RESEARCH ARTICLE

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Relationship Between Cyclic AMP-Dependent Protein Tyrosine Phosphorylation and Extracellular Calcium During Hyperactivation of Boar Spermatozoa

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SUMMARY

In mammalian spermatozoa, the state of protein tyrosine phosphorylation is modulated by protein tyrosine kinases and protein tyrosine phosphatases that are controlled via cyclic AMP (cAMP)-protein kinase A (PKA) signaling cascades. The aims of this study were to examine the involvement of cAMP-induced protein tyrosine phosphorylation in response to extracellular calcium and to characterize effects of pharmacological modulation of the cAMP-induced protein phosphorylation state and calmodulin activity during hyperactivation in boar spermatozoa. Ejaculated spermatozoa were incubated with cBiMPS (a cell-permeable cAMP analog) and $CaCl_2$ at 38.5°C to induce hyperactivation, and then used for Western blotting and indirect immunofluorescence of phosphorylated proteins and for the assessment of motility. Both cBiMPS and $CaCl_2$ were necessary for hyperactivation. The increase in hyperactivated spermatozoa exhibited a dependence on the state of cBiMPS-induced protein tyrosine phosphorylation in the connecting and principal pieces. The addition of calyculin A (an inhibitor for protein phosphatases 1/2A (PP1/PP2A), 50–100 nM) coincidently promoted hyperactivation and cAMP-induced protein tyrosine phosphorylation in the presence of cBiMPS and $CaCl_2$. Moreover, the addition of W-7 (a calmodulin antagonist, 2–4 μM) enhanced the percentages of hyperactivated spermatozoa after incubation with cBiMPS and $CaCl_2$, independently of protein tyrosine phosphorylation. These findings indicate that cAMP-induced protein tyrosine phosphorylation in the connecting and principal pieces is involved in hyperactivation in response to extracellular calcium, and that calmodulin may suppress hyperactivation via the signaling cascades that are independent of cAMP-induced protein tyrosine phosphorylation.

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INTRODUCTION

Hyperactivation is a pattern of sperm flagellar movement characterized by an intensive and asymmetric whiplash beating of the middle and principal pieces, which generates a strong driving force to penetrate extracellular matrix of oocytes (Yanagimachi, 1994). Mouse spermatozoa exhibit

Additional supporting information may be found in the online version of this article.

Abbreviations: cAMP, cyclic adenosine monophosphate; cBiMPS, Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3′,5′-monophoshorothioate; PKA, protein kinase A; PLC, phospholipase C; PP, protein phosphatase; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

hyperactivation after incubation in capacitation-supporting medium in vitro or in the female reproductive tract in vivo (Buffone et al., 2012). In pigs, however, only a small number of spermatozoa exhibit hyperactivation in the female reproductive tract in vivo (Suarez et al., 1992). Indeed, we have preliminary data indicating that it is difficult to induce hyperactivation uniformly and synchronously in vitro in boar spermatozoa populations by simple incubation in capacitation-supporting medium (Harayama, unpublished data). Moreover, a clear increase of tyrosine phosphorylation state was detectable in only limited proteins of spermatozoa that were incubated in capacitation-supporting medium by Western blotting techniques (Tardif et al., 2001; Kaneto et al., 2002; Bravo et al., 2005). These observations indicate that in vitro activation of intracellular signaling systems for hyperactivation and protein tyrosine phosphorylation is not synchronous among individual spermatozoa incubated in the capacitation-supporting medium, making it difficult to analyze any relationship between hyperactivation and protein tyrosine phosphorylation in in vitro-capacitated boar spermatozoa with biochemical tools. Instead, we found that the transition of motility from progressive to hyperactivation was effectively induced in boar ejaculated spermatozoa by incubating with the cell-permeable cyclic AMP (cAMP) analog cBiMPS (Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3′,5′-monophoshorothioate) for 180 min in vitro (Harayama, 2003; Harayama and Miyake, 2006; Harayama and Nakamura, 2008). During this incubation period, the capacitation state of the sperm head (as assessed by the chlortetracycline staining) and the tyrosine phosphorylation state of flagellar proteins were enhanced coincidently with the transition of motility states (Harayama et al., 2004b; Adachi et al., 2008; Harayama and Nakamura, 2008). These findings suggest that our simulation system can mimic intracellular changes leading to hyperactivation and protein tyrosine phosphorylation in in vitro-capacitated boar spermatozoa.

Our previous articles show results consistent with the hypothesis that there are several cAMP signaling cascades that regulate hyperactivation of boar spermatozoa in our simulation system. In brief, treatment with cBiMPS (i.e., increases intracellular cAMP) can rapidly induce serine/threonine phosphorylation by the activation of protein kinase A (PKA) and protein tyrosine phosphorylation, with a time lag of a few hours, via activation of protein tyrosine kinases [e.g., spleen tyrosine kinase (SYK)] in the connecting and principal pieces. Intriguingly, tyrosine phosphorylation is greatly enhanced in many flagellar proteins of boar spermatozoa coincident with hyperactivation (Harayama, 2003; Harayama et al., 2004a; Harayama and Nakamura, 2008). Possible functions of the cAMP-dependent protein tyrosine phosphorylation may include the activation of sperm phospholipase Cγ1 (PLCγ1), which is linked to the stimulation of flagellar calcium signaling (Harayama et al., 2005). Another cAMP-dependent signaling cascade appears to suppress tyrosine phosphorylation of flagellar proteins via the phosphatidyl-inositol-3 kinase (PI3K) and phosphoinositide-dependent protein kinase 1 (PDK1) in order to prevent the occurrence of precocious hyperactivation (Harayama and Nakamura, 2008). When viewed together with previous reports on the spermatozoa from other species (Si, 1999; Si and Okuno, 1999; Baker et al., 2006; Kaneto et al., 2008; Goto and Harayama, 2009; Harayama et al., 2010), these observations allowed us to postulate that cAMP-induced tyrosine phosphorylation of flagellar proteins is important in the transition from sperm motility to hyperactivation. Adenylyl cyclase (ADCY) 10 (soluble adenylyl cyclase: sAC) may generate cAMP to stimulate the above-mentioned signaling cascades as this enzyme has been found in the connecting piece (Tate et al., 2010) and principal pieces (Nada et al., unpublished data), although further experiments are necessary to test if this is the case in the boar spermatozoa.

There are also controversial observations regarding the relationship between flagellar protein phosphorylation and hyperactivation. For instance, treatment with procaine or caffeine immediately induced hyperactivation in uncapacitated spermatozoa by increasing intracellular calcium, but barely enhanced the tyrosine phosphorylation state of flagellar proteins (Marquez and Suarez, 2004; McPartlin et al., 2009). These observations indicate that procaine- or caffeine-induced hyperactivation might not be regulated by changes in the tyrosine phosphorylation state of flagellar proteins, although it should be noted that these chemically induced hyperactivation methods produced the phenotype before sperm capacitation could have occurred (usually several hours) (Marquez and Suarez, 2004; McPartlin et al., 2009). In order to harmonize this hypothesis with our model, we decided to pursue the relationship between cAMP-induced protein tyrosine phosphorylation and calcium signaling cascades in boar spermatozoa. In this study, we examined the involvement of cAMP-induced protein tyrosine phosphorylation of hyperactivation in response to extracellular calcium. Moreover, we investigated how pharmacological modulation of the cAMP-induced protein phosphorylation state and calcium-signaling components affected the occurrence of hyperactivation.

