Regulation of hyperactivation by PPP2 in hamster spermatozoa

Tatsuya Suzuki, Masakatsu Fujinoki1, Hiroaki Shibahara and Mitsuaki Suzuki

Department of Obstetrics and Gynecology, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke-shi, Tochigi 329-0498, Japan and 1Department of Physiology, School of Medicine, Dokkyo Medical University, Tochigi 321-0293, Japan

Correspondence should be addressed to T Suzuki; Email: statsuya@jichi.ac.jp

Abstract

It has been widely accepted that serine/threonine protein phosphatases (PPPs) are associated with the regulation of sperm hyperactivation. In the present study, we examined the types of PPPs associated with the regulation of hamster sperm hyperactivation. Protein phosphatases PPP1CA, PPP1CC, PPP2, and PPP3 are present in hamster sperm. In the experiments using several inhibitors, sperm hyperactivation was enhanced when PPP2 was inhibited at least, although inhibition of PPP1 also enhanced sperm hyperactivation. Interestingly, sperm were hyperactivated after PPP2 became an inactive form. And then, PPP1CA became an active form after sperm were hyperactivated. It has also been widely accepted that tyrosine phosphorylation is closely associated with the regulation of sperm hyperactivation. When PPP2 was inhibited, tyrosine phosphorylation was not enhanced at all. On the other hand, inhibition of PPP1 enhanced tyrosine phosphorylation. From the results, it is likely that PPP2 is closely associated with the regulation of sperm hyperactivation, although it is not associated with the regulation of tyrosine phosphorylation.

Reproduction (2010) 139 847–856

Introduction


Recent understanding is that sperm hyperactivation is closely associated with protein tyrosine phosphorylation (Visconti et al. 1995, 1998, 1999, Visconti & Kopf 1998, Fujinoki et al. 2001b, 2006, Jha & Shivaji 2001, Marín-Briggiler et al. 2005, Noguchi et al. 2008). Moreover, it is suggested that protein serine/threonine phosphorylation is also associated with the regulation of hyperactivation (Naz 1999, Jha & Shivaji 2002a, Fujinoki et al. 2004, 2006). On the other hand, it has been suggested that protein dephosphorylation is also associated with the regulation of hyperactivation (Fujinoki et al. 2006). It has been suggested that inhibitors of protein phosphatase 1/2A (PPP1/PPP2), which are okadaic acid and calyculin A, enhanced hyperactivation and tyrosine

© 2010 Society for Reproduction and Fertility
ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-08-0366
Online version via www.reproduction-online.org
Downloaded from Bioscientifica.com at 04/03/2022 2:35:22PM via free access
phosphorylation in rodent sperm (Si & Okuno 1999b, Goto & Harayama 2009). Moreover, Furuya et al. (1992, 1993) reported that okadaic acid induced rapid acrosome reaction of mouse sperm, and that calyculin A induced rapid acrosome reaction and enhanced phosphorylation of phosphoproteins of human sperm. And then, Baker et al. (2006, 2009) reported that PKA–PPP1 signals regulated tyrosine phosphorylation associated with sperm capacitation. These reports support the hypothesis that PPP1/PPP2 exists in sperm, and exerts the regulation of acrosome reaction and hyperactivation. Other recent studies have reported that PPP1CC2 is involved in the regulation of motility activation and/or hyperactivation in mammalian sperm (Smith et al. 1996, Huang et al. 2005, Chakrabarti et al. 2007, Goto & Harayama 2009). On the other hand, PPP3 (calcineurin) is also associated with the regulation of sperm motility and acrosome reaction (Tash et al. 1988, Tash & Bracho 1994, Ashizawa et al. 2004).

Generally, protein phosphatases are classified into two families: serine/threonine phosphatases (PPPs) and phosphotyrosine phosphatases (Barford et al. 1998). PPPs include PPP1, PPP2, and PPP3. In mammals, PPP1 has four catalytic subunit isoforms (PPP1CA, PPP1CB, PPP1CC1 and PPP1CC2) encoded by three genes (PPPICA, PPPICB and PPPICC), and many types of regulatory subunits (Ceulemans & Bollen 2004). PPP1CC1 and PPP1CC2 are the alternatively spliced variants generated from a single gene. PPP2 consists of one catalytic subunit and two regulatory subunits known as A subunit and B subunit, and PPP3 consists of one catalytic subunit and three regulatory subunits known as B subunit, calmodulin, and A-kinase anchoring protein 79 (AKAP79; Barford et al. 1998). Both PPP1 and PPP2 are negatively regulated by phosphorylation of the catalytic subunit, and PPP3 is regulated by calcium. In the cell cycle, PPP1CA is regulated by cyclin-dependent kinase via phosphorylation of Thr-320 (Kwon et al. 1997, Ceulemans & Bollen 2004). Activity of PPP2 is regulated by protein tyrosine kinase via phosphorylation of Tyr-307 and/or by autophosphorylation-activated protein kinase via phosphorylation of threonine residues (Chen et al. 1992, 1994, Guo & Damuni 1993).

