Suppression of progesterone-enhanced hyperactivation in hamster spermatozoa by estrogen

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Abstract

In this study, I examined whether sperm hyperactivation in hamster is regulated by steroid hormones such as estrogen (estradiol, E2) and progesterone. Although sperm hyperactivation was enhanced by progesterone, 17β-estradiol (17βE2) itself did not affect sperm hyperactivation. However, 17βE2 suppressed progesterone-enhanced hyperactivation in a concentration-dependent manner through non-genomic pathways when spermatozoa were exposed to 17βE2 at the same time or before exposure to progesterone. When spermatozoa were exposed to 17βE2 after exposure to progesterone, 17βE2 did not suppress progesterone-enhanced hyperactivation. Moreover, 17α-estradiol, an inactive isomer of E2, did not suppress progesterone-enhanced hyperactivation. Observations using a FITC-conjugated 17βE2 showed that it binds to the acrosome region of the sperm head. Binding of 17βE2 to spermatozoa was not inhibited by progesterone, although 17βE2 did not suppress progesterone-enhanced hyperactivation when spermatozoa were exposed to 17βE2 after exposure to progesterone. On the other hand, binding of progesterone to spermatozoa was also not inhibited by 17βE2 even if progesterone-enhanced hyperactivation was suppressed by 17βE2. Although tyrosine phosphorylations of sperm proteins were enhanced by progesterone, enhancement of tyrosine phosphorylations by progesterone was suppressed by 17βE2. Moreover, tyrosine phosphorylations were inhibited by 17βE2 when only 17βE2 was added to the medium. From these results, it is likely that 17βE2 competitively suppresses progesterone-enhanced hyperactivation through the inhibition of tyrosine phosphorylations via non-genomic pathways.


Introduction

After capacitation, mammalian spermatozoa fertilize eggs. Capacitated spermatozoa exhibit the acrosome reaction (AR) and hyperactivation. The AR is a modified exocytotic event involving the acrosome and the overlying sperm plasma membrane (Yudin et al. 1988, Yanagimachi 1994). The AR is required for penetration of the zona pellucida (ZP) of the egg and for sperm-egg plasma membrane fusion. Hyperactivation is a specialized movement of the sperm flagellum creating a propulsive force for passing through the ZP. Hyperactivated spermatozoa exhibit a large bend amplitude, whiplash and frenzied flagellar movements (Yanagimachi 1994, Fujinoki et al. 2001a, Suarez & Ho 2003).

Albumin, calcium and bicarbonate are important molecules in the capacitation process. Albumin promotes capacitation by removing cholesterol from the sperm plasma membrane (Langlais & Roberts 1985). Calcium and bicarbonate are involved in several intracellular signals such as the stimulation of adenylate cyclase, production of cAMP, and protein phosphorylation (Okamura et al. 1985, Visconti & Kopf 1998, Visconti et al. 1998, Ho & Suarez 2001, Ho et al. 2002). For protein phosphorylations, it has been suggested that tyrosine phosphorylations are closely associated with capacitation (Visconti et al. 1995, Fujinoki et al. 2001b, 2006, Jha & Shivaji 2001). Moreover, 80 and/or 85 kDa tyrosine phosphorylations are regulated by calcium/calmodulin-dependent signals (Carrera et al. 1996) and protein phosphatase 1 (Suzuki et al. 2010), and are identified as A-kinase anchoring protein (AKAP; Carrera et al. 1996, Jha & Shivaji 2002).

