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A Cyclic Adenosine 3′,5′-Monophosphate Stimulates Phospholipase Cγ1-Calcium Signaling via the Activation of Tyrosine Kinase in Boar Spermatozoa

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ABSTRACT: The aim of this study was to reveal a downstream part of the intracellular signaling that is mediated by cyclic adenosine monophosphate (cAMP)-dependent tyrosine kinases, including spleen tyrosine (Y) kinase (SYK), in boar spermatozoa. Ejaculated spermatozoa were incubated with cBiMPS (a cell-permeable cAMP analog; 0.1 mM) at 38.5°C for 180 minutes and then used for Western blot and indirect immunofluorescence. Incubation of spermatozoa with cBiMPS induced tyrosine phosphorylation at the linker region of SYK (which was essential to binding to phospholipase C [PLC]γ1) in the connecting and principal pieces, but the tyrosine phosphorylation was abolished by the addition of H-89 (a protein kinase A [PKA] inhibitor; 0.01–0.1 mM). Moreover, the cAMP-dependent tyrosine phosphorylation was also induced at the key regulatory residue of PLCγ1 in the same segments of spermatozoa, but it was inhibited by the addition of herbimycin A (a tyrosine kinase inhibitor; 5 μM). These results suggest that the sperm cAMP-dependent tyrosine kinases, including SYK, are linked to the activation of PLCγ1. Indirect immunofluorescence clearly detected both inositol 1,4,5-trisphosphate (IP_3) receptor and calreticulin in the connecting piece, indicating the presence of internal calcium store. Cell imaging with fluo-3/AM (a cell-permeable Ca^{2+} indicator) showed that incubation of spermatozoa with cBiMPS increased intracellular free calcium in the middle piece, but that it was reduced by the addition of U-73122 (a PLC inhibitor; 0.02 mM). Based on our findings, we conclude that the connecting piece of boar spermatozoa possesses the PLCγ1-IP_3 receptor-calcium signaling that is triggered by cAMP and mediated by PKA and herbimycin A–sensitive tyrosine kinases, including SYK.

Key words: Sperm, cAMP, SYK, PLC, calcium store.
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In mammalian spermatozoa, cyclic adenosine monophosphate (cAMP) has been considered to play a pivotal role in the expression of fertilizing ability. This second messenger is generated from adenosine triphosphate mainly by a soluble form of the adenylyl cyclase that is stimulated directly by the action of bicarbonate and calcium (Okamura et al, 1985; Kaupp and Weyand, 2000; Jaiswal and Conti, 2003). The cytoplasmic cAMP activates protein kinase A (PKA), which catalyzes protein phosphorylation at the serine and threonine residues. Interestingly, the protein serine/threonine phosphorylation leads to the protein tyrosine phosphorylation through the activation of protein tyrosine kinase, inactivation of protein tyrosine phosphatase, or both (Visconti and Kopf, 1998; Breitbart, 2002), though details of the mechanism remain to be determined. Recently, we (Harayama et al, 2004a) revealed that a spleen tyrosine (Y) kinase (SYK) tyrosine kinase is present in the connecting and principal pieces of boar spermatozoa, and that it is tyrosine phosphorylated at the activation loop of the kinase domain (Tyr518/519 residues) in response to the treatment with a cell-permeable cAMP analog. Moreover, the cAMP-dependent tyrosine phosphorylation of sperm SYK is inhibited by a PKA inhibitor (H-89). These findings suggest that the activation of sperm SYK is mediated via cAMP-PKA signaling. This regulatory mechanism for SYK in the spermatozoa is quite different from that in the lymphocytes (Dustin and Chan, 2000; Kurosaki and Tsukada, 2000; Wonerow and Watson, 2001) and platelets (Ichinohe et al, 1995; Wang et al, 1997).

In somatic cells, one of the substrates of SYK is phospholipase C (PLC)γ1, which hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG; Law et al, 1996; Di Bartolo et al, 1999; Williams et al, 1999). These second messengers initiate calcium signaling: the former induces release of calcium from the internal store by binding to IP_3 receptor (IP_3R; Taylor and Traynor, 1995; Singer et al, 1997), and the latter stimulates protein kinase C, which requires calcium for the activation (Nishizuka, 1984;

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Newton, 1995). The binding of SYK to PLCγ1 is regulated by tyrosine phosphorylation at the activation loop of kinase domain (Tyr518/519 residues in the pig; Taniguchi et al, 1991; Law et al, 1994, 1996). Moreover, the phosphorylation at the linker region joining the C-terminal SH2 domain and the kinase domain of SYK (Tyr348/352 residues in the human and Tyr341/345 residues in the pig) also plays an important role in binding to PLCγ1 (Taniguchi et al, 1991; Law et al, 1996). The enzymatic activity of PLCγ1 is stimulated by the tyrosine phosphorylation at the key regulatory residue of PLCγ1 (Tyr783 residue), which the active SYK catalyzes in the lymphocytes (Law et al, 1996). These strongly indicate that tyrosine phosphorylation is a key regulatory event for the activation of SYK-PLCγ1-calcium signaling in somatic cells.

In this article, we have shown data that support the hypothesis that cAMP stimulates the PLCγ1-calcium signaling via the activation of tyrosine kinases, including SYK, in boar spermatozoa. Our data suggest a new role of cAMP as a trigger for the activation of PLCγ1-calcium signaling in sperm flagella.

Materials and Methods

An Animal Use Ethics Statement

Our research plan and the feeding condition of our boars were investigated by the Committee of Laboratory Animal Experiments at Kobe University, Kobe, Japan. With the approval of this committee (approval #16-04-08), we undertook the following experiments.