In the present study, we examined which protein phosphatases are associated with the regulation and/or enhancement of hyperactivation and tyrosine phosphorylation using various protein phosphatase inhibitors. Furthermore, we also examined activities of PPP1 and PPP2.

**Results**

**PPPs present in hamster sperm**

Previous studies (Tash et al. 1988, Tash & Bracho 1994, Smith et al. 1996, Si & Okuno 1999b, Ashizawa et al. 2004, Huang et al. 2005, Chakrabarti et al. 2007, Goto & Harayama 2009) suggested that PPP1, PPP2, and PPP3 were associated with the regulation of sperm motility and hyperactivation. Moreover, PPP1CC was detected in sperm (Smith et al. 1996, Huang et al. 2005, Chakrabarti et al. 2007, Goto & Harayama 2009). At first, we examined which PPPs are present in hamster sperm. Although PPP1 has three isoforms of catalytic subunit, such as PPP1CA, PPP1CB, and PPP1CC, their molecular weights are different. Because the molecular weights of PPP1CA, PPP1CB, and PPP1CC are 38, 36, and 35 kDa, they are recognized as different bands. Using specific antibodies against each PPP1, as shown in Fig. 1, lanes c–h, PPP1CA and PPP1CC were detected in hamster sperm, and PPP1CB was not detected. As for PPP2 and PPP3, molecular weights of their catalytic subunits are 36/38 and 59 kDa respectively. As shown in Fig. 1, lanes i–l, they were also detected in hamster sperm using specific antibodies against catalytic subunits of PPP2 and PPP3. But reaction of PPP3 was very weak. Moreover, PPP1CA, PPP2, and PPP3 were mainly detected from the urea extracts, which include sperm proteins without the fibrous sheath and nucleus (Fujinoki et al. 2004). On the other hand, PPP1CC was detected from both the urea extracts and the urea-thiouria extracts, which include fibrous sheath proteins (Fujinoki et al. 2004).

**Effects of inhibition of PPP1 and PPP2 on sperm hyperactivation**

Si & Okuno (1999b) reported that 5 μM okadaic acid and 2 μM calyculin A significantly enhanced sperm hyperactivation in hamsters. They also reported that over 80% of the sperm had become fully hyperactivated within 40 min when 5 μM okadaic acid and 2 μM calyculin A were added to the modified Tyrode’s albumin lactate pyruvate (mTALP) medium, although incubation for

![Figure 1 Detection of PPP1, PPP2, and PPP3 in hamster sperm. Twenty-microliter samples were applied for SDS-PAGE. Lanes a and b show blotting membrane stained by CBB. Lanes c and d, lanes e and f, lanes g and h, lanes i and j, and lanes k and l show detection of PPP1CA (1:1000 dilution), PPP1CB (1:200 dilution), PPP1CC (1:200 dilution), PPP2 (1:1000 dilution), and PPP3 (1:1000 dilution) using specific antibodies respectively. Lanes a, c, e, g, i, and k show results obtained through the urea extracts, and lanes b, d, f, h, j, and l show results obtained through the urea–thiouria extracts. Numbers on the left side of lane a indicate the molecular weights of the standards.](image-url)
about 3 h was required to produce hyperactivated motility in the mTALP medium alone. Because both PPP1 and PPP2 were inhibited by 5 μM okadaic acid and 2 μM calyculin A in the previous experiment (Si & Okuno 1999b), we examined which PPPs are associated with the regulation of hyperactivation by using several concentrations of okadaic acid and calyculin A (okadaic acid: PPP1, IC50 = 10–15 nM, PPP2, IC50 = 0.1 nM; calyculin A: PPP1, IC50 = 2 nM, PPP2, IC50 = 0.5–1.0 nM). As for the percentage of motile sperm, both okadaic acid and calyculin A did not exert an effect (data not shown). However, they significantly increased sperm hyperactivation (Fig. 2A and B). As shown in Fig. 2A, 10–500 nM okadaic acid significantly increased hyperactivation at incubation for 1.5 h (versus control and 0.1 nM okadaic acid) and 2 h (versus control). At incubation for 2.5 h, 10 and 50 nM okadaic acid significantly increased hyperactivation compared with the control. But 500 nM okadaic acid significantly decreased hyperactivation at incubation for 2.5 h (versus 0.1 nM okadaic acid) and 4 h (versus control, and 0.1 and 10 nM okadaic acid). Then, 0.1 nM okadaic acid significantly increased hyperactivation at incubation for 2 and 2.5 h compared with the control. On the other hand, sperm hyperactivation was significantly increased at incubation for 0.5 h (versus control, and 0.5, 2, and 50 nM calyculin A), 1 h (versus control, and 0.5, 2, and 50 nM calyculin A), 1.5 h (versus control, and 0.5 and 2 nM calyculin A), 2 h (versus control and 0.5 nM calyculin A), and 2.5 h (versus control) when 500 nM calyculin A was added to the mTALP medium (Fig. 2B). Fifty-nanomolar calyculin A significantly increased hyperactivation at incubation for 1 h (versus control, and 0.5 and 2 nM calyculin A), 1.5 h (versus control, and 0.5 and 2 nM calyculin A), 2 h (versus control), and 2.5 h (versus control). At incubation for 3 h, 50 and 500 nM calyculin A significantly decreased hyperactivation compared with 0.5 nM calyculin A. However, 0.5 and 2 nM calyculin A significantly increased hyperactivation at incubation for 1.5, 2, 2.5, and 3 h compared with the control, and did not affect hyperactivation at incubation for 1 h.

