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The Addition of Mitogen-Activated Protein Kinase and p34^{cdc2} Kinase Substrate Peptides Inhibits the Flagellar Motility of Demembranated Fowl Spermatozoa

Koji Ashizawa,[1] Kei Hashimoto, Mari Higashio, and Yasuhiro Tsuzuki
Laboratory of Animal Reproduction, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan

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The motility of demembranated fowl spermatozoa was vigorous at 30°C, but decreased markedly following the addition of mitogen-activated protein (MAP) kinase or p34^{cdc2} kinase substrate peptide. Dephosphorylation of approximately 116, 86 and 79-kDa proteins of demembranated spermatozoa was observed after the addition of MAP kinase or p34^{cdc2} kinase substrate peptide. The activities of MAP kinase and histone H1 kinase of spermatozoa, estimated by measuring the phosphorylation of myelin basic protein and histone H1 as substrates, were 1.22 and 0.29 pmol/min/mg protein, respectively. Both enzymatic activities of spermatozoa were lower than those of chick brain, but higher than those of chick liver. These results suggest that the phosphorylation of axonemal and/or accessory cytoskeletal proteins mediated by MAP kinase and p34^{cdc2} kinase may be involved in the regulation of flagellar movement of fowl spermatozoa. © 1997 Academic Press

Although it is well recognized that the mechanochemical mechanism of flagellar movement of spermatozoa is based on the active sliding of microtubules as a result of ATPase activity on the dynein arms of the outer doublet microtubules (reviewed in [1-4]), much remains to be learned about how the sliding mechanism is regulated. To investigate this regulatory mechanism, fowl spermatozoa provide an excellent model, since they display the unique phenomenon of reversible temperature-dependent immobilization: in simple salt solutions they become immotile at the avian body temperature of 40-41°C, but motility is restored by decreasing the temperature [5-10]. It appears that the substance(s) involved in this regulatory system is closely associated with the axoneme and/or accessory cytoskeletal components, since the motility of demembranated spermatozoa is, as with intact spermatozoa, negligible at 40°C and restored at 30°C [11, 12] and physical damage to the demembranated spermatozoa, such as rapid freezing and thawing without cryoprotectants, is not enough to remove the substance(s) [13]. However, we know remarkably little about the actual molecular mechanisms of the immobilization and restoration of motility.

Spermatozoa are the terminal products of one of the most spectacular differentiation processes, yielding a cell that, although incapable of both transcription and protein synthesis, is still able to undergo a series of dynamic changes prior to fertilization. It is thus not illogical to believe that protein phosphorylation, a major mechanism for regulation of many cellular processes in eucaryotic systems, has a pivotal role in the sequence of integrated events in which spermatozoa are involved [14]. Substantial evidence implicates a role in protein phosphorylation by cAMP and cAMP-dependent protein kinase for the initiation and activation of sperm motility (reviewed in [15-19]). However, unlike that of other species, the motility of demembranated fowl spermatozoa was not restored by the addition of cAMP at 40°C [12, 20], and the presence of cAMP-dependent protein kinase substrate peptides did not appreciably affect the motility at 30°C [21, 22].

Recently, in contrast, it has been proposed that the phosphorylation of axonemal and/or accessory cytoskeletal protein(s) by myosin light chain kinase (MLCK) or an MLCK-like protein, rather than a cAMP-dependent protein kinase, may be involved in intracellular cascade systems for the regulation of fowl sperm motility, since the presence of not only 1-(5-chloronaphthalene-1-sulfonyl)-1H-hexahydro-1, 4-diazepine hydrochloride (ML-9), a specific inhibitor of MLCK, but also an MLCK substrate peptide, inhibited the motility of demembranated spermatozoa at 30°C [22].

Both MAP kinase and p34^{cdc2} kinase have been implicated in growth, differentiation and proliferation of

[1] To whom correspondence should be addressed. E-mail: a0c303u@cc.miyazaki-u.ac.jp.