Preparation of Sperm Samples

Sperm-rich fractions from ejaculates were obtained from 3 mature boars by a manual method. The spermatozoa were washed in an isotonic Percoll (Amersham Biosciences Corp, Piscataway, NJ) and then in phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich Co, St Louis, Mo) by centrifugation, as previously described (Harayama et al, 2004a,b). A basic incubation medium was a modified Krebs-Ringer Hepes medium (94.60 mM NaCl, 4.78 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 1.71 mM $CaCl_2$, 25.07 mM Hepes, 5.56 mM glucose, 0.50 mM sodium pyruvate, 21.58 mM sodium lactate, 50 μg/mL streptomycin sulfate, 100 IU/mL potassium penicillin G, and 2 μg/mL phenol red; pH 7.4), which was additionally supplemented with 0.1% PVA and 0.25 mM sodium orthovanadate (Na_3VO_4; Sigma-Aldrich) before use. Our previous report (Harayama et al, 2004a) suggested that the addition of sodium orthovanadate had an enhancing effect on the activity of sperm SYK. However, this inhibitor had a side effect on other events associated with phosphatases and, consequently, reduced sperm motility (see ''Results''). Therefore, in the experiments to examine possible relationships between the cAMP-SYK signaling and sperm motility, we used the medium without sodium orthovanadate. The cell-permeable, phosphodiesterase-resistant cAMP analog Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophoshorothioate (cBiMPS; Biomol Research Laboratories, Inc, Plymouth Meeting, Pa; Schaap et al, 1993) was dissolved in 10% (vol/vol) dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) as a 4-mM stock solution. Then, it was added to the incubation medium to adjust a final concentration to 0.1 mM. To the control samples without cBiMPS, the same volume of 10% (v/v) DMSO was added to equalize the concentration of solvent. The washed spermatozoa were resuspended in the incubation medium to adjust a final sperm concentration to 1.0×10^8 cells/mL and then were incubated in a water bath (38.5°C) up to 180 minutes. Immediately before and after incubation for 30 to 180 minutes, aliquots of the sperm suspensions were recovered and then used for the following experiments. In some experiments, H-89 (an inhibitor for PKA; Seikagaku Corp, Tokyo, Japan) or herbimycin A (an inhibitor for tyrosine kinase; Sigma-Aldrich) was dissolved in DMSO (100%) and added to the sperm suspension. The DMSO (100%) was also added to the control samples without these inhibitors to equalize the concentration of solvent among all the samples.

SDS-PAGE and Western Blot

The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and subsequent transfer of separated proteins to the polyvinylidene fluoride membrane (Immobilon P; Millipore, Bedford, Mass) were performed as previously described (Harayama et al, 2004a,b). The blotted membrane was blocked with 10% fetal calf serum (FCS; Dainippon Pharmaceutical Co, Ltd, Osaka, Japan) in PBS containing 0.1% Tween20 (PBS-Tween; Wako Pure Chemical Industries, Ltd, Osaka, Japan) for 60 minutes. Each of the primary antibodies was appropriately diluted with PBS-Tween containing 5% FCS and incubated with the membrane for 180 minutes. The primary antibodies used in this study were rabbit anti-phospho-SYK (Tyr352, a phosphotyrosine residue in the linker region) polyclonal antibody (1:1000; Cell Signaling Technology, Inc, Beverly, Mass), rabbit anti-phospho-PLCγ1 (Tyr783, a key regulatory phosphotyrosine residue) polyclonal antibody (1:1000; Cell Signaling), mouse anti-phosphotyrosine monoclonal antibody (4G10; 1:10 000; Upstate Cell Signaling Solutions, Charlottesville, Va) and mouse anti-α-tubulin monoclonal antibody (DM1A; 1:10 000; Sigma-Aldrich). After washing 3 times for 10 minutes each in PBS-Tween, the membrane was blocked in PBS-Tween containing 10% FCS for 60 minutes and then treated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulins (1:1000; Amersham) or HRP-conjugated goat anti-mouse immunoglobulins (1:2000 for anti-phosphotyrosine antibody or 1:10 000 for the anti-α-tubulin antibody; Dako Cytomation Denmark A/S, Glostrup, Denmark) in the blocking buffer for 60 minutes. After washing 3 times, peroxidase activity was visualized using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) and Hyperfilm-ECL (Amersham).

Indirect Immunofluorescence

All procedures, except the treatment with primary antibodies, were undertaken at room temperature. Each sperm suspension (5

$\times 10^5$ spermatozoa/preparation) was gently smeared on a glass slide and fixed in methanol for 10 minutes. The slides were gently rinsed with PBS twice, blocked with 5% bovine serum albumin (BSA; Serologicals Corp, Norcross, Ga) in PBS for 60 minutes, and then treated overnight at 4°C with each of the following primary antibodies that was diluted with PBS-BSA. The antibodies were rabbit anti-phospho-SYK polyclonal antibody (Tyr352, a phosphotyrosine residue in the linker region; 1:50), rabbit anti-phospho-PLCγ1 polyclonal antibody (Tyr783, a key regulatory phosphotyrosine residue; 1:50), rabbit anti-IP_3R type I polyclonal antibody (anti-IP_3R-I; 1:50; Sigma-Aldrich), and rabbit anti-calreticulin polyclonal antibody (1:10; Upstate). After being rinsed twice with PBS, the slides were treated with PBS-BSA for 60 minutes and then with fluorescein isothiocyanate–conjugated swine anti-rabbit immunoglobulins (1:50; Dako) for 60 minutes. After being rinsed twice with PBS, the slides were covered with 0.22-M 1,4-diazabicyclo [2,2,2] octane (Sigma-Aldrich) dissolved in glycerol:PBS (9:1) and coverslips. The sperm preparations were examined under a differential interference microscope equipped with epifluorescence (mirror unit U-MNIBA2: excitation filter BP470-490, dichroic mirror DM505, emission filter BA510-550; Olympus Optical Co Ltd, Tokyo, Japan).