RESULTS

Effects of cBiMPS and Extracellular Calcium on Protein Tyrosine Phosphorylation and Hyperactivation

In order to reveal the relationships between cAMP-induced protein tyrosine phosphorylation and extracellular calcium, we first examined the effects of removing cBiMPS (0.1 mM) or $CaCl_2$ (1.71 mM) from the capacitation medium on the motility rates, flagellar beating (Supplementary materials, movies 1–3), hyperactivation, and protein tyrosine phosphorylation state. The absence of either cBiMPS or $CaCl_2$ throughout the incubation period (270 min) dramatically suppressed the change of flagellar beating pattern from strong symmetrical beating to strong asymmetrical beating (hyperactivation) without a significant decrease in motility rates (Table 1). Protein tyrosine phosphorylation in the connecting and principal pieces was suppressed

TABLE 1. Effects of Deficiency of a Cell-Permeable cAMP Analog (cBiMPS) and $CaCl_2$ in the Medium on the Motility Rates, Flagellar Beating, and Hyperactivation in Boar Spermatozoa (n = 5)

	cBiMPS (mM)	$CaCl_2$ (mM)	Incubation time (min) 0	90	180	270
Motility rates[a]	0	0	67 ± 11	63 ± 8	59 ± 7[e]	47 ± 4
	0	1.71	67 ± 11	63 ± 8	62 ± 4[d]	51 ± 5
	0.1	0	67 ± 4	60 ± 0	48 ± 4[e]	43 ± 7
	0.1	1.71	69 ± 7	60 ± 0	58 ± 4[e]	51 ± 9
Patterns of flagellar beating[b]	0	0	SS (n = 5)	SS (n = 5)	SS (n = 5)	SS (n = 5)
	0	1.71	SS (n = 5)	SS (n = 5)	SS (n = 5)	SS (n = 5)
	0.1	0	SS (n = 5)	SS (n = 5)	SW (n = 1)/SS (n = 4)	SW (n = 1)/SS (n = 4)
	0.1	1.71	SS (n = 5)	SS & AS (n = 5)	SS & AS (n = 5)	SS & AS (n = 5)
Hyperactivated sperm[c]	0	0	− (n = 5)	− (n = 5)	− (n = 5)	− (n = 5)
	0	1.71	− (n = 5)	− (n = 5)	− (n = 5)	− (n = 5)
	0.1	0	− (n = 5)	− (n = 5)	− (n = 5)	− (n = 5)
	0.1	1.71	− (n = 5)	+ (n = 5)	+ (n = 1)/++ (n = 4)	+ (n = 4)/++ (n = 1)

[a]Values are means of percentages of motile spermatozoa ± standard deviation.
[b]SW, symmetrical beating (weak); SS, symmetrical beating (strong); and AS, asymmetrical beating (strong): see supplemental movies.
[c]−, 0–10%; +, 11–30%; ++, 31–50%; +++, >50%. [de]Values (the percentages of motile sperm) with different superscripts within the same column were significantly different (ANOVA and Tukey's multiple range tests, $P < 0.05$).

without cBiMPS in the medium, whereas the tyrosine phosphorylation state was barely affected by the absence of $CaCl_2$ (Fig. 1).

To examine the necessity of cBiMPS-induced protein tyrosine phosphorylation for the hyperactivation in response to extracellular calcium, washed spermatozoa were preincubated with either cBiMPS (0.1 mM) or $CaCl_2$ (1.71 mM) for 180 min, and then further incubated with both cBiMPS (0.09 mM) and $CaCl_2$ (1.67 mM) for 180 min (total of 360 min) at 38.5°C (see samples B and D; samples A and C were negative control samples for samples B and D, respectively; Table 2 and Fig. 2). The addition of $CaCl_2$ to the spermatozoa after a 180-min pretreatment with cBiMPS, which results in a high tyrosine phosphorylation profile, rapidly induced hyperactivation within the next 60 min, with a slight increase in the protein tyrosine phosphorylation state (see sample B). By contrast, the addition of cBiMPS after preincubation with $CaCl_2$ alone, which has a basal tyrosine phosphorylation profile, gradually induced hyperactivation during the subsequent 180-min incubation period (total of 360 min) coincident with the increase of protein tyrosine phosphorylation state (see sample D). This gradual induction of hyperactivation in parallel with an increase of protein tyrosine phosphorylation was similar to those observed in the spermatozoa incubated for 180 min with both cBiMPS and $CaCl_2$, as shown in Table 1 and Fig. 1 and our previous report (Harayama and Nakamura, 2008). These results indicate that cAMP-induced protein tyrosine phosphorylation is needed for calcium to induce hyperactivation.

Effects of Pharmacological Modulation of cBiMPS-Induced Protein Tyrosine Phosphorylation on Hyperactivation

Our previous articles (Harayama et al., 2004b; Harayama and Nakamura, 2008) showed that cAMP-induced protein tyrosine phosphorylation is associated with protein serine/threonine phosphorylation in boar spermatozoa. Thus, we examined effects of calyculin A (50 and 100 nM), an inhibitor for PP1/PP2A, on motility rates, flagellar beating, hyperactivation, and protein phosphorylation in spermatozoa incubated in capacitation medium containing cBiMPS (0.1 mM) and $CaCl_2$ (1.71 mM; Table 3 and Fig. 3). The addition of calyculin A clearly enhanced both the occurrence of hyperactivation and cBiMPS-induced protein tyrosine phosphorylation. Yet, effects of calyculin A on serine/threonine phosphorylation were barely distinguishable on the AGC kinase substrate proteins (molecular masses of >50 kDa) because protein phosphorylation was saturated by the action of cBiMPS alone. In addition, the effects of calyculin A on hyperactivation and protein tyrosine phosphorylation were barely noticeable in spermatozoa incubated with $CaCl_2$ (1.71 mM) and without cBiMPS (0.1 mM; Table 3 and Fig. 3). These results support the hypothesis that cAMP-induced protein tyrosine phosphorylation is involved in the occurrence of hyperactivation.

Effects of Pharmacological Inhibition of Calmodulin on Hyperactivation and cBiMPS-Induced Protein Tyrosine Phosphorylation

Finally, we investigated effects of the calmodulin antagonists W-7 and W-5 on motility rates, flagellar beating, hyperactivation, and protein tyrosine phosphorylation in spermatozoa incubated in capacitation medium containing cBiMPS (0.1 mM) and $CaCl_2$ (1.71 mM; Tables 4 and 5, Fig. 4). These antagonists were added to the sperm suspension at three concentration ranges (low concentration: 1–4 μM; middle concentration: 10–20 μM; and high concentration: 100–200 μM). In the case of the low concentration range, the addition of 2–4 μM W-7 apparently enhanced the