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cells [23]. The $p34^{cdc2}$ kinase, a component of maturation promoting factor (MPF) together with a regulatory cyclin, is specifically active during mitosis and has been linked to the profound changes preceding mitosis such as chromosome condensation, cytoskeletal rearrangement and nuclear membrane breakdown [24, 25]. MAP kinase and $p34^{cdc2}$ kinase may also be involved in the regulation of the contractile apparatus, since contractile regulation by thin-filament-associated proteins in smooth muscle, such as caldesmon and calponin, possibly through their phosphorylation by MAP kinase and/or other kinases, has been intensively studied, but remains to be conclusively proved [26]. In addition, it is suggested that a protein kinase containing $p34^{cdc2}$ has MLCK activity [27] and purified cyclin-$p34^{cdc2}$ kinase phosphorylates the myosin light chain of cytoplasmic and smooth muscle myosin-II *in vitro* [28].

With regard to spermatozoa, however, limited information is available concerning the effects of such kinases on the motility of vertebrate and invertebrate spermatozoa: only in human spermatozoa, the presence and possible involvement of $p34^{cdc2}$ kinase in capacitation and/or acrosome reaction are suggested [29]. In the following experiment, therefore, attempts were made to investigate the effects of MAP kinase and $p34^{cdc2}$ kinase substrate peptides on the motility and phosphorylation status of demembranated 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. New born chicks were also used for the analysis of MAP kinase and histone H1 kinase activities.

Semen was collected by the method of Bogdonoff and Shaffner [30]. Samples of semen pooled from four to six males were diluted approximately tenfold in 150 mM NaCl with 20 mM TES (*N*-Tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid) at pH 7.4 and centrifuged at $700 \times g$ for 15 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. Samples of 3-4 ml were poured into 30 ml Erlenmeyer flasks with a screw cap.

Chemicals. MAP kinase substrate peptide, Ala-Pro-Arg-Thr-Pro-Gly-Gly-Arg-Arg, including amino acid residues 95-98 in bovine myelin basic protein, and $p34^{cdc2}$ kinase substrate peptide, Pro-Lys-Thr-Pro-Lys-Lys-Ala-Lys-Lys-Leu, corresponding to amino acids 9-18 of histone H1 were purchased from Seikagaku Co., Ltd. (Tokyo, Japan). Calyculin A was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ATP, aprotinin, bovine serum albumin (BSA), cAMP-dependent protein kinase inhibitor (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp), dithiothreitol (DTT), β-glycerophosphate, Hepes, histone H1 (type III-S), leupeptin, myelin basic protein, potassium glutamate, sodium orthovanadate, TES and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO). BCA protein assay regent was from Pierce Chemical Co. (Rockford, IL). SDS-PAGE molecular weight standards were purchased from Bio-Rad Laboratories (Hercules, CA). [γ-^{32}P]ATP was from Du Pont-New England Nuclear (Boston, MA). Scintillation fluid (ACSII) was obtained from Amersham International plc. (Amersham, Buckinghamshire, U.K.). Other chemicals were of regent grade from Nacalai Tesque Inc. (Kyoto, Japan).

Measurement of motility of demembranated spermatozoa. Demembranation and reactivation of spermatozoa were performed at 30°C and 40°C according to the method described previously [12]. The extraction medium used consisted of 0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM DTT and 20 mM Tris-HCl buffer (pH 7.9). The reactivation medium consisted of 0.5 mM ATP, 200 mM sucrose, 25 mM potassium glutamate, 1.5 mM $MgSO_4$, 1 mM DTT and 20 mM Tris-HCl buffer (pH 7.9). To examine the effects of MAP kinase and $p34^{cdc2}$ kinase, the corresponded substrate peptides were added to the reactivation medium. The addition of EGTA and $CaCl_2$ or calyculin A, a specific inhibitor of protein phosphatase-type 1 and -type 2, were also performed.