Detection of Free Ca^{2+} in Sperm Flagella

The spermatozoa were loaded with fluo-3/AM (a cell-permeable Ca^{2+} indicator; 5 μM; Dojindo Laboratories, Kumamoto, Japan) in PVA-PBS containing 0.02% Pluronic F127 (Sigma-Aldrich), washed twice in PVA-PBS, and then incubated in the medium with or without cBiMPS (0.1 mM) as previously described (Harayama et al, 2004b). Immediately before and after incubation for 30 to 180 minutes, aliquots of each sample (2 μl) were recovered, placed on glass slides prewarmed to 37°C, and immediately examined under a differential interference microscope equipped with epifluorescence (mirror unit U-MNIBA2) at room temperature. Photographs of the spermatozoa were taken in the fluorescent field with a microscopic digital camera (DP50; Olympus). As a moderate fluorescence of fluo-3/AM was already observed in the middle piece of many spermatozoa before incubation, the exposure time of the photograph was adjusted to eliminate the fluorescence in the control samples before incubation (baseline reduction) in each experiment. This baseline reduction enabled us to detect a net increase of intracellular free calcium after incubation as an appearance of fluorescence.

In some experiments, a PLC inhibitor (U-73122; final concentration 0.02 mM; Sigma-Aldrich) was added to the sperm suspensions to confirm the involvement of the PCLγ1 in the cAMP-induced increase of the intracellular calcium.

Assessment for Sperm Viability

Sperm viability was assessed using a Live/Dead Sperm Viability kit (Molecular Probes, Inc, Eugene, Ore). Briefly, sperm suspensions were stained with 0.1 μM SYBR14 at 38.5°C for 5 minutes and, subsequently, with 12 μM propidium iodide (PI) at 38.5°C for 5 minutes. After staining, approximately 200 spermatozoa on each preparation were observed under a differential interference microscope equipped with epifluorescence (mirror unit U-MWIB2: excitation filter BP460-490, dichroic mirror DM505, emission filter BA510; Olympus) to determine the percentages of PI-positive spermatozoa (ie, dead spermatozoa).

Table 1. *Effects of incubation with a cAMP analog on viability of boar spermatozoa**

	Incubation Time (min)	
	0	180
cBiMPs	Propidium Iodide-Positive Sperm (Dead Sperm; %)†	
0 (control)	23 ± 9	28 ± 11
0.1	26 ± 11	29 ± 10

* Boar spermatozoa were incubated with 0.1% polyvinyl alcohol and 0.25 mM sodium orthovanadate (n = 3). cAMP indicates cyclic adenosine monophosphate; cBiMPS, Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3′,5′-monophoshorothioate.

† Values are means ± SD.

Assessment for Sperm Motility

Sperm motility was assessed by subjective observation. Briefly, the motility was observed in a 2-μl drop of sperm suspension on a heated stage (38.5°C) under the bright-field microscope. Spermatozoa showing any movement were considered motile cells, irrespective of their progressive motility. The percentages of spermatozoa showing hyperactivation were estimated, and the obtained results were classified into the following 4 categories: −, 0%–10%; +, 11%–30%; ++, 31%–50%; and +++, >50%.

Statistical Analysis

Percentages of PI-positive and motile spermatozoa were subjected to one-way analysis of variance (ANOVA) after arc-sine transformation. When *F*-test results were significant in ANOVA, individual means were further tested by Tukey's multiple range test (Motulsky, 1995).

Results

Viability of Boar Spermatozoa Before and After Incubation

At first, effects of cAMP analog (0.1 mM cBiMPS) on sperm viability were examined by the SYBR14-PI staining technique. As shown in Table 1, the 180-minute incubation with cBiMPS under our experimental condition had no significant influence on the percentages of PI-positive spermatozoa (ie, dead spermatozoa). This confirms that the cBiMPS-induced changes in boar spermatozoa (see the following) are not degenerative.

cAMP-Dependent Tyrosine Phosphorylation of Boar Sperm SYK at the Linker Region

The tyrosine phosphorylation of the SYK at the linker region is involved in binding to PLCγ1 in the lymphocytes (see ''Introduction''; Law et al, 1996). To examine the potential of sperm SYK to bind to PLCγ1, we observed the reactivity of this tyrosine kinase with the anti-