(Sabur et al. 1996, Baldi et al. 1998, 2009, Lösel & Wehling 2003, Luconi et al. 2004, Jang & Yi 2005). Moreover, it has been suggested that progesterone binds to the acrosome region, and PGR is localized to the same region in human (Gadkar et al. 2002) and hamster spermatozoa (Noguchi et al. 2008). Phospholipase C (PLC; Fukami et al. 2003) and/or protein kinase A (Harrison et al. 2000) are involved in progesterone-induced AR. Progesterone also changes motility parameters (Yang et al. 1994) and enhances hyperactivation in human (Sueldo et al. 1993) and hamster spermatozoa (Noguchi et al. 2008). Although progesterone enhances hyperactivation of human and hamster spermatozoa (Sueldo et al. 1993, Noguchi et al. 2008), the effective concentration of progesterone is different between humans and hamsters. For human spermatozoa, the effective concentration of progesterone for the enhancement of hyperactivation was several micrograms per milliliter, the same as the effective concentration for inducing the AR (Osman et al. 1989, Sueldo et al. 1993). On the other hand, for hamster spermatozoa, the effective concentration of progesterone for the enhancement of hyperactivation was 20 ng/ml (Noguchi et al. 2008). Moreover, at a concentration of 20 ng/ml, progesterone increased hamster sperm penetration and did not induce hamster sperm AR (Libersky & Boatman 1995b). At a concentration of 20 ng/ml, progesterone also enhanced and increased tyrosine phosphorylations associated with hyperactivation of hamster spermatozoa (Noguchi et al. 2008). The effective progesterone concentration for hamster sperm AR was also several micrograms per milliliter (Libersky & Boatman 1995b). Libersky & Boatman (1995a) measured progesterone concentration in fluids of reproductive organs, reporting that the progesterone concentration in hamster serum ranged from 5.56 to 12.85 ng/ml, the follicular fluid concentration ranged from 4.2 to 7.4 μg/ml, and the oviductal fluid concentration ranged from 44.04 to 175.06 ng/ml.

It has been suggested that 17β-estradiol (17βE2) is also associated with a regulation of the AR (Luconi et al. 1999, 2004, Baldi et al. 2000, 2009). In human spermatozoa, 17βE2 stimulates calcium influx and suppresses progesterone-induced AR (Luconi et al. 1999, Baldi et al. 2000). Estrogen receptors (ESRs) are also present in the cell membrane (Luconi et al. 1999, 2004, Baldi et al. 2000, 2009). Moreover, the suppression of progesterone-induced AR by 17βE2 is non-genomic (Luconi et al. 2004, Baldi et al. 2009).

Although the AR is regulated by estrogen (E2) and progesterone via non-genomic regulation, it is not known whether progesterone-enhanced hyperactivation is suppressed by 17βE2. In the present study, I have shown that progesterone-enhanced hyperactivation and tyrosine phosphorylations are suppressed by 17βE2 via non-genomic regulation.

**Results**

**Suppression of progesterone-enhanced hyperactivation by 17βE2**

An earlier study suggested that hamster sperm hyperactivation was enhanced by 20 ng/ml progesterone via non-genomic regulation (Noguchi et al. 2008). It has been suggested that the AR is induced by progesterone and suppressed by 17βE2 (Luconi et al. 1999, 2004, Baldi et al. 2000, 2009), so I examined whether hyperactivation was also enhanced by progesterone and suppressed by 17βE2 using hamster spermatozoa.

Although 17βE2 itself did not affect the percentage of motile spermatozoa and hyperactivation in hamster spermatozoa at all (Fig. 1), it affected progesterone-enhanced hyperactivation in a concentration-dependent manner (Figs 2 and 3). As shown in Fig. 2A and B, 17βE2 suppressed progesterone-enhanced hyperactivation in a concentration-dependent manner, although it did not affect the percentage of motile spermatozoa when spermatozoa were exposed to 20 ng/ml progesterone after exposure to 20 ng/ml down to 2 fg/ml 17βE2. When incubated for 1 (Fig. 2C), 1.5 (Fig. 2D), and 2 h (Fig. 2E), progesterone-enhanced hyperactivation was significantly suppressed by 20 ng/ml, 2 ng/ml, and 200 pg/ml 17βE2 respectively. On the other hand, no suppression of progesterone-enhanced hyperactivation was observed at a concentration from 2 pg/ml down...
to 2 fg/ml 17βE₂. Interestingly, the suppression of progesterone-enhanced hyperactivation by 20 pg/ml 17βE₂ was midway between progesterone-enhanced hyperactivation and vehicle alone, with no significant difference to either. When spermatozoa were exposed to 20 ng/ml to 2 fg/ml 17βE₂ and 20 ng/ml progesterone at the same time, 17βE₂ suppressed progesterone-enhanced hyperactivation in a concentration-dependent manner, although it did not affect the percentage of motile spermatozoa (Fig. 3A and B). When incubated for 1 (Fig. 3C), 1.5 (Fig. 3D), and 2 h (Fig. 3E), 20 and 2 ng/ml 17βE₂ significantly suppressed progesterone-enhanced hyperactivation. In contrast, 2 pg/ml to 1 fg/ml 17βE₂ did not suppress progesterone-enhanced hyperactivation. When incubated for 1 h (Fig. 3C), the suppression of progesterone-enhanced hyperactivation by 200 and 20 pg/ml 17βE₂ was midway between progesterone-enhanced hyperactivation and vehicle alone, with no significant difference to either. When incubated for 1.5 h (Fig. 3D), the suppression of progesterone-enhanced hyperactivation by 200 pg/ml 17βE₂ was midway between progesterone-enhanced hyperactivation and vehicle alone, with a significant difference to both. However, 20 pg/ml 17βE₂ did not suppress progesterone-enhanced hyperactivation. When incubated for 2 h (Fig. 3E), 200 pg/ml 17βE₂ significantly suppressed progesterone-enhanced hyperactivation, but 20 pg/ml 17βE₂ had no effect. When spermatozoa were exposed to 20 ng/ml to 2 fg/ml 17βE₂ after exposure to 20 ng/ml progesterone, 17βE₂ did not affect the percentage of motile spermatozoa or progesterone-enhanced hyperactivation at all (Fig. 4).

