Amersham.

Analysis of the acrosome reaction of spermatozoa

IPVLs were separated from laid fowl eggs (Robertson *et al.* 1997) and were homogenized using a Teflon glass homogenizer 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 PD 150606 or Y-27632 and the effects of the addition of CaCl_2 after the addition of PD 150606 or Y-27632 were also examined. The inhibition constant (K_i) values of PD 150606 for calpains are around 0.2–0.4 µmol/l (Wang *et al.* 1996) and the K_i value of Y-27632 for Rho-kinase is 0.14 µmol/l (Uehata *et al.* 1997). Ordinarily, 10- to 100-fold higher concentrations are required for the whole

cells. Therefore, both inhibitors were used in the range of 1–100 $\mu\text{mol/l}$ in this study.

Acrosome-reacted spermatozoa were identified using a fluorescent microscope at $\times 1000$ magnification and FITC-conjugated PNA – which binds to acrosome-reacted, but not acrosome-intact, spermatozoa – as described by Horrocks *et al.* (2000). The percentages of acrosome reaction were calculated from a total of approximately 100 spermatozoa distributed uniformly among three or more fields.

Analysis of motility of intact and demembrated spermatozoa

Sperm samples were pre-incubated aerobically in a water bath at 30 or 40°C for 10 min. After the pre-incubation, the dose–response and time course of motility of intact spermatozoa were measured at 30 or 40°C after addition of PD 150606 or Y-27632. The effects of the addition of CaCl_2 or calyculin A, after the addition of PD 150606 or Y-27632 were also examined at 30 and 40°C. The diluent for the incubation and measurement of sperm motility was TES/NaCl buffer without IPVL, as described above.

Demembration and reactivation of spermatozoa were performed at 30 and 40°C according to the method described previously (Ashizawa *et al.* 1989b). The extraction medium used consisted of 0.1% (v/v) TritonX-100, 200 mmol sucrose/l, 25 mmol potassium glutamate/l, 1 mmol MgSO_4 /l, 1 mmol dithiothreitol (DTT)/l and 20 mmol Tris–HCl buffer/l (pH 7.9). The reactivation medium consisted of 0.5 mmol ATP/l, 200 mmol sucrose/l, 25 mmol potassium glutamate/l, 1.5 mmol MgSO_4 /l, 1 mmol DTT/l and 20 mmol Tris–HCl buffer/l (pH 7.9). To examine the effects of PD 150606 or Y-27632, various concentrations of PD 150606 or Y-27632 were added to the reactivation medium. Addition of EGTA, CaCl_2 or calyculin A to inhibitor-treated spermatozoa was also performed.

The suspension of intact or demembrated 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 the 12 inch black and white monitor was approximately $\times 600$) at 30 or 40°C (Katz & Overstreet 1981). Measurements were made on a total of 200–300 spermatozoa, distributed uniformly among three or more fields, to determine the percentage of motile spermatozoa.

Analysis of ATP concentrations of intact spermatozoa

The ATP content of spermatozoa in the absence of IPVLs was assayed in boiled sperm extracts by firefly bioluminescence (Wishart 1982). The 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). The concentration of ATP was expressed in terms of nanomoles ATP/ 10^9 spermatozoa.

Western immunoblot analysis of calpain

Spermatozoa that had been washed as described above, and with concentrations adjusted to 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 μg protein were loaded onto 7.5% 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, proteins were transferred electrophoretically to a polyvinylidene difluoride membrane sheet (BioRad). After transfer, non-specific sites on the membranes were blocked by incubating them overnight at 4°C in 0.1% Tween 20 in Tris-buffered saline (TTBS) containing 5% skimmed milk powder. The blots were then incubated for 1.5 h at room temperature (20–25°C) with the antibody to calpain 12 (1:1000 dilution with TTBS containing 5% skimmed milk powder). For negative control, the blots were incubated in TTBS containing 5% skimmed milk powder alone. The blots were further incubated for 1 h at room temperature (20–25°C) with anti-rabbit immunoglobulins donkey serum conjugated with horseradish peroxidase (1:2000 dilution with TTBS containing 5% skimmed milk powder). After each incubation, the membranes were rinsed extensively in TTBS. Finally, blots were developed with the Amersham enhanced chemiluminescence (ECL) detection kit for 5 min. Immunocomplexes were detected with Amersham photoimager system (Typhoon 9410) exposures for around 15–20 min.