The suspension of demembranated 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 videomicroscopy (magnification on the 12-inch black and white monitor was approximately $\times$ 600) at 30°C or 40°C [31]. Measurements were made on a total of 200-300 spermatozoa, distributed uniformly among three or more fields, to determine the percentage of vigorously motile spermatozoa.

Phosphorylation of endogenous proteins and electrophoresis. Phosphorylation and electrophoresis on polyacrylamide gels of demembranated sperm proteins were carried out according to the methods described previously [32], but with some modifications. Briefly, demembranated spermatozoa were incubated for 2.5 min at 30°C with extraction-reactivation medium, containing 0.1% Triton X-100, 200 mM sucrose, 25 mM potassium glutamate, 1 mM $MgSO_4$, 1 mM DTT, 20 mM Tris-HCl buffer (pH 7.9), 0.1 mM ATP and approximately 7,000 cpm/pmol [γ-^{32}P]ATP. To examine the effects of MAP kinase and $p34^{cdc2}$ kinase on phosphorylation, these substrate peptides were added to the medium. At the end of the incubation, sodium pyrophosphate and unlabelled ATP, at final concentrations of 15 mM and 10 mM, respectively, were added to stop the reaction, and the samples were placed on ice. Each sample was centrifuged at $16{,}000 \times g$ for 10 min at 4°C, and the supernatant was discarded. Laemmli [33] sample buffer was added to the pellets and boiling for 5 min. Samples containing protein from approximately 2.2×10^6 spermatozoa were loaded on to 10% or 5-20% SDS-polyacrylamide slab gels, and subjected to electrophoresis. Autoradiography was performed at -70°C for 2-4 days exposure to X-ray film with an intensifying screen (Lightning plus, Du Pont, Wilmington, DE).

Measurment of MAP kinase and histone H1 kinase activities. Washed spermatozoa were suspended in the extraction buffer consisting of 50 mM Hepes, pH 7.4, 50 mM β-glycerophosphate, 25 mM NaF, 150 mM NaCl, 15 mM $MgCl_2$, 0.1% Triton X-100, 20 mM EGTA, 1 mM DTT, 25 μg leupeptin/ml, 25 μg aprotinin/ml and 0.2 mM sodium orthovanadate. The suspension was sonicated for 60 sec on ice with an ultrasonic processor with a microtip (Sonics & Materials, Inc., Danbury, CT) at 50 W. After removing cellular debris by centrifuging at $16{,}000 \times g$ for 20 min at 4°C, the supernatant was collected as a sample for the enzyme activity assay. New born chick brain and liver and rooster testes were also treated as the same manner, except the homogenization; in these cases teflon glass homogenizer was used. Protein concentration was determined using BCA protein assay reagent and BSA as a standard.

The activities of enzymes were measured, using myelin basic protein or histone H1 as a substrate for MAP kinase or histone H1 kinase, respectively. Ten μl of sample described above and 15 μl of substrate solution containing 33μM [γ-^{32}P]ATP (6,000-9,000 cpm/pmol), 50 mM Hepes, pH 7.4, 50 mM β-glycerophosphate, 25 mM NaF, 150 mM NaCl, 15 mM $MgCl_2$, 0.1% Triton X-100, 20 mM EGTA, 1 mM DTT, 25 μg leupeptin/ml, 25 μg aprotinin/ml, 0.2 mM sodium orthovanadate, 0.8 μM cAMP-dependent protein kinase inhibitor and with or without (for background incorporation) 1.7 mg substrate/ml

TABLE 1

Effects of MAP Kinase and p34^{cdc2} Kinase Substrate Peptides on the Motility of Demembranated Fowl Spermatozoa at 30°C and 40°C

Substrate peptide	Motility (%)	
	30°C	40°C
None (control)	79.5 ± 4.3[a]	0[a]
MAP kinase	0.5 ± 0.5[b]	0[a]
p34^{cdc2} kinase	1.9 ± 0.7[b]	0[a]