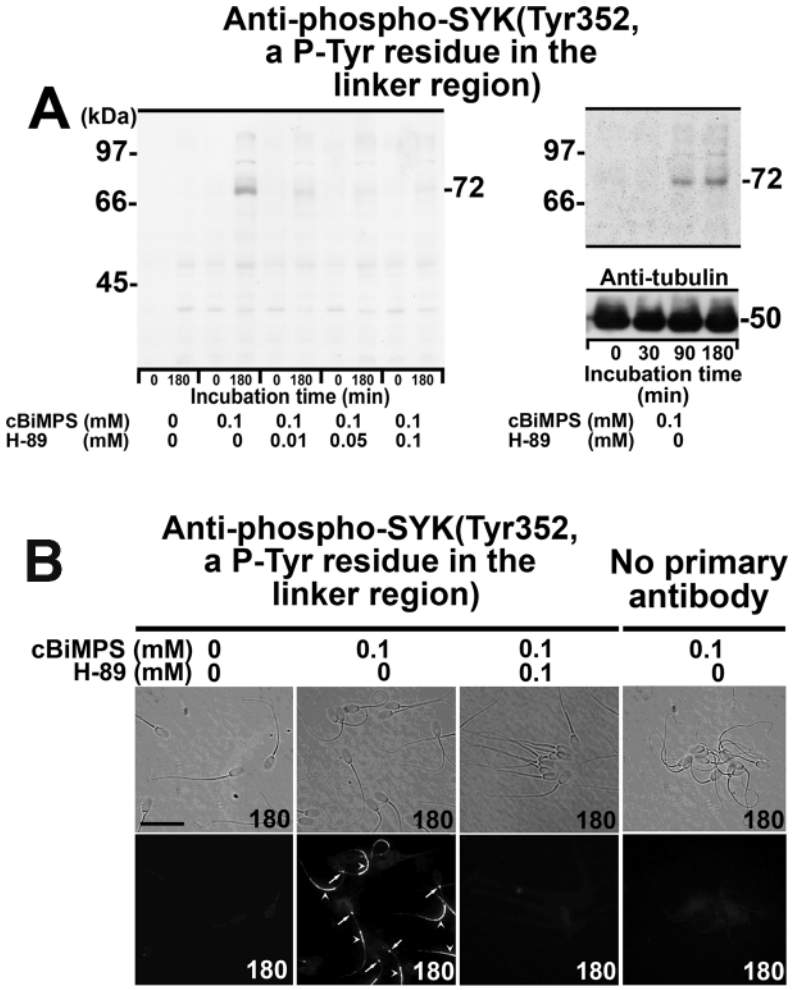

Figure 1. Immunodetection of cyclic adenosine monophosphate (cAMP)-dependent tyrosine phosphorylation at the linker region of SYK protein tyrosine kinase in boar spermatozoa. Washed spermatozoa were incubated with cBiMPS (a cell-permeable cAMP analog; 0 or 0.1 mM), Na_3VO_4 (an inhibitor for protein tyrosine phosphatase; 0.25 mM), and H-89 (an inhibitor for protein kinase A; 0–0.1 mM) up to 180 minutes at 38.5°C. Aliquots of each sperm suspension (1×10^6 spermatozoa/lane) were recovered immediately before and after incubation for 30 to 180 minutes, used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transblotting to the membranes, and then treated with the diluted anti-phospho-SYK antibody (Tyr352; 1:1000) or anti-α-tubulin (1:10 000) and, subsequently, with the horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulins (1:1000) or the HRP-conjugated anti-mouse immunoglobulins (1:10 000; **Panel A:** Western blot [a representative of 3 replicates]). Aliquots of each sperm suspension (5×10^5 spermatozoa/preparation) were immediately recovered before and after incubation for 180 minutes, fixed with methanol, and then treated with the diluted anti-phospho-SYK antibody (Tyr352; 1:50) or a blocking buffer without primary antibody (no primary antibody) and, subsequently, with fluorescein isothiocyanate (FITC)–conjugated anti-rabbit immunoglobulins (1:50; **Panel B:** indirect immunofluorescence [a representative of 3 replicates]). The numbers on the photographs (180) indicate incubation time in minutes **(Panel B).** In each set of photographs, the upper photograph is a differential interference and the lower photograph is an immunofluorescence. The photographs of spermatozoa immediately before incubation are omitted to save space. Arrows indicate connecting pieces; arrowheads, principal pieces. Scale bar = 25 μm **(Panel B).**

phospho-SYK antibody (Figure 1). Western blot analyses (Panel A) revealed that the anti-phospho-SYK antibody strongly recognized a band with a molecular mass of 72 kDa in the extracts from cBiMPS-incubated spermatozoa, but that the intensity of immunodetection was reduced by the addition of a PKA inhibitor (H-89; 0.01–0.1 mM). Moreover, appearance of this band required the incubation of spermatozoa with cBiMPS for more than 90 minutes. As shown by indirect immunofluorescence, the antibody had a specific reaction with the connecting and principal pieces of cBiMPS-incubated spermatozoa, but no reaction with those of spermatozoa after incubation without cBiMPS, after incubation with cBiMPS plus H-89 (0.1 mM), or before incubation (Panel B, but data were not shown for control spermatozoa before incubation). Additionally, in our previous report (Harayama et al, 2004a) we showed that another anti-phospho-SYK antibody (phosphotyrosine residues in the activation loop of the kinase domain) recognized the same segments (connecting and principal pieces) of cBiMPS-incubated spermatozoa, but hardly reacted with those of spermatozoa incubated without cBiMPS or with cBiMPS plus H-89. These results indicate that boar