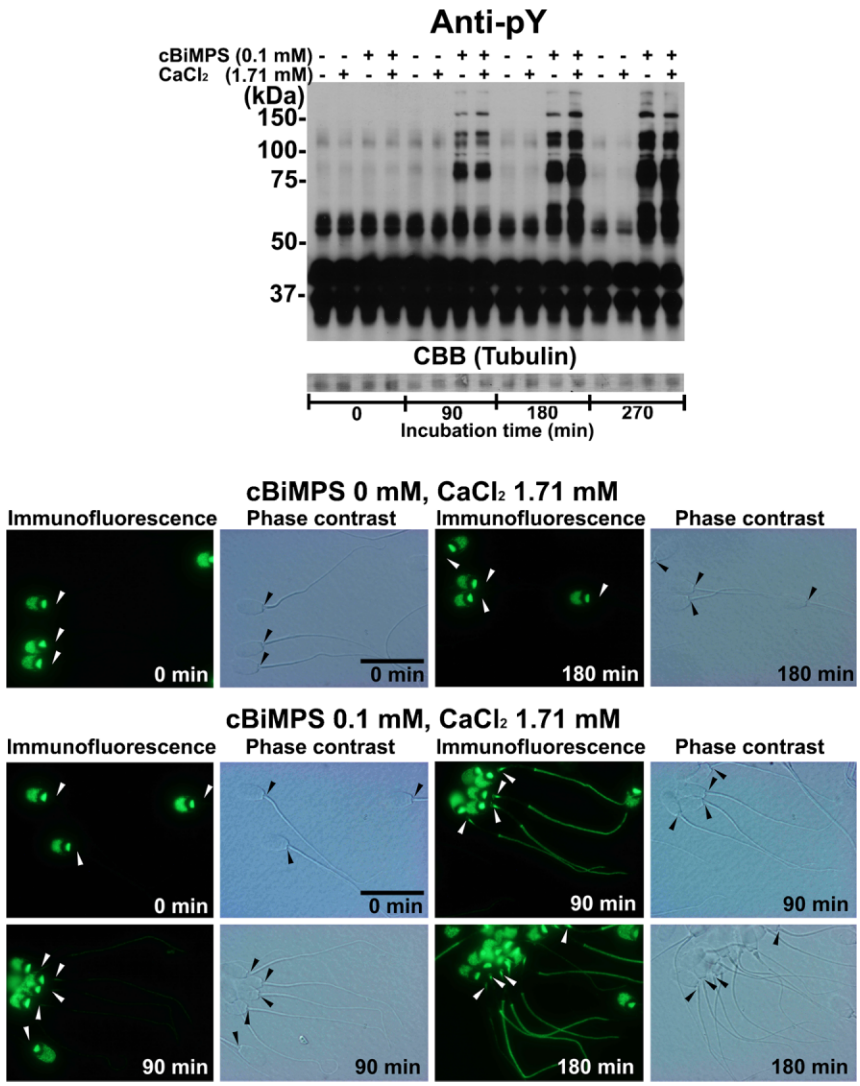

Figure 1. Effects of removing a cell-permeable cAMP analog (cBiMPS) and $CaCl_2$ from the medium on protein tyrosine phosphorylation in boar spermatozoa. Washed spermatozoa were incubated in media lacking either cBiMPS, $CaCl_2$, or both for 270 min. In the upper panel (Western blot, a representative of four replicates), aliquots of each sperm suspension (1×10^6 spermatozoa/lane) were recovered immediately before and after incubation, used in SDS–PAGE/Western blotting with mouse anti-phosphotyrosine monoclonal antibody (clone 4G10, 1:10,000) and subsequently with HRP-conjugated, goat anti-mouse immunoglobulin polyclonal antibody (1:10,000). After Western blotting, each membrane was stained with Coomasie Brilliant Blue G-250 to detect tubulin as the loading control [CBB (Tubulin)]. In the lower panel (indirect immunofluorescence: a representative of three replicates), aliquots of each sperm suspension (5×10^5 spermatozoa/preparation) were recovered immediately before and after the incubation, gently smeared on glass slides, and treated with methanol. The fixed and membrane-permeated spermatozoa were blocked and then treated overnight at 4°C with mouse anti-phosphotyrosine monoclonal antibody (Anti-pY, 1:1,000) followed by FITC-conjugated rabbit anti-mouse immunoglobulin polyclonal antibody (1:100). Arrowheads indicate the connecting pieces. Scale bar, 20 μm.

induction of hyperactivation without significant alterations in motility rates or the state of cBiMPS-induced protein tyrosine phosphorylation after incubation for 90 and 180 min. High concentrations of W-7 (100–200 μM), however, resulted in a severe reduction in motility rates and induction of hyperactivation. Moreover, both cBiMPS-induced protein tyrosine phosphorylation of high-molecular mass proteins (higher than 50-kDa) and basal tyrosine phosphorylation of low-molecular mass proteins (lower than 45-kDa) declined in the spermatozoa incubated with 100–200 μM W-7. The effects of middle-range concentrations of W-7 (10–20 μM) were varied among repeated

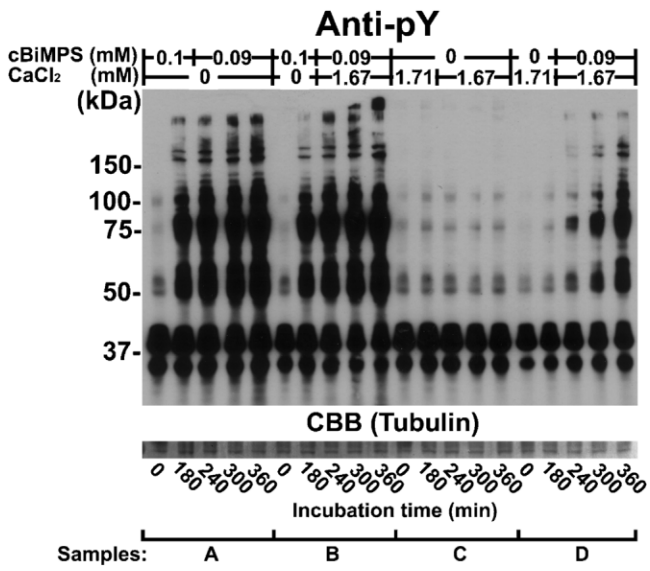

Figure 2. Western blots of tyrosine-phosphorylated proteins extracted from boar spermatozoa preincubated with either cBiMPS or $CaCl_2$, and then further incubated with both cBiMPS and $CaCl_2$. Washed spermatozoa were preincubated with either cBiMPS or $CaCl_2$ for 180 min, and then further incubated with both cBiMPS and $CaCl_2$ for 180 min (totally for 360 min) at 38.5°C (see samples B and D). Samples A and C were negative control samples for samples B and D, respectively. In each experiment (Anti-pY: a representative of three replicates), aliquots of each sperm suspension (1×10^6 spermatozoa/lane) were recovered immediately before and after incubation, used for SDS–PAGE/Western blotting with mouse anti-phosphotyrosine monoclonal antibody (clone 4G10, 1:10,000) followed by HRP-conjugated goat anti-mouse immunoglobulin polyclonal antibody (1:10,000). After Western blotting, each membrane was stained with Coomasie Brilliant Blue G-250 to detect tubulin as the loading control [CBB (Tubulin)].

experiments, and could not be determined exactly. Similarly, the less potent calmodulin antagonist W-5 had a tendency to enhance the induction of hyperactivation without significant alterations to the motility rates or the state of cBiMPS-induced protein tyrosine phosphorylation in boar spermatozoa after incubation with the middle-range concentrations (10–20 μM). These results are consistent with the suggestion that calmodulin may suppress hyperactivation independently of cBiMPS-induced protein tyrosine phosphorylation. Moreover, the addition of W-7 at the higher concentration could negatively affect the basal mechanisms supporting flagellar movement and protein tyrosine phosphorylation in boar spermatozoa.

DISCUSSION

Possible Relationship Between cAMP-Induced Protein Tyrosine Phosphorylation and Extracellular Calcium in the Connecting and Principal Pieces

Ho et al. (2002) reported that calcium could solely switch on hyperactivation of flagellar movement in detergent-

TABLE 2. Effects of Preincubation With Either cBiMPS or $CaCl_2$ on the Boar Sperm Hyperactivation Induced by Additional Incubation in the Presence of Both cBiMPS and $CaCl_2$ (n = 3)

		Preincubation: 0–180 min		Incubation: 180–360 min		Incubation time (min)					
	Samples	cBiMPS (mM)	$CaCl_2$ (mM)	cBiMPS (mM)	$CaCl_2$ (mM)	0	180	240	300	360
Motility rates[a]	A	0.1	0	0.09	0	75 ± 5	72 ± 13	65 ± 9	63 ± 6	50 ± 10
	B	0.1	0	0.09	1.67	75 ± 5	72 ± 13	65 ± 9	65 ± 9	58 ± 8
	C	0	1.71	0	1.67	75 ± 5	67 ± 6	67 ± 6	63 ± 6	55 ± 9
	D	0	1.71	0.09	1.67	75 ± 5	67 ± 6	67 ± 6	63 ± 6	58 ± 8
Patterns of flagellar beating[b]	A	0.1	0	0.09	0	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 1)/SS (n = 2)	SW (n = 1)/SS (n = 2)	SW (n = 2)/SS (n = 1)
	B	0.1	0	0.09	1.67	SS (n = 3)	SW (n = 1)/SS (n = 2)	SS & AS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	C	0	1.71	0	1.67	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 1)/SS (n = 2)	SW (n = 1)/SS (n = 2)	SW (n = 1)/SS (n = 2)
	D	0	1.71	0.09	1.67	SS (n = 3)	SW (n = 1)/SS (n = 2)	SS (n = 3)	SS (n = 2)/SS & AS (n = 1)	SS & AS (n = 3)
Hyperactivated sperm[c]	A	0.1	0	0.09	0	– (n = 3)	– (n = 3)	– (n = 3)	– (n = 3)	– (n = 3)
	B	0.1	0	0.09	1.67	– (n = 3)	– (n = 3)	++ (n = 3)	++ (n = 2)/+++ (n = 1)	++ (n = 3)
	C	0	1.71	0	1.67	– (n = 3)	– (n = 3)	– (n = 3)	– (n = 3)	– (n = 3)
	D	0	1.71	0.09	1.67	– (n = 3)	– (n = 3)	– (n = 3)	– (n = 2)/+ (n = 1)	+ (n = 1)/++ (n = 2)

[a]Values are means of percentages of motile spermatozoa ± standard deviation.
[b]SW, symmetrical beating (weak); SS, symmetrical beating (strong) and AS, asymmetrical beating (strong); see supplemental movies.
[c]–, 0–10%; +, 11–30%; ++, 31–50%; +++, >50%.