Each peptide added in the reactivation medium was 0.5 mM. Each value represents the mean (±S.E.M.) of five samples of spermatozoa. Within columns, values with different superscripts differ significantly ($P < 0.01$) from each other.

were mixed and incubated at 30°C for 30 min. The reaction was stopped by the addition of 400 μl of 20% trichloroacetic acid; 50 μl of 1% BSA was also added as a carrier protein for precipitation. Following centrifugation at 16,000 × *g* for 10 min at 4°C, the precipitates were washed twice with 400 μl of 20% trichloroacetic acid and dissolved in 400 μl of 1 M NaOH. The solution was then transferred to scintillation vials with 3 ml of scintillation fluid, and radioactivity was counted using a liguid scintillation counter (Pharmacia, Wallace 1410, Turku, Finland). Kinase activities were expressed as pmoles of phosphate incorporated per minute per mg protein.

Statistical analysis. Percentage of motility was transformed using arc sine transformation. The results were analyzed by DuncanÕs multiple-range tests [34].

RESULTS

Effects of MAP Kinase and p34^{cdc2} Kinase Substrate Peptides on the Motility of Demembranated Fowl Spermatozoa

In the presence of ATP, the percentage of motility of demembranated spermatozoa without substrate peptide was high at 30°C, but negligible at 40°C. At 30°C, in contrast, inhibition of motility of demembranated spermatozoa was observed following the addition of either MAP kinase or p34^{cdc2} kinase substrate peptide. At 40°C, no stimulation or inhibition of motility of demembranated spermatozoa was observed after the addition of any substrate peptides (Table 1).

Table 1 shows the percentage of motility at 5 min after the addition of substrate peptides. The time course of the effects of substrate peptides are shown in Figs. 1 and 2. The presence of calyculin A, a specific inhibitor of protein phosphatase-type 1 and -type 2, permitted reactivation of demembranated spermatozoa at 40°C. However, even in the presence of calyculin A, no stimulation of motility was observed by the addition of MAP kinase or p34^{cdc2} kinase substrate peptide at both 30°C and 40°C (Fig. 1a, b).

Sperm motility was inhibited immediately after the addition of 1 mM EGTA at 30°C and was restored by the subsequent addition of 2 mM $CaCl_2$ without the addition of substrate peptide. However, restoration of motility by Ca^{2+} was not observed in the presence of either MAP kinase or p34^{cdc2} kinase substrate peptide (Fig. 2).

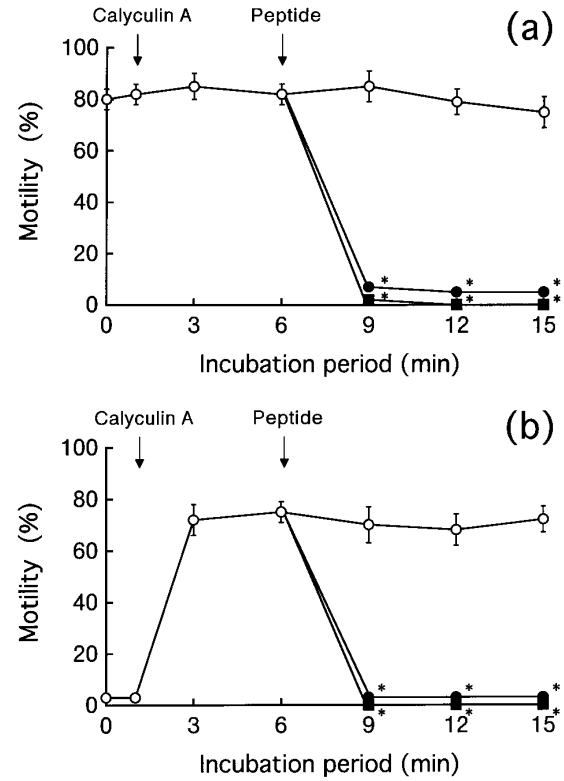

FIG. 1. The time course of motility of demembranated fowl spermatozoa in the reactivation medium at (a) 30°C and (b) 40°C following addition of 100 nM calyculin A and 0.5 mM substrate peptide (○; no addition, ●; MAP kinase, ■; p34^{cdc2} kinase). Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *$P<0.01$ compared with value of control (no addition of substrate peptide) at each period.