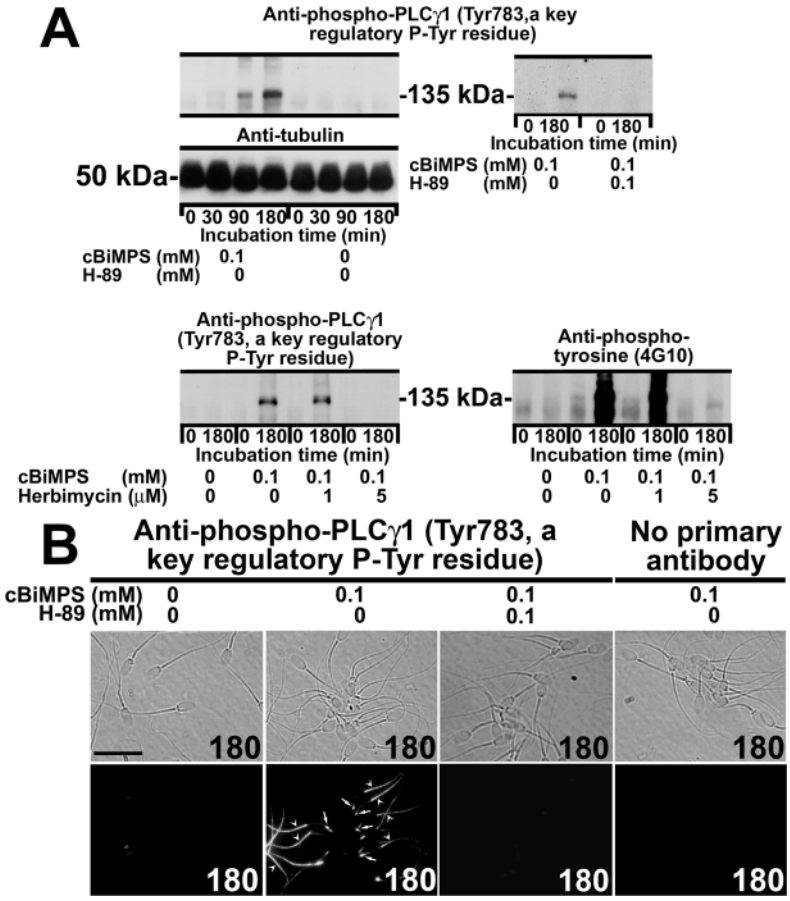

Figure 2. Immunodetection of cAMP-dependent tyrosine phosphorylation at the key regulatory residue of phospholipase Cγ1 (PLCγ1) in boar spermatozoa. Washed spermatozoa were incubated with cBiMPS (0 or 0.1 mM) and Na_3VO_4 (0.25 mM) up to 180 minutes at 38.5°C. In some experiments, H-89 (0 or 0.1 mM) or herbimycin A (an inhibitor for protein tyrosine kinase; 0–5 μM) was added to the sperm samples. In **Panel A** (Western blot; anti-phospho-PLCγ1 [Tyr783; 1:1000], a representative of 3 replicates; anti-α-tubulin [1:10 000], a representative of 3 replicates; and anti-phosphotyrosine [4G10; 1:10 000], a representative of 3 replicates), aliquots of each sperm suspension (1×10^6 spermatozoa/lane) were immediately recovered before and after the incubation for 30 to 180 minutes, used for SDS-PAGE and transblotting, and then treated with either of the previously mentioned primary antibodies and, subsequently, with HRP-conjugated anti-rabbit immunoglobulins (1:1000 for anti-phospho-PLCγ1) and HRP-conjugated anti-mouse immunoglobulins (1:2000 for anti-phosphotyrosine or 1:10 000 for anti-α-tubulin). In **Panel B** (indirect immunofluorescence, a representative of 3 replicates), aliquots of each sperm suspension (5×10^5 spermatozoa/preparation) were recovered immediately before and after incubation, fixed with methanol, and then treated with the anti-phospho-PLCγ1 antibody (Tyr783; 1:50) and, subsequently, with FITC-conjugated anti-rabbit immunoglobulins (1:50). The numbers on the photographs (180) indicate incubation time in minutes **(Panel B).** In each set of photographs, the upper photograph is a differential interference and the lower photograph is an immunofluorescence. The photographs of spermatozoa immediately before incubation are omitted to save space. Arrows indicate connecting pieces; arrowheads, principal pieces. Scale bar = 25 μm **(Panel B).**

sperm SYK is tyrosine phosphorylated at the linker region as well as at the activation loop of the kinase domain in response to the activation of cAMP-PKA signaling.

cAMP-Dependent Tyrosine Phosphorylation of Boar Sperm PLCγ1 at the Key Regulatory Residue

In the lymphocytes, the active SYK stimulates PLCγ1 by phosphorylation at the key regulatory residue (Law et al, 1996). To examine if the cAMP-activated sperm tyrosine kinase stimulates PLCγ1, we observed the effects of cBiMPS on the phosphorylation state of sperm PLCγ1 at the regulatory residue (Figure 2). The anti-phospho-PLCγ1 antibody detected a 135-kDa band in the sperm samples incubated with cBiMPS for 90 minutes, and its detection level was enhanced by the incubation prolonged to time 180 minutes (Panel A). However, this reactivity was abolished by the addition of the PKA inhibitor (H-89; 0.1 mM) or tyrosine kinase inhibitor (herbimycin A; 5 μM; Panel A). Moreover, the anti-phospho-PLCγ1 antibody had a strong reaction with the same segments (connecting and principal pieces) of the cBiMPS-incubated spermatozoa (Panel B) as the anti-phospho-SYK antibody (a phosphotyrosine residue in the linker region; Figure 1) and another anti-phospho-SYK antibody (phosphotyrosine residues in the activation loop of the kinase domain; Harayama et al, 2004a), but no or slight reaction with those of the spermatozoa after incubation without cBiMPS, after incubation with cBiMPS plus H-89 (0.1 mM), or be-

fore incubation (Panel B, but data were not shown for control spermatozoa before incubation). These findings suggest that the cAMP-dependently tyrosine-phosphorylated protein with a molecular mass of 135 kDa is PLCγ1, and that herbimycin A–sensitive tyrosine kinases, which are regulated by the cAMP-PKA signaling, stimulate the PLCγ1 by the tyrosine phosphorylation at the key regulatory residue.