In the next step, we used other inhibitors of PPPs such as tautomycin (PPP1, IC50 = 1 nM; PPP2, IC50 = 10 nM; Fig. 2C). As for the percentage of motile sperm, tautomycin did not exert an effect (data not shown). As shown in Fig. 2C, however, both 1 and 10 nM tautomycin significantly increased sperm hyperactivation at incubation for 2 and 2.5 h compared with the control. Moreover, 1 nM tautomycin also significantly increased hyperactivation at incubation for 3 h compared with the control.

**Effects of inhibition of PPP3 on sperm hyperactivation**

Because PPP3 is also associated with the regulation of sperm motility (Tash et al. 1988, Tash & Bracho 1994) and acrosome reaction (Ashizawa et al. 2004), we examined whether PPP3 affected the regulation of hyperactivation using deltamethrin (IC50 = 100 pM) and fenvalerate (IC50 = 2–4 nM; Fig. 3). When 100 pM to 1 μM deltamethrin was added to the mTALP medium,
no effect on hyperactivation was observed (Fig. 3A). In addition, 4 nM to 4 µM fenvalerate had no effect on hyperactivation (Fig. 3B). As for the percentage of motile sperm, they also did not exert an effect (data not shown).

**Status of activities of PPP1CA and PPP2 during sperm hyperactivation**

In many cases, enzyme activities are thought to be regulated by post-translational modifications by other enzymes such as phosphorylation, dephosphorylation, and methylation. Activities of PPP1CA and PPP2 are negatively regulated by the phosphorylation of PPP1CA and PPP2 (Chen et al. 1992, 1994, Guo & Damuni 1993, Kwon et al. 1997). Activity of PPP1CA was inhibited by threonine phosphorylation (Kwon et al. 1997). As shown in Fig. 4, several phosphoprotein bands were detected using anti-phospho-PPP1CA (Thr-320) antibody. But one of them corresponded to PPP1CA. As shown in Fig. 4C, PPP1CA was phosphorylated at incubation for 0, 0.5, 1, and 2 h. Interestingly, PPP1CA was dephosphorylated at incubation for 3 and 4 h. Therefore, it seems that PPP1CA is inactivated by phosphorylation during hyperactivation, and is activated by dephosphorylation in hyperactivated sperm.

On the other hand, PPP2 is inactivated by tyrosine phosphorylation (Chen et al. 1992, 1994, Guo & Damuni 1993). As shown in Fig. 5, tyrosine phosphorylation corresponding to PPP2 was detected in the urea extracts. Tyrosine phosphorylation of PPP2 did not occur at incubation for 0 h (Fig. 5C, lane a), but it occurred at incubation for 0.5, 1, 2, 3, and 4 h (Fig. 5C, lanes b–f). Therefore, it seems that PPP2 was activated in activated sperm at incubation for 0 h, and was inactivated by phosphorylation during hyperactivation and in hyperactivated sperm.