Statistical analysis

The 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 PD 150606 or Y-27632 on the acrosome reaction of fowl spermatozoa

In Fig. 1a and c, various concentrations of PD 150606 or Y-27632 alone were added at the start of incubation and spermatozoa were incubated for 30 min at 40°C. In contrast, in Fig. 1b and d, 15 min after the start of the 30 min incubation, CaCl_2 was added in the PD 150606- or Y-27632-treated spermatozoa, to stimulate the acrosome reaction. During the incubation of spermatozoa for 30 min at 40°C, the acrosome reaction of spermatozoa with or without IPVL was almost negligible in the absence of Ca^{2+} in TES/NaCl buffer (Fig. 1a and c). When 2 mmol CaCl_2 /l was added, the acrosome reaction was stimulated in the presence, but not in the absence of IPVL. However, no inhibition or stimulation of acrosome reaction was

Figure 1 Effects of PD 150606 (a, b) or Y-27632 (c, d) on the acrosome reaction of fowl spermatozoa in the absence (a, c) and in the presence (b, d) of 2 mmol CaCl₂/l incubated with or without the IPVLs at 40°C. PD 150606 or Y-27632 was added at the start of incubation and spermatozoa were incubated for 30 min; 15 min after the start of incubation, CaCl₂ was added to the inhibitor-treated spermatozoa. Each value represents the mean (± S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly from each other ($P < 0.05$).

observed in the presence of PD 150606 or Y-27632 within the range of 1–100 μmol/l (Fig. 1b and d).

Effects of PD 150606 or Y-27632 on the motility of fowl spermatozoa

The motility of intact spermatozoa at 30°C was unaffected at 10 min after the addition of Y-27632, within the range 0–100 μmol/l. Around 65–70% of spermatozoa were motile. In contrast, the addition of PD 150606 inhibited sperm motility by approximately 20, 40 and 70% following exposure to concentrations of 1, 10 and 100 μmol/l respectively, compared with the control (no addition of PD 150606) spermatozoa (about 70% of spermatozoa were motile). At 40°C, the addition of PD 150606 or Y-27632 alone did not appreciably affect the motility of spermatozoa, which remained almost negligible.

The time course of motility in the presence of CaCl₂ or calyculin A, an inhibitor of PP1 and PP2A, after the addition of PD 150606 or Y-27632 at 30 and 40°C is shown in Fig. 2a–d. Even the presence of CaCl₂ after

the addition of PD 150606 could not prevent the inhibition of motility of intact spermatozoa at 30°C, although no inhibition of motility of spermatozoa was observed after the addition of Y-27632 (Fig. 2a). At 40°C, the motility of intact spermatozoa was negligible, and the addition of PD 150606 or Y-27632 retained the immotility, but the motility was immediately restored by the subsequent addition of 2 mmol CaCl₂/l in the control (no addition of inhibitor) and Y-27632-treated spermatozoa. In contrast, restoration of Ca²⁺-supplemented sperm motility was not observed in the presence of PD 150606 (Fig. 2b). In the absence of inhibitors (control), the addition of calyculin A did not appreciably affect the intact sperm motility at 30°C (Fig. 2c), but permitted restoration of motility of at 40°C (Fig. 2d). Similar effects were observed with the addition of Y-27632 (Fig. 2c and d). In contrast, the presence of calyculin A after the addition of PD 150606 could not prevent the inhibition of motility of intact spermatozoa at 30 and 40°C (Fig. 2c and d).

No stimulation or inhibition of motility of demembrated spermatozoa was observed at 5 min after the

Figure 2 The time course of motility of intact fowl spermatozoa after addition of: 100 μ mol PD 150606/l and 2 mmol CaCl_2 /l (□), 100 μ mol Y-27632/l and 2 mmol CaCl_2 /l (○) or 2 mmol CaCl_2 /l alone (control, ●) at (a) 30 °C and (b) 40 °C; 100 μ mol PD 150606/l and 100 nmol calyculin A/l (□), 100 μ mol Y-27632/l and 100 nmol calyculin A/l (○) or 100 nmol calyculin A/l alone (control, ●) at (c) 30 °C and (d) 40 °C. Each point represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. * P < 0.05 compared with value when no inhibitor was added (control) at each period.

addition of PD 150606 or Y-27632 alone, within the range 0–100 μ mol/l, at 30 and 40 °C. About 70% of spermatozoa were motile at 30 °C and the motility remained almost negligible at 40 °C.

The effect of more prolonged exposure is shown in Fig. 3a and b. At 30 °C, the motility was inhibited immediately after the addition of 2 mmol EGTA/l. Then, motility was restored by the subsequent addition of 2 mmol CaCl_2 /l without the addition of inhibitors (control). As well as control spermatozoa, restoration of motility by Ca^{2+} was observed in the presence of either PD 150606 or Y-27632 (Fig. 3a). The presence of calyculin A permitted reactivation of demembranated spermatozoa at 40 °C, and this effect was maintained after addition of PD 150606 or Y-27632 as well as in the control (no addition) (Fig. 3b).