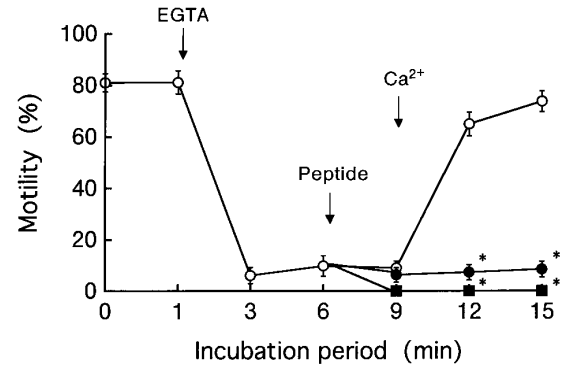

FIG. 2. The time course of motility of demembranated fowl spermatozoa in the reactivation medium at 30°C following addition of 1 mM EGTA, 0.5 mM substrate peptide (○; no addition, ●; MAP kinase, ■; p34^{cdc2} kinase) and 2 mM $CaCl_2$. Each point represents the mean (±S.E.M.) of five samples of spermatozoa. *$P<0.01$ compared with value of control (no addition of substrate peptide) at each period.

Effects of MAP Kinase and p34^{cdc2} Kinase Substrate Peptides on the Phosphorylation State of Proteins of Demembranated Fowl Spermatozoa

In the control samples, six major phosphorylated protein bands of molecular weights approximately 79, 50, 44, 33, 29 and 18-kDa were identified, together with several minor phosphorylated proteins. After the addition of p34^{cdc2} kinase substrate peptide, dephosphorylation of 116, 86 and 79-kDa proteins (Fig. 3a, b, denoted by arrows) and increased phosphorylation of 29 and 28-kDa proteins were observed. The similar dephosphorylation pattern was found after the addition of MAP kinase substrate peptide with additional increased phosphorylation of 50 and 44-kDa proteins.

MAP Kinase and Histone H1 Kinase Activities of Fowl Spermatozoa and Different Tissues

Since it is suggested that p34^{cdc2} kinase and histone H1 kinase are the same entity [35], we measured the activity of histone H1 kinase in this experiment, using histone H1 as a substrate. Among 4 kinds of cells, the highest activity was obtained for the chick brain in both MAP kinase and histone H1 kinase. Both enzymatic activities of spermatozoa were lower than those of chick brain, but significantly higher than those of chick liver ($P<0.01$) (Table 2).

DISCUSSION

It is proposed that MAP kinase is activated by p34^{cdc2} kinase during meiotic maturation in *Xenopus,* since the activity of MAP kinase increased shortly after the increase in histone H1 kinase activity and was activated by purified p34^{cdc2} kinase in *Xenopus* extracts [36, 37]. In contrast, MAP kinase may not be downstream of p34^{cdc2} kinase in the cascade of phosphorylation in mouse oocytes, since MAP kinase activity increased before the activation of histone H1 kinase during meiotic maturation [38]. In any case, both MAP kinase and p34^{cdc2} kinase appear to be closely related and play a critical role in the signal transduction pathway of eukaryotic cell cycles. The present study showed that the addition of MAP kinase and p34^{cdc2} kinase substrate peptides completely inhibited the percentage of motile demembranated spermatozoa at 30°C. Furthermore, our results indicate that fowl spermatozoa, even though they are incapable of both transcription and protein synthesis, possessed the activities of these kinases, estimated by measuring the phosphorylation of myelin basic protein and histone H1 as substrates: the activities of spermatozoa were lower than those of chick brain, but higher than those of chick liver. These results suggest that the activities of MAP kinase and p34^{cdc2} kinase, present in the axoneme and/or accessory cytoskeletal components, may be involved in the maintenance of fowl sperm motility.