**Effects of inhibition of PPP2 on sperm protein tyrosine phosphorylation**

Si & Okuno (1999b) reported that high concentrations of okadaic acid and calyculin A significantly enhanced 80 kDa tyrosine phosphorylation. In our previous study (Fujinoki et al. 2006), we could detect four tyrosine phosphorylations, which were designated pY80u, pY80ut, pY85u, and pY85ut, in hamster sperm. Because these four proteins were phosphorylated at tyrosine residues during hyperactivation, we examined the effects of low concentrations of okadaic acid (Fig. 6) and calyculin A (Fig. 7) on tyrosine phosphorylation.

As shown in Figs 6A and F and 7A and F, tyrosine phosphorylation of pY80u, pY80ut, pY85u, and pY85ut was detected in both the urea extracts and the urea-thiourea extracts. Reactivity increased in a time-dependent manner (Figs 6K–N and 7K–N). When 0.1 nM okadaic acid was added to mTALP medium, it was not enhanced (Fig. 6B, G, and K–N). When 10 and 50 nM okadaic acid were added to the mTALP medium, incubation for 3 and 4 h. Therefore, it seems that PPP1CA is inactivated by phosphorylation during hyperactivation, and is activated by dephosphorylation in hyperactivated sperm.

On the other hand, PPP2 is inactivated by tyrosine phosphorylation (Chen et al. 1992, 1994, Guo & Damuni 1993). As shown in Fig. 5, tyrosine phosphorylation corresponding to PPP2 was detected in the urea extracts. Tyrosine phosphorylation of PPP2 did not occur at incubation for 0 h (Fig. 5C, lane a), but it occurred at incubation for 0.5, 1, 2, 3, and 4 h (Fig. 5C, lanes b–f). Therefore, it seems that PPP2 was activated in activated sperm at incubation for 0 h, and was inactivated by phosphorylation during hyperactivation and in hyperactivated sperm.

**Effects of inhibition of PPP2 on sperm protein tyrosine phosphorylation**

Si & Okuno (1999b) reported that high concentrations of okadaic acid and calyculin A significantly enhanced 80 kDa tyrosine phosphorylation. In our previous study (Fujinoki et al. 2006), we could detect four tyrosine phosphorylations, which were designated pY80u, pY80ut, pY85u, and pY85ut, in hamster sperm. Because these four proteins were phosphorylated at tyrosine residues during hyperactivation, we examined the effects of low concentrations of okadaic acid (Fig. 6) and calyculin A (Fig. 7) on tyrosine phosphorylation.

As shown in Figs 6A and F and 7A and F, tyrosine phosphorylation of pY80u, pY80ut, pY85u, and pY85ut was detected in both the urea extracts and the urea-thiourea extracts. Reactivity increased in a time-dependent manner (Figs 6K–N and 7K–N). When 0.1 nM okadaic acid was added to mTALP medium, it was not enhanced (Fig. 6B, G, and K–N). When 10 and 50 nM okadaic acid were added to the mTALP medium,
tyrosine phosphorylation of pY80u and pY85u obtained from the urea extracts was not enhanced (Fig. 6C, D, H, I, K, and L). But tyrosine phosphorylation of pY80ut and pY85ut obtained from the urea–thiourea extracts was significantly enhanced after incubation for 0.5, 1, and 2 h compared with the control. Therefore, it is interesting that okadaic acid is a highly specific inhibitor of PPP2, and has 100-fold specificity against PPP2 rather than against other related kinases.

### Discussion

Hyperactivation is the change of sperm flagellar movement that occurs during capacitation, and it is regulated through protein phosphorylation signals (Morisawa 1994, Yanagimachi 1994). In protein phosphorylation signal-regulated hyperactivation, 80 kDa tyrosine phosphorylations are important events (Visconti et al. 1995, 1998, 1999, Visconti & Kopf 1998, Si & Okuno 1999b, Fujinoki et al. 2001b, 2006, Iha & Shivaji 2001, Marin-Briggeler et al. 2005), and one of them is identified as AKAP (Iha & Shivaji 2002a). It has been suggested that 80 kDa tyrosine phosphorylation is regulated by cAMP signals (Visconti et al. 1999), calcium/calmodulin signals (Carrera et al. 1996), and PPP1/PPP2 (Si & Okuno 1999b).

Several studies have reported that PPP1CC is involved in the regulation of motility activation and hyperactivation in mammalian sperm (Smith et al. 1996, Huang et al. 2005, Chakrabarti et al. 2007, Goto & Harayama 2009). On the other hand, it has been reported that PPP3 is also associated with the regulation of sperm motility and acrosome reaction (Tash et al. 1988, Tash & Bracho 1994, Ashizawa et al. 2004). In the present study, we have suggested that hamster sperm have PPP1CA, PPP1CC, PPP2, and PPP3 (see Fig. 1).