TABLE 3. Effects of a Cell-Permeable Inhibitor (calyculin A) for Protein Phosphatase (PP) 1/PP2A on Motility Rates, Flagellar Beating, and Hyperactivation in Boar Spermatozoa Incubated With or Without cBiMPS

	cBiMPS (mM)	Calyculin A (nM)	Incubation time (min) 0	90	180
Motility rates[a] (n = 5)	0.1	0	76 ± 9	65 ± 10	62 ± 8
	0.1	50	76 ± 9	62 ± 14	60 ± 12
	0.1	100	76 ± 9	63 ± 12	61 ± 8
	0	0	68 ± 5	60 ± 14	52 ± 13
Patterns of flagellar beating[b] (n = 5)	0.1	0	SS (n = 5)	SS (n = 1)/SS & AS (n = 4)	SS & AS (n = 5)
	0.1	50	SS (n = 5)	SS & AS (n = 5)	SS & AS (n = 5)
	0.1	100	SS (n = 5)	SS & AS (n = 5)	SS & AS (n = 5)
	0	0	SS (n = 5)	SW (n = 4)/SS (n = 1)	SW (n = 4)/SS (n = 1)
Hyperactivated sperm[c] (n = 5)	0.1	0	− (n = 5)	− (n = 1)/+ (n = 4)	+ (n = 2)/++ (n = 3)
	0.1	50	− (n = 5)	+ (n = 3)/++ (n = 2)	++ (n = 4)/+++ (n = 1)
	0.1	100	− (n = 5)	+ (n = 3)/++ (n = 1)/+++ (n = 1)	++ (n = 3)/+++ (n = 2)
	0	0	− (n = 5)	− (n = 5)	− (n = 5)

	cBiMPS (mM)	Calyculin A (nM)	Incubation time (min) 0	90	180
Motility rates[a] (n = 3)	0	0	67 ± 6	60 ± 17	53 ± 16
	0	50	67 ± 6	63 ± 12	57 ± 14
	0	100	67 ± 6	63 ± 12	58 ± 12
Patterns of flagellar beating[b] (n = 3)	0	0	SS (n = 3)	SW (n = 2)/SS (n = 1)	SW (n = 2)/SS (n = 1)
	0	50	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 2)/SS (n = 1)
	0	100	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 1)/SS (n = 2)
Hyperactivated sperm[c] (n = 3)	0	0	− (n = 3)	− (n = 3)	− (n = 3)
	0	50	− (n = 3)	− (n = 3)	− (n = 3)
	0	100	− (n = 3)	− (n = 3)	− (n = 3)

[a]Values are means of percentages of motile spermatozoa ± standard deviation.
[b]SW, symmetrical beating (weak); SS, symmetrical beating (strong); and AS, asymmetrical beating (strong): see supplemental movies.
[c]−, 0–10%; +, 11–30%; ++, 31–50%; +++, >50%.

demembranated bull spermatozoa by enabling curvature of the principal bends to increase in the presence of sufficient ATP and under an alkaline environment, and that cAMP was not required for hyperactivation of detergent-demembranated spermatozoa under these conditions. Qi et al. (2007) demonstrated that hyperactivation is under the control of extracellular calcium entry through CatSper (Cation channel, sperm-associated) channels in the plasma membrane of the principal piece of mouse membrane-intact spermatozoa, though roles for other types of calcium channels during hyperactivation are poorly understood (Olson et al., 2011). Finally, Marquez and Suarez (2007) indicated that hyperactivation is dependent on intracellular alkalization that stimulates extracellular calcium entry mainly through CatSper channels in the principal piece of membrane-intact bull spermatozoa. In this study, we find that cAMP-induced protein tyrosine phosphorylation in the connecting and principal pieces facilitates the transition to hyperactivation in response to extracellular calcium in membrane-intact boar spermatozoa (Tables 1 and 2, Figs. 1 and 2). Moreover, enhancement of cAMP-induced protein tyrosine phosphorylation with calyculin A was accompanied by the acceleration of hyperactivation in membrane-intact boar spermatozoa (Table 3, Fig. 3). In previous reports (Harayama et al., 2005), we suggested that PLCγ1 within the connecting piece of spermatozoa may be activated by cAMP-induced phosphorylation at a key regulatory tyrosine residue, and that calcium was consequently released from internal store though the inositol 1,4,5-triphosphate receptor (IP_3R; Fig. 5). These findings are consistent with the hypothesis that boar spermatozoa need to exert the action of protein tyrosine phosphorylation in the connecting and principal pieces in order to be capable of transitioning to hyperactivation in response to the calcium recruited from the extracellular space and internal stores (Fig. 5). In addition, Aitken and co-workers (Aitken et al., 1998; Ecroyd et al., 2003) demonstrated that cAMP-dependent protein tyrosine phosphorylation is the redox-regulated event associated with capacitation. Breitbart et al. (2005) indicated that this protein tyrosine phosphorylation is linked to filamentous-actin (F-actin) formation, which is important for the translocation of PLC from the cytoplasm to the plasma membrane in the sperm head. Subsequently, zona-induced increases in intracellular calcium lead to the depolymerization of the F-actin, which allows the acrosome reaction to take place.

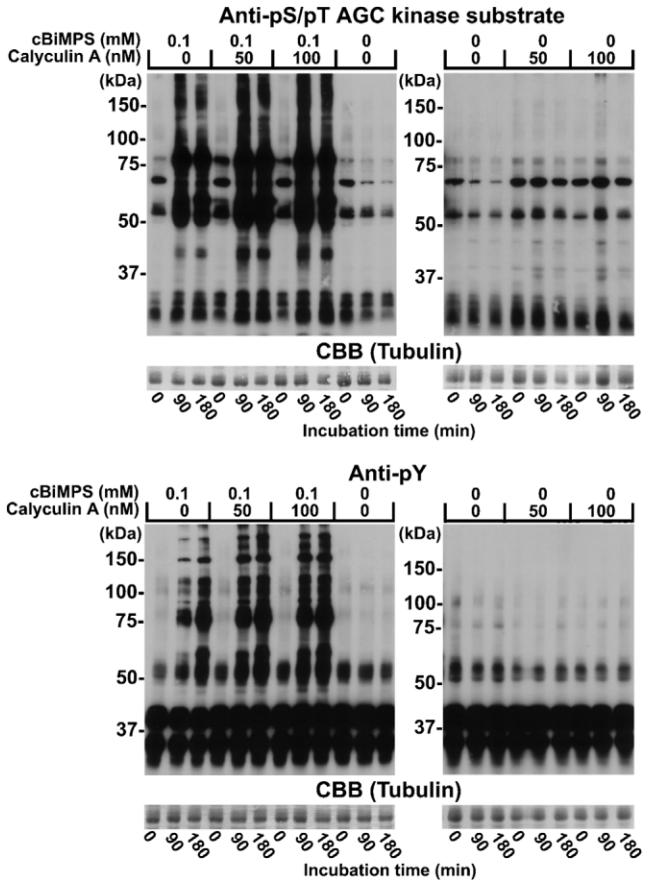

Figure 3. Effects of calyculin A, an inhibitor for protein phosphatase 1 (PP1)/protein phosphatase 2A (PP2A), on serine/threonine phosphorylation of AGC kinase substrates and tyrosine phosphorylation of proteins in boar spermatozoa incubated with or without cBiMPS. Washed spermatozoa were incubated with calyculin A in the presence or absence of cBiMPS for up to 180 min at 38.5°C. Immediately before and after incubation, aliquots of the sperm suspensions (1×10^6 spermatozoa/lane) were recovered and used for the SDS–PAGE/Western blotting with the diluted rabbit anti-phospho-AGC kinase substrate polyclonal antibody (Anti-pS/pT AGC kinase substrate, 1:5,000, left panel) or mouse anti-phosphotyrosine monoclonal antibody (Anti-pY, 1:10,000, right panel), followed by HRP-conjugated donkey anti-rabbit immunoglobulin polyclonal antibody (1:1,000–1:2,000) or HRP-conjugated goat anti-mouse immunoglobulin polyclonal antibody (1:10,000). Each blot is a representative of three replicates. After Western blotting, each membrane was stained with Coomasie Brilliant Blue G-250 to detect tubulin as the loading control [CBB (Tubulin)].