In human spermatozoa, p34^{cdc2} kinase seems to act

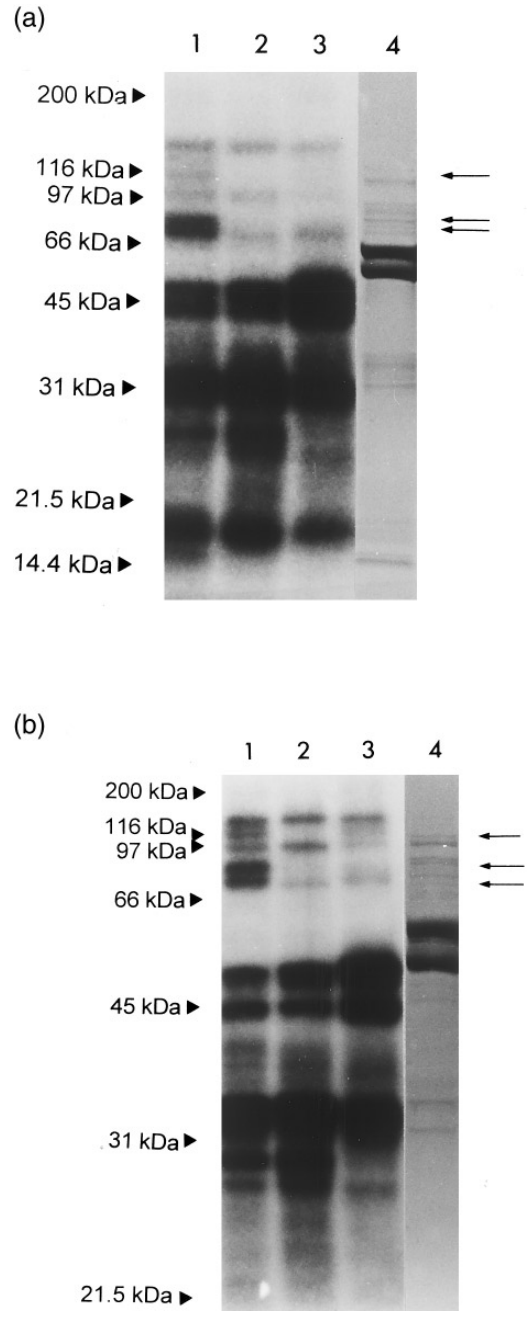

FIG. 3. (a) 5–20% and (b) 10% SDS–PAGE profile of demembranated fowl sperm proteins stained with the Coomassie blue (lane 4) and corresponding autoradiography of phosphorylated proteins (lanes 1–3). Triton X-100-extracted and reactivated samples were incubated for 2.5 min at 30°C. Approximately 7000 cpm/pmol [γ-^{32}P]ATP and 0.1 mM ATP were added in the medium. Samples containing protein from approximately 2.2×10^6 spermatozoa were loaded. Lane 1, no treatment (control); lane 2, presence of 0.5 mM p34^{cdc2} kinase substrate peptide; lane 3, presence of 0.5 mM MAP kinase substrate peptide.