Previously, Si & Okuno (1999b) reported that over 80% of the sperm had become fully hyperactivated within 40 min when 5 μM okadaic acid and 2 μM calyculin A were added to the mTALP medium. However, 5 μM okadaic acid and 2 μM calyculin A were much higher than the IC50 concentration of these inhibitors. Therefore, in the present experiment, we have shown that the IC50 concentration of okadaic acid, calyculin A, and tautomycin significantly enhanced sperm hyperactivation (see Fig. 2), although enhancement of hyperactivation differed from the results reported by Si & Okuno (1999b).

Okadaic acid, calyculin A, and tautomycin are inhibitors of PPP1 and PPP2 (Hardie 1993). Okadaic acid is a highly specific inhibitor of PPP2, and has 100-fold specificity against PPP2 rather than against
PPP1. Calyculin A is a less specific inhibitor of PPP2, and has fourfold specificity against PPP2 rather than against PPP1. Calyculin A sometimes inhibits PPP1 rather than PPP2 in an intact cell. Tautomycin is a low specificity inhibitor of PPP1, and has tenfold specificity against PPP1 rather than against PPP2. Because these inhibitors are fat soluble and enter the cell easily, PPPs associated with functions are able to be reasoned by comparison among the effects of inhibitors (Hardie 1993). By understanding the differences in their IC50 values against PPP1 and PPP2, we examined which PPPs are associated with the regulation of hyperactivation (see Fig. 2). Sperm hyperactivation was significantly enhanced when only PPP2, at least, was inhibited by okadaic acid and calyculin A, although we could not show which PPPs were associated with the regulation of hyperactivation when we used tautomycin. On the other hand, it has been suggested that PPP3 is also associated with the regulation of sperm function (Tash et al. 1988, Tash & Bracho 1994, Ashizawa et al. 2004). However, inhibitors of PPP3 had no effect on sperm hyperactivation (see Fig. 3).

It is widely accepted that tyrosine phosphorylation is closely associated with the regulation of hyperactivation (Si & Okuno 1999b, Fujinoki et al. 2001b, 2006, Jha & Shivaji 2001, Marín-Briggiler et al. 2005). In our previous studies (Fujinoki et al. 2006), four tyrosine phosphorylations, namely pY80u, pY80ut, pY85u, and pY85ut, were significantly increased during sperm hyperactivation. Additionally, these tyrosine

Figure 6 Effects of okadaic acid on time-dependent protein tyrosine phosphorylation during hyperactivation. Sperm were incubated in modified TALP medium alone (A and F) or in the presence of 0.1 nM (B and G), 10 nM (C and H), 50 nM (D and I), and 500 nM (E and J) okadaic acid. Photographs A–E show gels stained by CBB, and photographs F–J show western blotting using anti-phosphotyrosine MAB (1:1000 dilution). In SDS-PAGE for CBB stain, 10-μl samples were applied. On the other hand, 5-μl samples were applied in SDS-PAGE for western blotting. Lanes a–f show western blotting of the urea extracts, and lanes g–l show western blotting of the urea–thiourea extracts. Lanes a and g, lanes b and h, lanes c and i, lanes d and j, lanes e and k, and lanes f and l indicate 0, 0.5, 1, 2, 3, and 4 h respectively. Arrows indicate 80 and 85 kDa tyrosine phosphoproteins. Tyrosine phosphorylations of 80 and 85 kDa obtained from the urea extracts correspond to pY80u and pY85u described previously (Fujinoki et al. 2006). On the other hand, 80 and 85 kDa tyrosine phosphorylations obtained from the urea–thiourea extracts correspond to pY80ut and pY85ut described previously (Fujinoki et al. 2006). Tyrosine phosphorylations of pY85u (K) and pY80u (L) obtained from the urea extracts and of pY85ut (M) and pY80ut (N) obtained from the urea–thiourea extracts were analyzed using a densitometer. ‘a’ indicates significant differences compared with the control (P<0.05); ‘b’ indicates significant differences compared with 0.1 nM okadaic acid (P<0.05); ‘c’ indicates significant differences compared with 10 nM okadaic acid (P<0.05); and ‘d’ indicates significant differences compared with 50 nM okadaic acid (P<0.05).
phosphorylations were significantly enhanced when hyperactivation was enhanced by supplying progesterone (Noguchi et al. 2008). Although protein tyrosine kinase was found in mammalian sperm (Uma Devi et al. 2000), no evidence suggests that it is present during tyrosine phosphorylation associated with the regulation of hyperactivation. On the other hand, it was suggested that cAMP was associated with the regulation of hyperactivation (Visconti & Kopf 1998, Visconti et al. 1998, 1999). Moreover, cAMP-dependent tyrosine phosphorylation was also detected (Morisawa 1994, Fujinoki et al. 2001b). Because cAMP-dependent protein kinase is generally a serine/threonine kinase, it has been proposed that spermatozoa have a cAMP-dependent tyrosine kinase or a tyrosine kinase regulated by cAMP-dependent protein kinase. Recently, several studies have suggested that serine/threonine phosphorylation is associated with the regulation of hyperactivation (Naz 1999, Jha & Shivaji 2002b, Fujinoki et al. 2004, 2006). Serine/threonine phosphorylation occurred before tyrosine phosphorylation occurred (Fujinoki et al. 2006). Therefore, it seems that tyrosine phosphorylation is generally regulated by serine/threonine phosphorylation. On the other hand, serine/threonine phosphorylation of a 10 kDa protein occurred during hyperactivation after it was phosphorylated at the tyrosine residues (Fujinoki et al. 2004). Moreover, tyrosine phosphorylation of the 10 kDa protein occurred during hyperactivation after it was phosphorylated at the tyrosine residues (Fujinoki et al. 2004). Moreover, tyrosine phosphorylation partially occurred downstream of tyrosine phosphorylation during sperm hyperactivation.