Effects of PD 150606 or Y-27632 on the ATP concentrations of fowl spermatozoa

As with the effect on motility, the ATP concentrations of spermatozoa were fairly constant following exposure to Y-27632, CaCl_2 or calyculin A and their mixtures at 30 °C. In spite of the inhibition of motility in PD 150606-treated spermatozoa, the ATP concentrations increased slightly, compared with those of the other treated samples, but there was no difference among the nine groups (Fig. 4a). Additionally, at 40 °C, the ATP concentrations of spermatozoa after the addition of PD 150606 or Y-27632 were almost the same values compared with those of untreated spermatozoa (control). However, the ATP concentrations of spermatozoa decreased in the presence of Ca^{2+} or calyculin A alone and Ca^{2+} or calyculin A with Y-27632, presumably due to the restoration of motility (Fig. 4b).

Figure 3 The time course of motility of demembrated fowl spermatozoa after addition of: (a) 2 mmol EGTA/l and 2 mmol CaCl_2 /l alone (control, ●) and 100 μmol PD 150606/l (□) or 100 μmol Y-27632/l (○) at 30°C; (b) 100 nmol calyculin A/l alone (control, ●) and 100 μmol PD 150606/l (□) or Y-27632/l (○) at 40°C. Each point represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. There was no significant difference between the presence of inhibitors and control (absence of inhibitors) at each period.

Immunoblot identification of calpain in fowl spermatozoa

No appreciable immunoreactive protein was detected in the negative control lane (no antibody). In contrast, a protein of approximately 80 kDa was recognized by the anti-calpain 12 antibody which corresponds to the molecular weight of the large subunit of calpain 12, even though the visible immunoreactive band was faint (Fig. 5).

Discussion

Substantial evidence indicates that smooth muscle contraction is regulated by the cytosolic Ca^{2+} concentration and by the Ca^{2+} sensitivity of myofilaments: the former activates myosin light chain phosphorylation mediated by the Ca^{2+} -calmodulin-dependent myosin light chain kinase pathway and the latter is achieved partly by inhibition of myosin light chain phosphatase activity (Somlyo & Himpsen 1989, Kitazawa *et al.* 1991, Somlyo & Somlyo 1994). The small GTPase Rho and its target protein, Rho-kinase, seem to participate in this latter mechanism *in vitro* (Hirata *et al.* 1992, Gong *et al.* 1996, Kimura *et al.* 1996). It appears that Rho-kinase phosphorylates myosin light chain phosphatase, thus inactivating it *in vitro* (Kimura *et al.* 1996). Moreover, it has been demonstrated that the addition of a Rho-kinase inhibitor, Y-27632, inhibits smooth muscle contraction selectively by inhibiting the phosphorylation of myosin light chain phosphatase and then the Ca^{2+} sensitization (Uehata *et al.* 1997).

The regulatory serine/threonine protein phosphatases, such as myosin light chain phosphatase, are classified into four main enzymes: type 1 (PP1), type 2A (PP2A), type 2B (PP2B) and type 2C (PP2C); myosin light chain phosphatase activity in smooth muscle is classified as PP1 (Cohen 1989). On the other hand, PP1 appears to be dominant in

the temperature-dependent inhibition of flagellar movement of fowl spermatozoa at body temperatures of 40°C, since the motility of demembrated fowl spermatozoa at 40°C was stimulated by the addition of calyculin A or okadaic acid (specific inhibitors of PP1 and PP2A), and inhibitors 1 and 2 (small heat-stable proteins which inhibit PP1 activity) (Ashizawa *et al.* 1994b). In addition, the motility of demembrated fowl spermatozoa at 30°C decreased markedly following the addition of recombinant PP1 supplemented with Mn^{2+} (Ashizawa *et al.* 1997). These results, together with the Rho-kinase involvement in smooth muscle contraction by phosphorylating PP1 (Velasco *et al.* 2002), invoke the following hypothesis: if Rho-kinase is involved in the reversible temperature-dependent immobilization of fowl spermatozoa, then the addition of Y-27632 would not permit the restoration of motility at 40°C, because there would be no inhibition of PP1 activity. In the present study, however, no inhibition of motility of spermatozoa by the presence of Y-27632 was observed after the addition of stimulators, such as CaCl_2 or calyculin A at 40°C (Fig. 2b and d). Furthermore, the vigorous motility of spermatozoa at 30°C was not inhibited following the addition of Y-27632 (Fig. 2a and c). Therefore, Rho-kinase cannot be demonstrated to be involved in the regulation of motility of fowl spermatozoa *in vitro*.