Thus, it will be of interest to analyze the relationship of protein tyrosine phosphorylation and calcium level with changes in flagellar actin during hyperactivation of boar spermatozoa in the future.

Complicated Calcium Signaling Cascades Regulating the Hyperactivation

Calmodulin is a ubiquitous molecule that regulates calcium signaling (Klee et al., 1980). In mammalian spermatozoa, calmodulin appears to delay the ability of sperm to fertilize an oocyte as its antagonists accelerate the process of capacitation in spermatozoa (Adeoya-Osiguwa and Fraser, 1996; Leclerc et al., 1998). Indeed, it has been reported that the decrease in the concentration and binding affinity of calmodulin is important for capacitation of bull spermatozoa (Leclerc et al., 1992; Leclerc and Goupil, 2000). In this study, the addition of the calmodulin antagonists (W-7 at 2 or 4 μM and W-5 at 10 or 20 μM) enhanced the occurrence of hyperactivation in the presence of cBiMPS and $CaCl_2$ without changes to cAMP-induced protein tyrosine phosphorylation (Tables 4 and 5, Fig. 4). The action of extracellular calcium was required for hyperactivation after cAMP-induced protein tyrosine phosphorylation, (Tables 1 and 2, Figs. 1 and 2). Our previous paper (Harayama et al., 2005) suggested that PLCγ1 is activated by this cAMP-induced tyrosine phosphorylation, and that resultant release of calcium from internal stores may be linked to transition to hyperactivation. Thus, boar spermatozoa possess complicated calcium signaling cascades that regulate hyperactivation, including a promoting system downstream of cAMP signaling cascades but also a calmodulin-mediated suppressing system (Fig. 5). The calmodulin-mediated suppressing system might prevent precocious hyperactivation in uncapacitated spermatozoa with slightly injured plasma membrane in response to uncontrolled entry of extracellular calcium.

CONCLUSIONS

As summarized in Figure 5, we found that cAMP-induced protein tyrosine phosphorylation is connected to calcium signaling cascades that promote hyperactivation in the connecting and principal pieces of boar spermatozoa. Calmodulin may exert effects on signaling cascades, leading to the suppression of hyperactivation. In conclusion, we hypothesize that these signaling cascades must be orchestrated during the capacitation process for the full development of flagellar movement during hyperactivation in boar spermatozoa.

MATERIALS AND METHODS

An Animal Use Ethics Statement

This study was approved by the Institutional Animal Care and Use Committee (Permission number: 16-04-08, 22-05-14) and carried out according to the Guidelines of Animal Experiments of Kobe University.

Preparation of Sperm Samples

Sperm-rich fractions from ejaculates were obtained from six mature boars (three Large White boars, one Duroc boar, and two Meishan boars) by a manual method. The spermatozoa were washed in isotonic Percoll (GE Healthcare UK Ltd., Buckinghamshire, UK) and then in phosphate-buffered saline (PBS) containing 0.1% polyvinyl alcohol

TABLE 4. Effects of a Cell-Permeable Calmodulin Antagonist (W-7 hydrochloride) on the Motility Rates, Flagellar Beating, and Hyperactivation in Boar Spermatozoa Incubated With cBiMPS (n = 3)

			Incubation time (min)		
	cBiMPS (mM)	W-7 (μM)	0	90	180
Motility rates[a]	0.1	0	78 ± 4	75 ± 9	68 ± 6
	0.1	1	80 ± 0	77 ± 6	70 ± 5
	0.1	2	80 ± 0	77 ± 6	70 ± 5
	0.1	4	77 ± 6	70 ± 10	67 ± 8
	0	0	73 ± 12	63 ± 15	63 ± 15
	0	1	77 ± 6	67 ± 15	63 ± 15
	0	2	75 ± 9	65 ± 15	65 ± 13
	0	4	73 ± 12	67 ± 15	63 ± 15
Patterns of flagellar beating[b]	0.1	0	SS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	0.1	1	SS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	0.1	2	SS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	0.1	4	SS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	0	0	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 3)
	0	1	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 3)
	0	2	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 3)
	0	4	SS (n = 3)	SS (n = 3)	SW (n = 3)
Hyperactivated sperm[c]	0.1	0	− (n = 3)	+ (n = 3)	++ (n = 3)
	0.1	1	− (n = 3)	+ (n = 3)	++ (n = 3)
	0.1	2	− (n = 3)	+ (n = 1)/++ (n = 2)	+++ (n = 3)
	0.1	4	− (n = 3)	+ (n = 1)/++ (n = 2)	+++ (n = 3)
	0	0	− (n = 3)	− (n = 3)	− (n = 3)
	0	1	− (n = 3)	− (n = 3)	− (n = 3)
	0	2	− (n = 3)	− (n = 3)	− (n = 3)
	0	4	− (n = 3)	− (n = 3)	− (n = 3)

			Incubation time (min)		
	cBiMPS (mM)	W-7 (μM)	0	90	180
Motility rates[a]	0.1	0	77 ± 10	67 ± 6	57 ± 6
	0.1	10	77 ± 10	63 ± 12	48 ± 10
	0.1	20	75 ± 9	60 ± 10	43 ± 15
	0	0	75 ± 9	70 ± 10	63 ± 12
	0	10	73 ± 12	67 ± 15	57 ± 15
	0	20	73 ± 12	63 ± 12	53 ± 12
Patterns of flagellar beating[b]	0.1	0	SS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	0.1	10	SS (n = 3)	SS & AS (n = 3)	SW (n = 1)/SS & AS (n = 2)
	0.1	20	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SW (n = 1)/SS & AS (n = 2)
	0	0	SS (n = 3)	SS (n = 3)	SS (n = 3)
	0	10	SS (n = 3)	SW (n = 1)/SS (n = 2)	SW (n = 1)/SS (n = 2)
	0	20	SS (n = 3)	SS (n = 3)	SW (n = 1)/SS (n = 2)
Hyperactivated sperm[c]	0.1	0	− (n = 3)	+ (n = 3)	+ (n = 1)/++ (n = 2)
	0.1	10	− (n = 3)	+ (n = 2)/++ (n = 1)	− (n = 1)/+ (n = 1)/++ (n = 1)
	0.1	20	− (n = 3)	− (n = 1)/+ (n = 1)/++ (n = 1)	− (n = 1)/+ (n = 1)/++ (n = 1)
	0	0	− (n = 3)	− (n = 3)	− (n = 3)
	0	10	− (n = 3)	− (n = 3)	− (n = 3)
	0	20	− (n = 3)	− (n = 3)	− (n = 3)

			Incubation time (min)		
	cBiMPS (mM)	W-7 (μM)	0	90	180
Motility rates[a]	0.1	0	75 ± 13[d]	65 ± 13[d]	65 ± 13[d]
	0.1	100	63 ± 12[de]	18 ± 16[de]	5 ± 5[e]
	0.1	200	60 ± 10[de]	8 ± 8[e]	3 ± 3[e]
	0	0	75 ± 13[d]	68 ± 16[d]	68 ± 16[d]
	0	100	63 ± 12[de]	33 ± 15[de]	7 ± 8[e]
	0	200	50 ± 17[e]	13 ± 13[e]	2 ± 3[e]
Patterns of flagellar beating[b]	0.1	0	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0.1	100	SW (n = 1)/SS (n = 2)	SW (n = 3)	SW (n = 3)
	0.1	200	SW (n = 1)/SS (n = 2)	SW (n = 3)	SW (n = 3)
	0	0	SS (n = 3)	SS (n = 3)	SW (n = 1)/SS (n = 2)
	0	100	SW (n = 1)/SS (n = 2)	SW (n = 3)	SW (n = 3)
	0	200	SW (n = 1)/SS (n = 2)	SW (n = 3)	SW (n = 3)
Hyperactivated sperm[c]	0.1	0	− (n = 3)	− (n = 1)/+(n = 2)	+ (n = 1)/++ (n = 2)
	0.1	100	− (n = 3)	− (n = 3)	− (n = 3)
	0.1	200	− (n = 3)	− (n = 3)	− (n = 3)
	0	0	− (n = 3)	− (n = 3)	− (n = 3)
	0	100	− (n = 3)	− (n = 3)	− (n = 3)
	0	200	− (n = 3)	− (n = 3)	− (n = 3)

[a]Values are means of percentages of motile spermatozoa ± standard deviation.
[b]SW, symmetrical beating (weak); SS, symmetrical beating (strong); and AS, asymmetrical beating (strong): see supplemental movies.
[c]−, 0–10%; +, 11–30%; ++, 31–50%; +++.>50%. [de]Values (the percentages of motile sperm) with different superscripts within the same column were significantly different (ANOVA and Tukey's multiple range tests, $P < 0.05$).