Presence of IP_3R and Calreticulin in the Connecting Piece of Boar Spermatozoa

The active PLC generates IP_3 and DAG from PIP_2, and then the increased IP_3 triggers release of free calcium from the internal store by binding to IP_3R (Rebecchi and Pentyala, 2000; Rhee, 2001). To examine if the cAMP-activated PLCγ1 is connected to the IP_3R-calcium signaling, we tried to detect IP_3R and calreticulin (markers of the internal calcium store) in washed boar spermatozoa by indirect immunofluorescence (Figure 3). The anti-IP_3R-I antibody (Panel A) had a strong reaction with the connecting piece and acrosome. Moreover, the anti-calreticulin antibody (Panel B) recognized only the connecting piece. These suggest the presence of both IP_3R and internal calcium store in the connecting piece of boar spermatozoa.

cAMP-Dependent Increase of Intracellular Free Calcium in the Middle Piece of Boar Spermatozoa

Calcium imaging with flou-3/AM (Figure 4) revealed that treatment of boar spermatozoa with cBiMPS (0.1 mM) increased the free calcium in the middle piece, as well as in the head, after incubation for more than 90 minutes. However, the addition of a PLC inhibitor (U-73122; 0.02 mM) abolished this increase. These results indicate that the cAMP-activated PLCγ1 is associated with the increase of intracellular free calcium in the middle piece.

cAMP-Dependent Induction of Hyperactivation in Boar Spermatozoa

In our preliminary experiments, we found that the addition of sodium orthovanadate to the medium reduced motility of boar spermatozoa. Therefore, in the assessment to examine the potential of cBiMPS to induce hyperactivation in boar spermatozoa, we used the medium lacking sodium orthovanadate. As shown in Table 2, more than 70% of spermatozoa were motile during the 180-minute incubation period, irrespective of the addition of cBiMPS (0.1 mM). However, many of the motile spermatozoa exhibited the hyperactivation in the samples incubated with cBiMPS for 90 minutes. These results indicate that the 90-minute incubation with cBiMPS can switch on the cAMP-signaling cascades leading to hyperactivation in boar spermatozoa. Moreover, this timing at occurrence of cAMP-dependent hyperactivation was consistent both

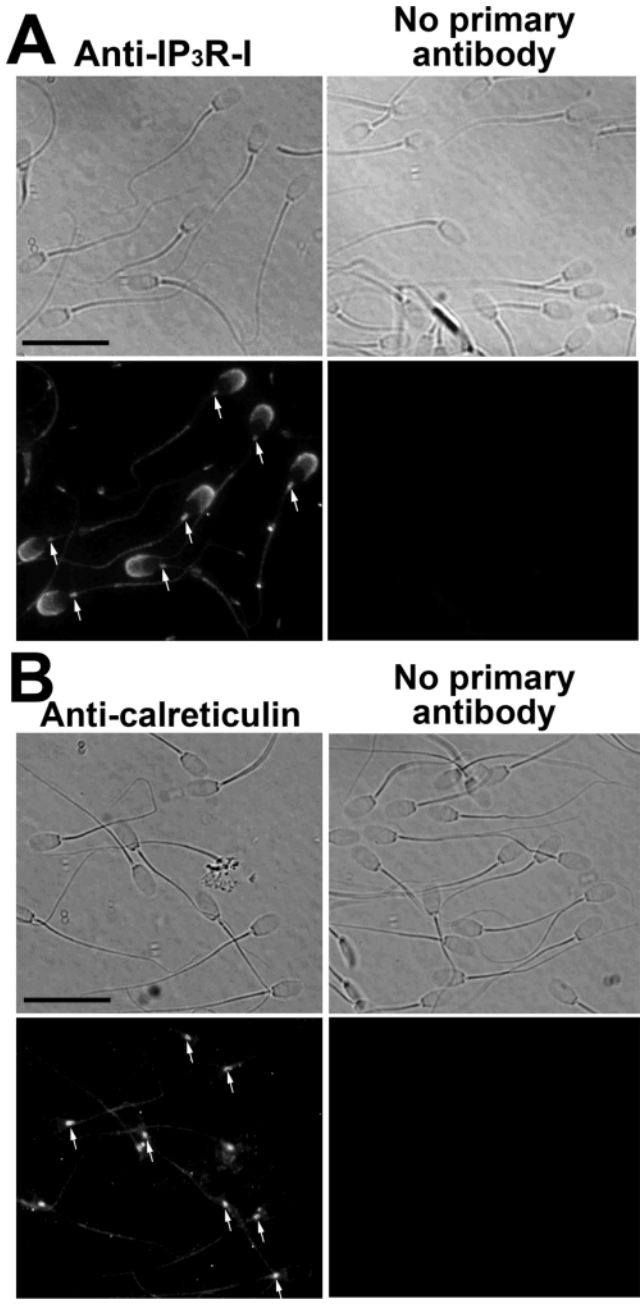

Figure 3. Indirect immunofluorescence of inositol 1,4,5-trisphosphate receptor (IP_3R) and calreticulin in boar spermatozoa (**Panel A:** anti-IP_3R type 1 [anti-IP_3R-I; a representative of 3 replicates; 1:50]; **Panel B:** anti-calreticulin [a representative of 3 replicates; 1:10]). Aliquots of washed spermatozoa (5×10^5 spermatozoa/preparation) immediately before incubation were fixed with methanol and then treated with either of diluted primary antibodies or with a blocking buffer without primary antibody (no primary antibody) and, subsequently, with FITC-conjugated anti-rabbit immunoglobulins (1:50). In each set of photographs, the upper photograph is a differential interference and the lower photograph is an immunofluorescence. The arrows indicate the connecting piece of spermatozoa. Scale bars = 25 μm.