It has been suggested that the acrosome reaction of spermatozoa is caused by the increase of intracellular Ca^{2+} concentration and Ca^{2+} sensitization (for review see Fraser 1995, Benoff 1998, Baldi *et al.* 2000, Guraya 2000, Topfer-Petersen *et al.* 2000). As mentioned earlier, Y-27632 appears to inhibit the Ca^{2+} sensitization mechanism in smooth muscle (Uehata *et al.* 1997). Thus, if Rho-kinase is involved in the acrosome reaction of spermatozoa, the addition of Y-27632 may inhibit the induction of

Figure 4 Effects of PD 150606 (100 μ mol/l), Y-27632 (100 μ mol/l), CaCl_2 (2 mmol/l) and calyculin A (100 nmol/l) on the ATP concentrations of fowl spermatozoa incubated at (a) 30°C and (b) 40°C. PD 150606 or Y-27632 was added at the start of incubation and then 15 min after the start of incubation, CaCl_2 or calyculin A was added. Spermatozoa were incubated for a total of 30 min. Each value represents the mean ($\pm$ S.E.M.) of five samples of spermatozoa. Values with different superscripts differ significantly from each other ($P < 0.05$).

the acrosome reaction. However, during incubation for 30 min at 40°C, the presence of Y-27632 within the range 1–100 μ mol/l could not inhibit the CaCl_2 /IPVL-induced acrosome reaction (Fig. 1d). Therefore, as with motility, Rho-kinase does not appear to be involved in the regulation of the acrosome reaction of fowl spermatozoa *in vitro* or Rho-kinase may not exist in fowl spermatozoa.

Calpain, a Ca^{2+} -dependent cysteine protease, has been found in mammalian spermatozoa (Schollmeyer 1986, Rojas *et al.* 1999, Rojas & Moretti-Rojas 2000, Yudin *et al.* 2000, Aoyama *et al.* 2001), and this protease appears to be involved in the regulation of sperm motility (Aoyama *et al.* 2001, Ozaki *et al.* 2001), the acrosome reaction (Schollmeyer 1986, Yudin *et al.* 2000, Aoyama *et al.* 2001) and the cell fusion process that takes place during

Figure 5 Immunoblot analysis of fowl sperm calpain 12. Lane 1, fowl sperm lysate incubated without a polyclonal antibody of calpain 12 (control); lane 2, fowl sperm lysate incubated with a rabbit polyclonal antibody raised against a synthetic peptide based on the amino-terminal end of domain-I in the large subunit of calpain 12.

penetration of the oocyte (Rojas *et al.* 1999, Rojas & Moretti-Rojas 2000).

In the study reported here, immunoblot analysis of fowl sperm proteins showed that a protein of approximately 80 kDa was recognized by polyclonal antibodies raised against a synthetic peptide based on the amino-terminal end of domain-I in the large subunit of calpain 12 (Fig. 5), suggesting that calpain 12 may be present in fowl spermatozoa. The present study also demonstrated that unlike in mammalian spermatozoa, calpain appears not to be involved in the regulation of the acrosome reaction of fowl spermatozoa, since the presence of PD 150606 before the addition of CaCl_2 did not inhibit the acrosome reaction within the range of 1–100 μ mol/l during incubation for 30 min at 40°C (Fig. 1b). However, as for mammalian spermatozoa, the flagellar movement of fowl spermatozoa may be controlled by calpain, since even in the presence of CaCl_2 or calyculin A, the motility of intact spermatozoa at 40°C remained inhibited following the addition of PD 150606 (Fig. 2b and d). Under all of these circumstances, sperm ATP concentrations were not reduced by the addition of PD 150606 (Fig. 4). Thus, it appears that the addition of PD 150606 was not simply affecting membrane damage or inhibiting energy production in these spermatozoa, but may be acting on some part of the regulatory cascade of flagellar movement. Furthermore, it seems that calpain might be present in the cytoplasmic matrix and/or the plasma membrane, but not retained in the axoneme and/or accessory cytoskeletal components, since the motility of demembrated spermatozoa was not affected by the addition of the same concentrations of PD 150606 (Fig. 3). This suggestion was consistent with the observations that calpain 2 seems to be associated with porcine sperm membranes (Schollmeyer 1986) and both calpains 1 and 2 were localized at the ultrastructural level to the region between the plasma membrane and the outer acrosomal membrane of cynomolgus macaque spermatozoa (Yudin *et al.* 2000).

In conclusion, Rho-kinase could not be shown to be involved in the regulation of the acrosome reaction or motility of fowl spermatozoa, but calpain appears to be involved in the calcium-initiated cascade that regulates flagellar movement of fowl spermatozoa, but not in that which induces the acrosome reaction.

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