TABLE 5. Effects of a Cell-Permeable Calmodulin Antagonist (W-5 Hydrochloride) on the Motility Rates, Flagellar Beating, and Hyperactivation in Boar Spermatozoa Incubated With cBiMPS (n = 3)

			Incubation time (min)		
	cBiMPS (mM)	W-5 (μM)	0	90	180
Motility rates[a]	0.1	0	73 ± 6	63 ± 6	63 ± 6
	0.1	1	73 ± 6	63 ± 6	63 ± 6
	0.1	2	73 ± 6	63 ± 6	63 ± 6
	0.1	4	73 ± 6	63 ± 6	60 ± 10
	0	0	70 ± 0	70 ± 0	63 ± 6
	0	1	70 ± 0	70 ± 0	63 ± 6
	0	2	73 ± 6	70 ± 0	60 ± 10
	0	4	73 ± 6	70 ± 0	63 ± 6
Patterns of flagellar beating[b]	0.1	0	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0.1	1	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0.1	2	SS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	0.1	4	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0	0	SS (n = 3)	SS (n = 3)	SW (n = 2)/SS (n = 1)
	0	1	SS (n = 3)	SS (n = 3)	SW (n = 2)/SS (n = 1)
	0	2	SS (n = 3)	SS (n = 3)	SW (n = 2)/SS (n = 1)
	0	4	SS (n = 3)	SS (n = 3)	SW (n = 1)/SS (n = 2)
Hyperactivated sperm[c]	0.1	0	− (n = 3)	− (n = 1)/+ (n = 2)	+ (n = 1)/++ (n = 2)
	0.1	1	− (n = 3)	− (n = 1)/+ (n = 2)	+ (n = 1)/++ (n = 2)
	0.1	2	− (n = 3)	+ (n = 3)	+ (n = 2)/++ (n = 1)
	0.1	4	− (n = 3)	− (n = 1)/+ (n = 1)/++ (n = 1)	+ (n = 1)/++ (n = 2)
	0	0	− (n = 3)	− (n = 3)	− (n = 3)
	0	1	− (n = 3)	− (n = 3)	− (n = 3)
	0	2	− (n = 3)	− (n = 3)	− (n = 3)
	0	4	− (n = 3)	− (n = 3)	− (n = 3)

			Incubation time (min)		
	cBiMPS (mM)	W-5 (μM)	0	90	180
Motility rates[a]	0.1	0	77 ± 6	70 ± 10	70 ± 10
	0.1	10	77 ± 6	70 ± 10	70 ± 10
	0.1	20	77 ± 6	70 ± 10	70 ± 10
	0	0	77 ± 6	73 ± 6	67 ± 6
	0	10	77 ± 6	73 ± 6	67 ± 6
	0	20	77 ± 6	73 ± 6	67 ± 6
Patterns of flagellar beating[b]	0.1	0	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0.1	10	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0.1	20	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0	0	SS (n = 3)	SS (n = 3)	SW (n = 2)/SS (n = 1)
	0	10	SS (n = 3)	SS (n = 3)	SW (n = 2)/SS (n = 1)
	0	20	SS (n = 3)	SS (n = 3)	SW (n = 2)/SS (n = 1)
Hyperactivated sperm[c]	0.1	0	− (n = 3)	− (n = 1)/+ (n = 2)	+ (n = 1)/++ (n = 2)
	0.1	10	− (n = 3)	− (n = 1)/+ (n = 1)/++ (n = 1)	+ (n = 1)/+++ (n = 2)
	0.1	20	− (n = 3)	− (n = 1)/++ (n = 2)	++ (n = 1)/+++ (n = 2)
	0	0	− (n = 3)	− (n = 3)	− (n = 3)
	0	10	− (n = 3)	− (n = 3)	− (n = 3)
	0	20	− (n = 3)	− (n = 3)	− (n = 3)

			Incubation time (min)		
	cBiMPS (mM)	W-5 (μM)	0	90	180
Motility rates[a]	0.1	0	70 ± 10	63 ± 15	57 ± 12
	0.1	100	73 ± 6	60 ± 20	50 ± 0
	0.1	200	73 ± 6	60 ± 20	50 ± 0
	0	0	63 ± 15	63 ± 6	63 ± 6
	0	100	63 ± 15	63 ± 6	63 ± 6
	0	200	63 ± 15	63 ± 6	57 ± 6
Patterns of flagellar beating[b]	0.1	0	SS (n = 3)	SS & AS (n = 3)	SS & AS (n = 3)
	0.1	100	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0.1	200	SS (n = 3)	SS (n = 1)/SS & AS (n = 2)	SS & AS (n = 3)
	0	0	SS (n = 3)	SS (n = 3)	SS (n = 3)
	0	100	SS (n = 3)	SS (n = 3)	SS (n = 3)
	0	200	SS (n = 3)	SS (n = 3)	SS (n = 3)
Hyperactivated sperm[c]	0.1	0	− (n = 3)	+ (n = 2)/+ (n = 1)	+ (n = 2)/++ (n = 1)
	0.1	100	− (n = 3)	− (n = 1)/+ (n = 1)/+++ (n = 1)	+ (n = 1)/++ (n = 2)
	0.1	200	− (n = 3)	− (n = 1)/+ (n = 1)/++ (n = 1)	+ (n = 1)/++ (n = 2)
	0	0	− (n = 3)	− (n = 3)	− (n = 3)
	0	100	− (n = 3)	− (n = 3)	− (n = 3)
	0	200	− (n = 3)	− (n = 3)	− (n = 3)

[a]Values are means of percentages of motile spermatozoa ± standard deviation.
[b]SW, symmetrical beating (weak); SS, symmetrical beating (strong); and AS, asymmetrical beating (strong): see supplemental movies.
[c]−, 0–10%;+, 11–30%; ++, 31–50%, +++, >50%.