Figure 7 Effects of calyculin A on time-dependent protein tyrosine phosphorylation during hyperactivation. Sperm were incubated in modified TALP medium alone (A and F) or in the presence of 0.5 nM (B and G), 2 nM (C and H), 50 nM (D and I), and 500 nM (E and J) calyculin A. Photographs A–E show gels stained by CBB, and photographs F–J show western blotting using anti-phosphotyrosine MAB (1:1000 dilution). In SDS-PAGE for CBB stain, 10-μl samples were applied. In SDS-PAGE for western blotting, 5-μl samples were applied. Photographs A–J show western blotting of the urea extracts, and lanes g–l show western blotting of the urea–thiourea extracts. Arrows indicate 80 and 85 kDa tyrosine phosphoproteins. Tyrosine phosphorylations of 80 and 85 kDa obtained from the urea extracts correspond to pY80u and pY85u described previously (Fujinoki et al. 2006). On the other hand, 80 and 85 kDa tyrosine phosphorylations obtained from the urea–thiourea extracts correspond to pY80ut and pY85ut described previously (Fujinoki et al. 2006). pY85u (K) and pY80u (L) obtained from the urea extracts, and pY85ut (M) and pY80ut (N) obtained from the urea–thiourea extracts were analyzed using a densitometer. ‘a’ indicates significant differences compared with the control (P<0.05).
Si & Okuno (1999b) also reported that 5 μM okadaic acid and 2 μM calyculin A significantly enhanced 80 kDa tyrosine phosphorylation. In the present experiment, however, 0.1 nM okadaic acid, which is IC50 value of PPP2, did not enhance four tyrosine phosphorylations, such as 80u, 85u, 80ut, and 85ut, at all (see Fig. 6). Moreover, 10, 50, and 500 nM okadaic acid enhanced and/or increased two tyrosine phosphorylations obtained from the urea–thiourea extracts such as 80ut and 85ut, although they did not enhance and/or increase two tyrosine phosphorylations obtained from the urea extracts such as 80u and 85u. In contrast, calyculin A enhanced and/or increased four tyrosine phosphorylations after incubation for 0.5, 1, and 2 h (see Fig. 7). The results obtained through okadaic acid suggested that enhancement of hyperactivation by inhibition of PPP2 did not depend on the enhancement of tyrosine phosphorylation. But the results obtained through calyculin A suggested that enhancement of hyperactivation depends on the enhancement of tyrosine phosphorylation. When IC50 values of PPP1 and PPP2 are compared between okadaic acid and calyculin A, differences of okadaic acid are very large (100-fold), but those of calyculin A are very small (fourfold). Therefore, 0.5 nM calyculin A may slightly suppress both PPP1 and PPP2. And then, 10 nM okadaic acid is the IC50 value of PPP1, and may suppress both PPP1 and PPP2. So, it seems that tyrosine phosphorylation was associated with the enhancement of hyperactivation when PPP1 or both PPP1 and PPP2 are inhibited. From the results obtained through okadaic acid and calyculin A (see Figs 2, 6, and 7), it is likely that hyperactivation is enhanced by inhibition of PPP2 in a tyrosine phosphorylation-independent manner. And then, inhibition of PPP1 or both PPP1 and PPP2 enhanced and/or increased hyperactivation and tyrosine phosphorylation. Therefore, the question remains whether PPP1 and PPP2 suppress sperm hyperactivation. Previous studies suggested that PPP1CC2 was phosphorylated with motility activation and/or hyperactivation in mouse sperm (Smith et al. 1996, Huang et al. 2005, Chakrabarti et al. 2007, Goto & Harayama 2009). In two of these studies (Huang et al. 2005, Goto & Harayama 2009), phosphorylation of PPP1CC2 was examined using anti-phospho-PPP1CA antibody. At the same time, the authors described that anti-phospho-PPP1CA antisera might cross-react with phospho-PPP1CC2 (Huang et al. 2005, Goto & Harayama 2009). PPP1CA is negatively regulated by threonine phosphorylation (Kwon et al. 1997). In the present study (see Fig. 4), PPP1CA was phosphorylated at threonine residues before and during hyperactivation, and was dephosphorylated in hyperactivated sperm. Therefore, it seems that PPP1CA is inactive when sperm change from activation to hyperactivation. We could not examine phosphorylation of PPP1CC because we could not obtain anti-phospho-PPP1CC antisera. To examine phosphorylation of PPP1CA, we purchased anti-phospho-PPP1CA antibody from Cell Signaling Technology (Tokyo, Japan). In the explanatory leaflet, it is described that the anti-phospho-PPP1CA antibody may cross-react with PPP1CC, although this antibody is basically phospho-PPP1CA specific. Goto & Harayama (2009) used this anti-phospho-PPP1CA antibody purchased from Cell Signaling Technology to examine phosphorylation of PPP1CC in mouse sperm. Huang et al. (2005) also prepared antiserum against the same epitope as this anti-phospho-PPP1CA antibody, and examined phosphorylation of PPP1CC2. Although they described that anti-phospho-PPP1CA antisera might cross-react with phospho-PPP1CC (Huang et al. 2005, Goto & Harayama 2009), we could not detect PPP1CC in hamster sperm using this anti-phospho-PPP1CA antibody purchased from Cell Signaling Technology (data not shown).