TABLE 2

The Activity of MAP Kinase and Histone H1 Kinase in Fowl Spermatozoa and Various Tissues at 30°C

Tissue	Activity (pmol/min/mg protein)	
	MAP kinase	Histone H1 kinase
Fowl spermatozoa	1.22 ± 0.10^{a}	0.29 ± 0.02^{a}
Fowl testes	1.15 ± 0.14^{a}	0.62 ± 0.03^{b}
Chick liver	0.42 ± 0.07^{b}	0.14 ± 0.02^{c}
Chick brain	11.89 ± 0.94^{c}	1.90 ± 0.15^{d}

Each value represents the mean (±S.E.M.) of five different samples. Within columns, values with different superscripts differ significantly ($P < 0.01$) from each other.

differently: the addition of antibodies to cyclins and $p34^{cdc2}$ kinase did not have any significant effect on the percentage of motile cells, although they significantly affected various sperm motility parameters involved in hyperactivation phenomenon, such as velocity, amplitude of lateral head displacement and beat cross frequency. Thus, the presence and possible involvement of cyclin and $p34^{cdc2}$ kinase in the capacitation and/or acrosome reaction of human spermatozoa has been suggested [29].

As described above, it seems likely that MAP kinase and $p34^{cdc2}$ kinase are involved in the regulation of fowl and mammalian spermatozoa. To date, however, no identification of the substrate protein(s) has been reported. In the present study, autoradiography of demembranated sperm proteins revealed that the inhibition of fowl sperm motility was associated with dephosphorylation of Mr 116, 86 and 79-kDa proteins. Interestingly, these proteins were also dephosphorylated in the presence of recombinant protein phosphatase type 1 (PP-1) and Mn^{2+} in the demembranated fowl spermatozoa [39]. These results suggest that dephosphorylation of some of these proteins may be involved in the inhibition of motility. Furthermore, it appears that these proteins are present in the axoneme and/or accessory cytoskeletal components, but not retained in the plasma membrane and/or cytoplasm.

The activation of PP-1, one of the serine/threonine phosphatases, present in the axoneme and/or accessory cytoskeletal components of fowl spermatozoa, appears to be involved in the temperature-dependent inhibition of motility, since the immobilization of demembranated spermatozoa at 40°C can be reversed by the addition of inhibitors of PP-1, such as okadaic acid, calyculin A, inhibitors 1 and 2 [40]. Furthermore, the addition of recombinant PP-1 supplemented with Mn^{2+} inhibited the motility of demembranated spermatozoa at 30°C [39]. In the study reported here, stimulation of the motility of demembranated spermatozoa at 40°C by calyculin A was inhibited by the subsequent addition of MAP kinase and $p34^{cdc2}$ kinase substrate peptides. Thus, it is possible that MAP kinase and $p34^{cdc2}$ kinase may be acting on some part of the regulatory cascade initiated by calyculin A. Recent work has demonstrated that PP-1 of rabbit skeletal muscle, as well as recombinant PP-1α and PP-1γ1, but not PP-2A, was phosphorylated and inhibited their activity by cyclin-dependent kinases such as $p34^{cdc2}$/cyclin A and $p34^{cdc2}$/cyclin B [41]. From these results together with our study, it may be hypothesized that when exogenous $p34^{cdc2}$ kinase substrate peptide is added to the demembranated fowl spermatozoa, PP-1, present in axoneme and/or accessory cytoskeletal components, is activated due to the inhibition of phosphorylation of PP-1 by endogenous $p34^{cdc2}$ kinase. Then, Mr 116, 86 and 79-kDa proteins are dephosphorylated, resulting the inhibition of motility.

In the present study, restoration of motility by Ca^{2+} was not observed in the presence of $p34^{cdc2}$ kinase substrate peptide, suggesting another possibility that if $p34^{cdc2}$ kinase possesses MLCK activity, one of Ca^{2+}/calmodulin-dependent protein kinases [27], then the addition of $p34^{cdc2}$ kinase substrate peptide inhibits the phosphorylation of substrate protein(s) by endogenous MLCK, or an MLCK-like protein which seems to be essential for the maintenance of fowl sperm motility [22], resulting the inhibition of motility. However, with our present state of knowledge, functional roles for these novel enzymes in the signal transduction pathway leading to the regulation of fowl sperm motility awaits further experimental investigation.

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