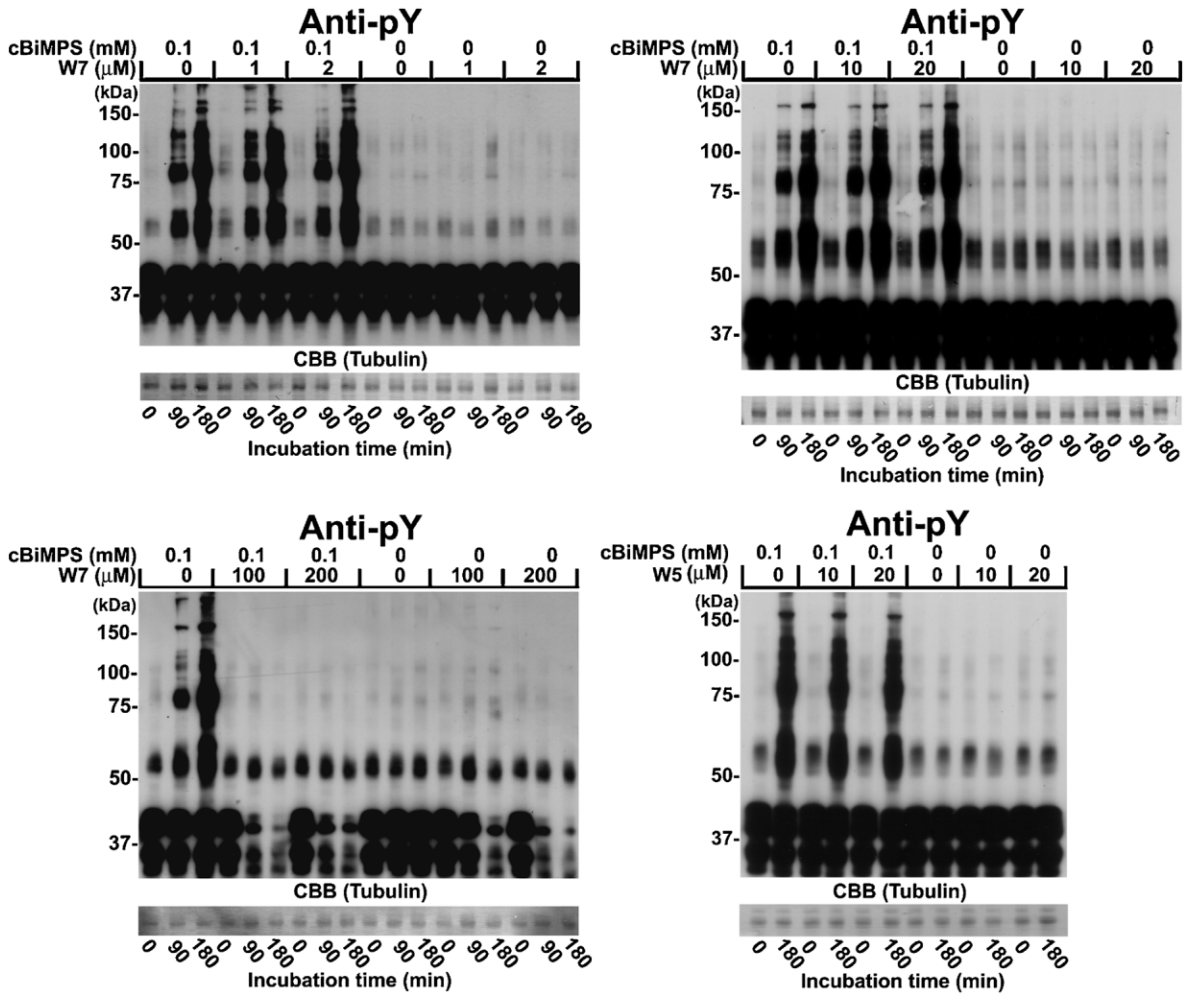

Figure 4. Effects of calmodulin antagonists W-7 and W-5 on protein tyrosine phosphorylation in boar spermatozoa incubated with cBiMPS. Washed spermatozoa were incubated with W-7 or W-5 in the presence of cBiMPS and $CaCl_2$, up to 180 min at 38.5°C. Immediately before and after incubation, aliquots of sperm suspensions (1×10^6 spermatozoa/lane) were recovered and used for the SDS–PAGE/Western blotting with the diluted mouse anti-phosphotyrosine monoclonal antibody (Anti-pY, 1:10,000), and then with HRP-conjugated goat anti-mouse immunoglobulin polyclonal antibody (1:10,000). Each blot was a representative of three replicates. After Western blotting, each membrane was stained with Coomasie Brilliant Blue G-250 to detect tubulin as the loading control [CBB (Tubulin)].

(PVA, Sigma–Aldrich Co., St. Louis, MO; PVA–PBS) by centrifugation, as described previously (Harayama et al., 2004ab). The basic incubation medium was a modified Krebs-Ringer Hepes medium (mKRH: 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, 27.64 mM glucose, 50 μg/ml streptomycin sulfate, 100 IU/ml potassium penicillin G and 2 μg/ml phenol red: pH 7.4) supplemented with 0.1% PVA before use.

A cell-permeable, phosphodiesterase-resistant cAMP analog Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3′,5′-monophoshorothioate (cBiMPS; Biomol Research Laboratories, Inc., Plymouth Meeting, PA; Schaap et al., 1993) was dissolved in 10% (v/v) dimethyl sulfoxide (DMSO, Wako Pure Chemical Industries Ltd., Osaka, Japan) to a 4 mM stock solution, and then added to the incubation medium to a final concentration of 0.1 mM. To the control samples without cBiMPS, the same volume of 10% DMSO was added to equalize the concentration of solvent. The washed spermatozoa were resuspended in incubation medium to a final sperm concentration of 1.0×10^8 cells/ml, and then were incubated in a water bath (38.5°C) for up to 360 min. Before and after incubation for 90–360 min, sperm suspensions were mixed well and then aliquots were recovered for use in the following experiments. To examine effects of cAMP-induced protein phosphorylation state and calmodulin on the occurrence of hyperactivation, calyculin A (an inhibitor for PP1/PP2A, Sigma–Aldrich), *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) hydrochloride (a calmodulin antagonist, Wako) and *N*-(6-aminohexyl)-1-naphthalene sulfonamide (W-5) hydrochloride (a calmodulin antagonist, Wako) were

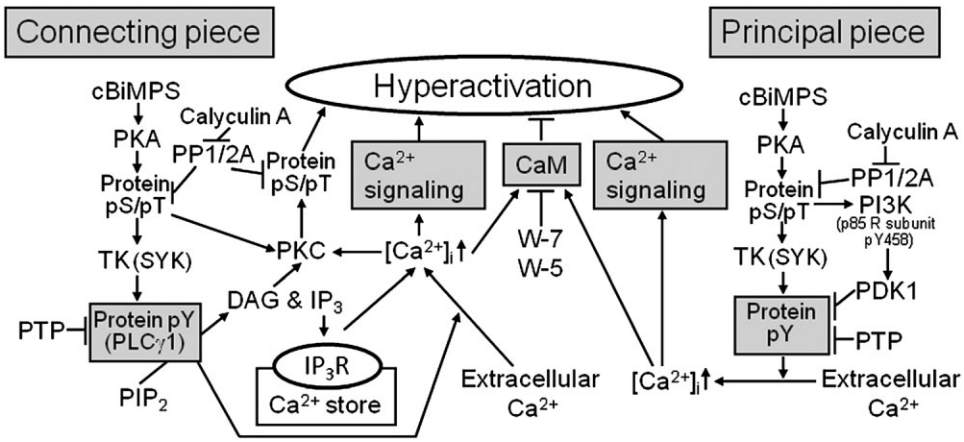

Figure 5. Possible segment-specific signaling cascades regulating the transition of flagellar movement to hyperactivation in boar spermatozoa. CaM, calmodulin; DAG, 1,2-diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphatidylinositol-3 kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A (cAMP-dependent protein kinase); PKC, protein kinase C; PLC, phospholipase C; PP, protein phosphatase; pS/pT, serine/threonine phosphorylation; PTP, protein tyrosine phosphatase; pY, tyrosine phosphorylation; SYK, spleen tyrosine kinase; TK, tyrosine kinase.

dissolved in DMSO (100%) and then added to the sperm suspension. DMSO (100%) was also added to the control samples without these inhibitors to equalize the concentration of solvent among all samples.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting were performed as described previously (Harayama and Miyake, 2006; Harayama and Nakamura, 2008).The antibodies used in this study were rabbit anti-phospho-AGC kinase substrate (specific to a phosphoserine or phosphothreonine residue with arginine at the −3 position or a phosphoserine residue with arginine at the −2 position) polyclonal antibody [Cell Signaling Technology, Inc., Beverly, MA, Cat#9621 named phospho-(Ser/Thr) PKA substrate antibody, 1:5,000], mouse anti-phosphotyrosine monoclonal antibody (clone 4G10, Upstate Cell Signaling Solutions, Charlottesville, VA, Cat.#05-321, 1:10,000), horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulins (GE Healthcare, Cat.#NA934V, 1:1,000–1:2,000) and HRP-conjugated goat anti-mouse immunoglobulins (Dako Cytomation Denmark A/S, Glostrup, Denmark, Cat.#P0447, 1:10,000).

Indirect Immunofluorescence

Immunofluorescence procedures were described previously (Harayama and Miyake, 2006). Briefly, sperm suspensions were gently smeared on glass slides and fixed in methanol for 10 min. The slides were gently rinsed with PBS twice, blocked with 5% bovine serum albumin (BSA, Cohn fraction V, pH 7.0, Wako) in PBS (PBS–BSA) for 60 min, and then treated with the anti-phosphotyrosine antibody (1:1,000) overnight at 4°C. After two washes in PBS, slides were treated with FITC-conjugated rabbit anti-mouse immunoglobulin polyclonal antibody (Dako, Cat.#F0261, 1:100). After two washes with PBS, the slides were covered with VECTASHIELD Mounting Medium (Vector Laboratories, Inc., Burlingame, CA) and then coverslips. The preparations were observed under a differential interference microscope equipped with epifluorescence (U-MNIBA2 mirror unit composed of BP470-490 excitation filter, DM505 dichroic mirror and BA510-550 emission filter, Olympus Corporation, Tokyo, Japan).