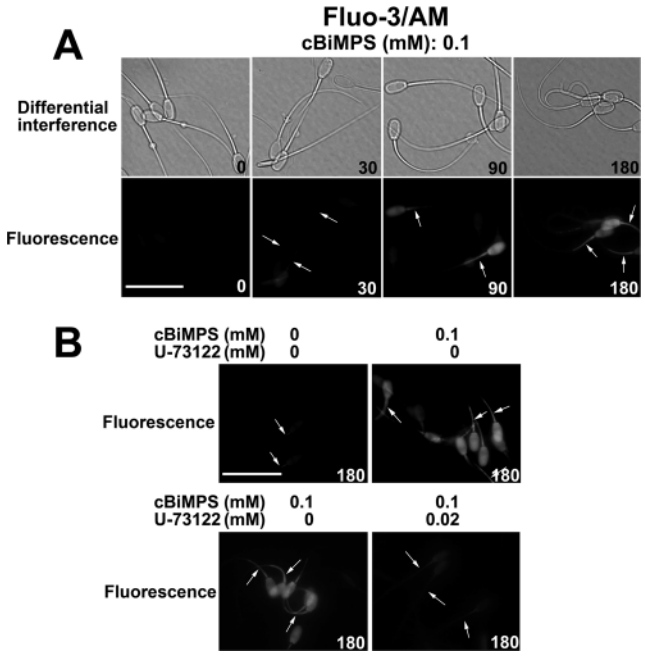

Figure 4. Detection of intracellular free calcium in the middle piece of boar spermatozoa. Washed spermatozoa were loaded with a cell-permeable Ca^{2+} indicator fluo-3/AM (5 μM in the presence of 0.02% Pluronic F127) in the dark for 30 minutes at 38.5°C. Subsequently, spermatozoa were washed twice and then incubated with cBiMPS (0 or 0.1 mM) and Na_3VO_4 (0.25 mM) up to 180 minutes at 38.5°C (**Panel A:** a representative of 3 replicates). In some experiments (**Panel B:** a representative of 3 replicates), an inhibitor for PLC (U-73122; 0.02 mM) was added to the sperm suspensions. Aliquots of each sperm suspension were immediately recovered before and after incubation and then observed with a differential interference microscope equipped with epifluorescence. Photographs that were taken under the bright field were omitted to save space **(Panel B).** Arrows indicate the middle piece of spermatozoa; numbers on the photographs (0, 30, 90, and 180), incubation time in minutes. Scale bars = 25 μm.

with the timing at cAMP-dependent phosphorylation of SYK and PLCγ1 (Figures 1 and 2) and with the timing at occurrence of cAMP-dependent increase of intracellular free calcium in the middle pieces (Figure 4), suggesting the possible existence of a relationship among these events. This suggestion is supported by our observation that the addition of a PLC inhibitor blocked the cAMP-dependent hyperactivation of boar spermatozoa (Table 3).

Table 3. *Effects of phospholipase C inhibitor U-73122 on motility of boar spermatozoa incubated with a cAMP analog**

		Incubation Time (min)	
U-73122 (mM)		0	180
0 (control)	Motile sperm (%)†	77 ± 6‡	77 ± 6‡
	Hyperactivation§	−	+++
0.02	Motile sperm (%)†	73 ± 6‡	50 ± 10‖
	Hyperactivation§	−	−

* Boar spermatozoa were incubated with 0.1% polyvinyl alcohol and 0.1 mM cBiMPS, but without sodium orthovanadate (n = 3).
† Values are means ± SD.
‡ Values differ significantly ($P < .05$).
§ Percent of spermatozoa exhibiting the hyperactivation (−, 0%–10%; +, 11%–30%; ++, 31%–50%; and +++, >50%).
‖ Values differ significantly ($P < .05$).

Discussion

Regulation of PLCγ1 by cAMP-Activated Tyrosine Kinase

It has been demonstrated that tyrosine phosphorylation is a key event that mediates the PLCγ activation. The PLCγ often forms a complex with a receptor protein tyrosine kinase, including epidermal growth factor (EGF) receptor. When extracellular stimuli bind to and activate the receptor protein tyrosine kinase, the PLCγ1 is phosphorylated at the Tyr771, Tyr783, and Tyr1245 residues (Margolis et al, 1989; Kim et al, 1991). According to the suggestion of Breitbart (2002), EGF seems to promote sperm capacitation through this EGF receptor–PLCγ signaling. In the lymphocytes (Kolanus et al, 1993; Takata et al, 1994), however, binding of antigens to receptors activates the nonreceptor protein tyrosine kinases including SYK and, subsequently, the active kinases catalyze the phosphorylation of PLCγ1 at the Tyr771 residue and the key regulatory residue Tyr783. In the in vitro experiments to examine the relationship between the tyrosine phosphorylation state and kinase function in the SYK (Law et al, 1996), substitution of Tyr525/526 residues (in the human; equivalent to Tyr518/519 residues in the pig) with Phe525/526 residues in the activation loop results in re-

Table 2. *Time-related changes in the motility of boar spermatozoa during incubation with a cAMP analog**

		Incubation Time (min)			
cBiMPS (mM)		0	30	90	180
0 (control)	Motile sperm (%)†	76 ± 6	72 ± 8	72 ± 8	72 ± 8
	Hyperactivation‡	−	−	−	−
0.1	Motile sperm (%)†	76 ± 6	74 ± 9	74 ± 9	74 ± 9
	Hyperactivation‡	−	−	++	+++