Activity of PPP2 is negatively regulated by tyrosine phosphorylation (Chen et al. 1992, 1994, Guo & Damuni 1993). In the present study, PPP2 was phosphorylated at tyrosine residues during hyperactivation and in hyperactivated sperm (see Fig. 5). Since PPP2 was not phosphorylated before the sperm began to be hyperactivated, it is likely that they begin to be hyperactivated by inactivation of PPP2.

The regulatory mechanism of hyperactivation is not yet understood by us. But we suggest that sperm were hyperactivated by inactivation of PPP2 in the present study. Furthermore, tyrosine phosphorylations of 80 and 85 kDa proteins were not associated with the regulation of hyperactivation by inactivation of PPP2, although they were enhanced/increased by inhibition of PPP1. Moreover, it was also suggested that PPP1CA was inactive during hyperactivation, and was active on hyperactivation. On the other hand, the regulation of hyperactivation is associated with several types of protein kinases (Morisawa 1994, Yanagimachi 1994, Fujinoki 2009). In future studies, we plan to examine the relationship between protein kinases and protein phosphatases, and between serine/threonine phosphorylations and dephosphorylations on sperm hyperactivation.

Materials and Methods

Reagents

Anti-phosphotyrosine MAB (PT-66) was purchased from Sigma Chemical Company. Anti-PPP1CA antibody, anti-phospho-PPP1CA (Thr-320) antibody, anti-PPP2 C subunit antibody, and anti-Pan-calcineurin A antibody were purchased from Cell Signaling Technology Japan, K.K. Anti-PPP1CB antibody and anti-PP1CC antibody were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Okadaic acid, calyculin A, tautomycin, deltamethrin, and fenvalerate were purchased from Calbiochem Corporation (La Jolla, CA, USA). PVDF membrane was purchased from Millipore (Bedford, MA, USA). ECL plus kit and hyperfilm for ECL were purchased from...
Amersham Biosciences. Other chemicals were of reagent grade from Wako Pure Chemical Industries (Osaka, Japan). Stock solutions of each inhibitor were prepared in DMSO, and were stored at −20 °C. In the experiment, inhibitors and controls of these agents had 0.1% DMSO.

**Animals and preparation of hyperactivated sperm**

Sexually mature male golden hamsters (*Mesocricetus auratus*) were used as experimental animals. Hamsters were housed in accordance with the guidelines of the Dokkyo Medical University and the Laboratory Animal Research Center in Dokkyo Medical University of the care and use of laboratory animals.