Assessment for Sperm Motility

Sperm motility was assessed by subjective observation. Briefly, the motility was observed in a 5-μl drop of sperm suspension on a heated stage (38.5°C) under bright-field microscopy. Spermatozoa showing any movement were considered ''motile,'' irrespective of their progressive motility. Predominant patterns of sperm flagellar beating were classified into three categories [SW, symmetrical beating (weak); SS, symmetrical beating (strong); and AS, asymmetrical beating (strong)], as shown in Supplementary materials (movies 1–3). The percentages of spermatozoa showing hyperactivation were estimated and the obtained results were classified into four categories: (−), 0–10%; (+), 11–30%; (++), 31–50%; (+++), >50% of total spermatozoa.

Statistical Analysis

Percentages of 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).

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REFERENCES

Adachi J, Tate S, Miyake M, Harayama H. 2008. Effects of protein phosphatase inhibitor calyculin A on the postacrosomal protein serine/threonine phosphorylation state and acrosome reaction in boar spermatozoa incubated with a cAMP analog. J Reprod Dev 54:171–176.

Adeoya-Osiguwa SA, Fraser LR. 1996. Evidence for Ca^{2+}-dependent ATPase activity, stimulated by decapacitation factor and calmodulin, in mouse sperm. Mol Reprod Dev 44:111–120.

Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. 1998. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. J Cell Sci 111:645–656.

Baker MA, Hetherington L, Aitken RJ. 2006. Identification of SRC as a key PKA-stimulated tyrosine kinase involved in the capacitation-associated hyperactivation of murine spermatozoa. J Cell Sci 119:3182–3192.

Bravo MM, Aparicio IM, Garcia-Herreros M, Gil MC, Peña FJ, Garcia-Marin LJ. 2005. Changes in tyrosine phosphorylation associated with true capacitation and capacitation-like state in boar spermatozoa. Mol Reprod Dev 71:88–96.

Breitbart H, Cohen G, Rubinstein S. 2005. Role of actin cytoskeleton in mammalian sperm capacitation and the acrosome reaction. Reproduction 129:263–268.

Buffone MG, Ijiri TW, Cao W, Merdiushev T, Aghajanian HK, Gerton GL. 2012. Heads or tails? Structural events and molecular mechanisms that promote mammalian sperm acrosomal exocytosis and motility. Mol Reprod Dev 79:4–18.

Ecroyd HW, Jones RC, Aitken RJ. 2003. Endogenous redox activity in mouse spermatozoa and its role in regulating the tyrosine phosphorylation events associated with sperm capacitation. Biol Reprod 69:347–354.

Goto N, Harayama H. 2009. Calyculin A-sensitive protein phosphatases are involved in maintenance of progressive movement in mouse spermatozoa *in vitro* by suppression of autophosphorylation of protein kinase A. J Reprod Dev 55:327–334.

Harayama H. 2003. Viability and protein phosphorylation patterns of boar spermatozoa agglutinated by the treatment with a cell-permeable cyclic adenosine 3′,5′-monophosphate analog. J Androl 24:831–842.

Harayama H, Miyake M. 2006. A cyclic adenosine 3′,5′-monophosphate-dependent protein kinase C activation is involved in the hyperactivation of boar spermatozoa. Mol Reprod Dev 73:1169–1178.

Harayama H, Nakamura K. 2008. Changes of PKA and PDK1 in the principal piece of boar spermatozoa treated with a cell-permeable cAMP analog to induce flagellar hyperactivation. Mol Reprod Dev 75:1396–1407.

Harayama H, Muroga M, Miyake M. 2004a. A cyclic adenosine 3′,5′-monophosphate-induced tyrosine phosphorylation of Syk protein tyrosine kinase in the flagella of boar spermatozoa. Mol Reprod Dev 69:436–447.

Harayama H, Sasaki K, Miyake M. 2004b. A unique mechanism for cyclic adenosine 3′,5′-monophosphate-induced increase of 32-kDa tyrosine-phosphorylated protein in boar spermatozoa. Mol Reprod Dev 69:194–204.

Harayama H, Murase T, Miyake M. 2005. A cyclic adenosine 3′,5′-monophosphate stimulates a phospholipase Cγ1-calcium signaling via the activation of tyrosine kinase in boar spermatozoa. J Androl 26:732–740.

Harayama H, Nishijima K, Murase T, Sakase M, Fukushima M. 2010. Relationship of protein tyrosine phosphorylation state with tolerance to frozen storage and the potential to undergo cyclic AMP-dependent hyperactivation in the spermatozoa of Japanese Black bulls. Mol Reprod Dev 77:910–921.

Ho HC, Granish KA, Suarez SS. 2002. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca^{2+} and not cAMP. Dev Biol 250:208–217.

Kaneto M, Harayama H, Miyake M, Kato S. 2002. Capacitation-like alterations in cooled boar spermatozoa: Assessment by the chlortetracycline staining assay and immunodetection of tyrosine-phosphorylated sperm proteins. Anim Reprod Sci 73:197–209.

Kaneto M, Krisfalusi M, Eddy EM, O'Brien DA, Miki K. 2008. Bicarbonate-induced phosphorylation of p270 protein in mouse sperm by cAMP-dependent protein kinase. Mol Reprod Dev 75:1045–1053.

Klee CB, Crouch TH, Richman PG. 1980. Calmodulin. Annu Rev Biochem 49:489–515.

Leclerc P, Goupil S. 2000. Distribution and localization of calmodulin-binding proteins in bull spermatozoa. Biol Reprod 62: 1875–1881.

Leclerc P, Sirard MA, Chafouleas JG, Lambert RD. 1992. Decrease in calmodulin concentrations during heparin-induced capacitation in bovine spermatozoa. J Reprod Fertil 94: 23–32.

Leclerc P, de Lamirande E, Gagnon C. 1998. Interaction between Ca^{2+}, cyclic 3′,5′ adenosine monophosphate, the superoxide anion, and tyrosine phosphorylation pathways in the regulation of human sperm capacitation. J Androl 19:434–443.

Marquez B, Suarez SS. 2004. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. Biol Reprod 70:1626–1633.

Marquez B, Suarez SS. 2007. Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca^{2+} influx. Biol Reprod 76: 660–665.

McPartlin LA, Suarez SS, Czaya CA, Hinrichs K, Bedford-Guaus SJ. 2009. Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. Biol Reprod 81:199–206.

Motulsky H. 1995. Intuitive Biostatistics. New York: Oxford University Press.

Olson SD, Fauci LJ, Suarez SS. 2011. Mathematical modeling of calcium signaling during sperm hyperactivation. Mol Hum Reprod 17:500–510.

Qi H, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, Kirichok Y, Ramsey IS, Quill TA, Clapham DE. 2007. All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. Proc Natl Acad Sci USA 104:1219–1223.

Schaap P, van Ments-Cohen M, Soede RD, Brandt R, Firtel RA, Dostmann W, Genieser HG, Jastorff B, van Haastert PJ. 1993. Cell-permeable non-hydrolyzable cAMP derivatives as tools for analysis of signaling pathways controlling gene regulation in Dictyostelium. J Biol Chem 268:6323–6331.

Si Y. 1999. Hyperactivation of hamster sperm motility by temperature-dependent tyrosine phosphorylation of an 80-kDa protein. Biol Reprod 61:247–252.

Si Y, Okuno M. 1999. Role of tyrosine phosphorylation of flagellar proteins in hamster sperm hyperactivation. Biol Reprod 61:240–246.

Suarez SS, Dai XB, DeMott RP, Redfern K, Mirando MA. 1992. Movement characteristics of boar sperm obtained from the oviduct or hyperactivated in vitro. J Androl 13: 75–80.

Tardif S, Dúbe C, Chevalier S, Bailey JL. 2001. Capacitation is associated with tyrosine phosphorylation and tyrosine kinase-like activity of pig sperm proteins. Biol Reprod 65: 784–792.

Tate S, Nakamura K, Suzuki C, Noda T, Lee J, Harayama H. 2010. Evidence of the existence of adenylyl cyclase 10 (ADCY10) ortholog proteins in the heads and connecting pieces of boar spermatozoa. J Reprod Dev 56:271–278.

Yanagimachi R. 1994. Mammalian fertilization. In: Knobil E, Neill JD, editors. The Physiology of Reproduction, 2nd edition. New York: Raven Press. pp 189–317.

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