* Boar spermatozoa were incubated with 0.1% polyvinyl alcohol, but without sodium orthovanadate (n = 5).
† Values are means ± SD.
‡ Percent of spermatozoa exhibiting the hyperactivation (−, 0%–10%; +, 11%–30%; ++, 31%–50%; and +++, >50%).

duction of its kinase activity and its binding to C-terminal SH2 domain of PLCγ1. This result has demonstrated the requirement of tyrosine phosphorylation at the activation loop of the kinase domain for the ability to bind to PLCγ1, as well as for the kinase activation. Moreover, substitution of Tyr348/352 residues (in the human; equivalent to Tyr341/345 residues in the pig) with Phe348/352 residues in the linker region joining the C-terminal SH2 domain and the kinase domain did not affect the kinase activity but almost completely eliminated its binding to the PLCγ1 SH2 domain and, consequently, eliminated its ability to induce tyrosine phosphorylation of PLCγ1. These findings indicate that the tyrosine phosphorylation in SYK, not only at the activation loop of the kinase domain but also at the linker region, is important for its ability to bind to PLCγ1 and to phosphorylate the key regulatory tyrosine residue of PLCγ1. As previously described (Harayama et al, 2004a), the SYK of boar sperm connecting and principal pieces is tyrosine phosphorylated at the activation loop of kinase domain via the activation of cAMP-PKA signaling. In this study, Western blot and indirect immunofluorescence have revealed that this tyrosine kinase is also tyrosine phosphorylated at the linker region via the activation of the same cAMP-PKA signaling (Figure 1). These results indicate the potential of sperm cAMP-activated SYK to bind to PLCγ1 and to phosphorylate it at the key regulatory residue. Moreover, our analyses on the PLCγ1 (Figure 2) have revealed that herbimycin A–sensitive, cAMP-activated tyrosine kinases phosphorylate the PLCγ1 at the key regulatory residue in the connecting and principal pieces of boar spermatozoa. These findings have been interpreted as showing that the activation of boar sperm PLCγ1 is triggered by cAMP and may be mediated by PKA and tyrosine kinases, including SYK. In addition, Schmidt et al (2001) have recently proposed a novel PLCε-calcium signaling that is triggered by cAMP and mediated by a small GTPase of the Rap family in somatic cells. By contrast, the activity of PLCβ3 is inhibited by the phosphorylation at its Ser1105 residue by the cAMP-activated PKA (Yue et al, 1998).

Function of cAMP-Activated PLCγ1 in the Calcium Signaling

Several articles (eg, Roldan and Murase, 1994; Spungin et al, 1995; Fukami et al, 2001) have revealed that PLC is involved in the acrosomal exocytosis that is apparently regulated by the calcium signaling. Especially for PLCγ1, Tomes et al (1996) have shown that this isoform is located in the head of mouse spermatozoa and that its enzymatic activity is enhanced by the treatment of spermatozoa with solubilized zona pellucida glycoprotein ZP3. This glycoprotein is a physiologic inducer of acrosomal exocytosis. In this study on boar spermatozoa, however, an immunostaining study has shown that phosphorylated PLCγ1 is restricted to the connecting and principal pieces, but not to the head (Figure 2). These suggest different roles of sperm PLCγ1 between mice and boars. It is possible that boar sperm PLCγ1 may be linked to the regulation of motility, including hyperactivation and metabolic activity in the flagella. Indeed, the timing at occurrence of cAMP-dependent hyperactivation is consistent with that at cAMP-dependent phosphorylation of PLCγ1 (Table 2; Figure 2).

Our immunocytochemical observation that boar spermatozoa possess IP_3R (Type I) and calreticulin in the connecting piece (Figure 3) is in agreement with results obtained in bull spermatozoa (Ho and Suarez, 2001, 2003). This result indicates the existence of calcium store in the connecting piece. Recently, it has been proposed that a redundant nuclear envelope has the function of the calcium store in the connecting pieces of bull spermatozoa (Ho and Suarez, 2003). Moreover, the stored calcium is released through the IP_3R to the middle piece when bull spermatozoa are treated with thimerosal (Ho and Suarez, 2001). As shown in Figure 4, treatment with the cAMP analog increases the intracellular free calcium in the middle piece of boar spermatozoa as well as in the head, but this increase is abolished by the addition of the PLC inhibitor (U-73122; 0.02 mM). Thus, it is likely that this cAMP-dependent increase of intracellular free calcium in the middle piece of boar spermatozoa is regulated by the PLCγ1-IP_3R signaling in the connecting piece.

Conclusions

Based on our findings, we have concluded that the connecting piece of boar spermatozoa possesses the PLCγ1-IP_3R-calcium signaling that is triggered by cAMP and mediated by PKA and herbimycin A–sensitive tyrosine kinases. Moreover, this sperm signaling seems to include a nonreceptor tyrosine kinase SYK that has been shown to activate the PLCγ1 in the lymphocytes (Law et al, 1996). To our knowledge, the positive regulation of sperm PLCγ1 by cAMP-PKA signaling is quite distinct from the mechanism for control of the enzymatic activity that has been shown in somatic cells, including lymphocytes (Ichinohe et al, 1995; Law et al, 1996; Wang et al, 1997; Williams et al, 1999; Dustin and Chan, 2000; Kurosaki and Tsukada, 2000). These findings are consistent with the indication that sperm cAMP has a unique role as a trigger for the activation of PLCγ1-calcium signaling.

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