Sperm were obtained from the caudal epididymis. Hyperactivated sperm were prepared according to a method described previously with a mTALP medium ([Si & Okuno 1999b](#)). An aliquot of caudal epididymal sperm was placed in a culture dish. Several milliliters of the mTALP medium were then carefully added to the culture dish. The culture dish was incubated for 5 min at 37 °C to allow sperm to swim up, and was incubated for 4 h at 37 °C under 5% CO₂ in air to accomplish hyperactivation.

**Measurement of motile sperm and hyperactivated sperm**

Percentage of motile sperm and that of hyperactivated sperm were calculated according to the method described in our previous study ([Noguchi et al. 2008](#)). Hamster sperm suspended in mTALP medium were diluted to tenfold of the medium, and placed in a culture plate (35-mm dish). Sperm were recorded on a videotape by CCD camera (Progressive 3CCD, Sony, Tokyo, Japan) attached to a microscope (IX70, Olympus, Tokyo, Japan) with phase contrast illumination and a 3CCD, Sony, Tokyo, Japan) attached to a microscope (IX70, Olympus, Tokyo, Japan) with phase contrast illumination and a small CO₂ incubator (MI-IBC, Olympus). Each observation was carried out at 37 °C, recorded for 5 min, and analyzed by counting the number of total sperm, motile sperm, and hyperactivated sperm in 30 different fields. Motile sperm that exhibited an asymmetric and whirlflash flagellar movement ([Fujinoki et al. 2001a](#)) were classified as hyperactivated sperm. Percentage of motile sperm (%) and that of hyperactivated sperm (%) were respectively defined as (number of motile sperm/number of total sperm)×100 and (number of hyperactivated sperm/number of total sperm)×100.

**Preparation of sperm protein extracts**

Sperm proteins were extracted according to the method described in our previous study ([Fujinoki et al. 2006](#)). At first, collected sperm that were incubated to be hyperactivated were suspended at 30 mg sperm/ml in a urea solution containing 7 M urea and 10% 2-mercaptoethanol (2-ME). After pipetting, the suspension was incubated on ice for 10 min. After centrifugation at 15 000 g for 10 min at 4 °C, the supernatants were used as the urea–thiourea extracts. The precipitate was resuspended in the same volume of the urea–thiourea solution containing 5 M urea, 1 M thiourea, 10% 2-ME, and 2% nonidet P-40. After pipetting, the resuspension was incubated on ice for 10 min. After centrifugation at 15 000 g for 10 min at 4 °C, the supernatants were used as the urea–thiourea extracts.

**SDS-PAGE**

SDS-PAGE was carried out according to the method of Laemmli ([1970](#)). The separating gel was 10% (w/v) polyacrylamide containing 0.1% (w/v) SDS. Mixtures (1:1) of above extracts and SDS buffer containing 10% SDS, 10% 2-ME, 10% glycerol, 0.01% bromophenol blue, and 0.5 M Tris–HCl (pH 6.8) were used as samples for SDS-PAGE.

**Western blotting**

Western blotting was carried out according to the method described by Towbin et al. ([1979](#)). After SDS-PAGE, the proteins were electroblotted onto a PVDF membrane. The blotted membrane was blocked with 5% (w/v) BSA in Tris-buffered saline (TBS) containing 0.15 M NaCl and 20 mM Tris–HCl (pH 7.4) for 1 h at 20 °C, and was incubated with the primary antibodies with 5% (w/v) BSA in TBS for 1 h at 20 °C. After washing with TBS, it was incubated with secondary antibodies (1:5000 dilution) conjugated to HRP with 5% (w/v) BSA in TBS. A color reaction was carried out with ECL plus kit. Reactivity of western blotting was measured by means of a densitometer (GS-800 densitometer, Bio-Rad Laboratories), and analyzed using Quantity One Software version 4.6.1 (Bio-Rad Laboratories).

**Statistical analysis**

Data were analyzed using Tukey's post-hoc test of ANOVA. Values were considered statistically significant when *P*<0.05.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This work was supported by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan to T Suzuki. (No. 17791134 and 20791159) and M Fujinoki (No. 15790860 and 18791135), and by a Jichi Medical University Young Investigator Award to T Suzuki.

**References**


Fujinoki M, Ohtake H & Okuno M 2001a Serum phosphorylation of flagellar proteins associated with the motility activation of hamster spermatozoa. Biomedical Research 22 45–58.


Jha K & Shivaji S 2001 Capacitation-associated changes in protein tyrosine phosphorylation, hyperacrosome and acrosome reaction in hamster spermatozoa. Andrologia 33 95–104.