The two isomers of E₂ are 17α-estradiol (17αE₂) and 17βE₂, of which 17βE₂ alone has biological activity. When spermatozoa were exposed to 20 ng/ml progesterone after exposure to 20 ng/ml to 200 pg/ml 17αE₂, 17αE₂ had no effect on the percentage of motile spermatozoa or progesterone-enhanced hyperactivation.
progesterone were added to mTALP medium. The percentage of hyperactivation is shown as an overview of effects (B) and after incubation for 1 (C), 17

Reproduction (2010)

17

E2 before exposure to 20 ng/ml pro-

2 pg/ml 17

Hyperactivation (%)

Motile spermatozoa (%)

100

75

50

25

0

Vehicle

20 ng/ml E

200 pg/ml E

20 pg/ml E

2 pg/ml E

200 fg/ml E

20 fg/ml E

Figs 2 and 6D, 17

E2 suppressed progesterone-enhanced hyperactivation (Fig. 2B). As shown in Figs 2 and 6D, 17

E2 before exposure to 20 ng/ml pro-
gesterone. However, the suppression of progesterone-enhanced hyperactivation by 17

E2 was significantly inhibited by tamoxifen when spermatozoa were exposed to 1

μmol/l tamoxifen before exposure to 20 ng/ml 17

E2 and 20 ng/ml progesterone (Fig. 6D). Under the same condition (Fig. 6C), tamoxifen had no effect on the percentage of motile spermatozoa.

Suppression of progesterone-enhanced hyperactivation by 17

E2 via non-genomic regulation

Figs 2 and 6D, 17

E2 suppressed progesterone-enhanced hyperactivation when spermatozoa were exposed to 20 ng/ml 17

E2 before exposure to 20 ng/ml pro-
gesterone. However, the suppression of progesterone-enhanced hyperactivation by 17

E2 was significantly inhibited by tamoxifen when spermatozoa were exposed to 1

μmol/l tamoxifen before exposure to 20 ng/ml 17

E2 and 20 ng/ml progesterone (Fig. 6D). Under the same condition (Fig. 6C), tamoxifen had no effect on the percentage of motile spermatozoa.

Sperm hyperactivation is enhanced by progesterone via non-genomic regulation associated with PGR (Noguchi et al. 2008). In the next step, I used FITC/BSA-17

E2 to determine whether hamster sperm hyperactivation was regulated by 17

E2 via non-genomic regulation. Generally, 17

E2 enters the cell, binds to the intracellular ESRs, and induces gene expression (Baldi et al. 2004). However, FITC/BSA-17

E2 cannot enter the cell because BSA blocks the entry of 17

E2 into the cell. It follows that the effects of FITC/BSA-17

E2 occur through non-genomic signals.

As shown in Fig. 7A and B, 7.4 nmol/l, 740 pmol/l, and 74 pmol/l FITC/BSA-17

E2, which are converted into ~20 ng/ml, 2 ng/ml, and 200 pg/ml 17

E2 respectively, did not affect the percentage of motile spermatozoa and hyperactivation, as with 17

E2 (Fig. 1). When spermatozoa were exposed to 20 ng/ml progesterone after exposure to 7.4 nmol/l, 740 pmol/l, and 74 pmol/l

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FITC/BSA-17βE₂, progesterone-enhanced hyperactivation was significantly suppressed by 7.4 nmol/l, 740 pmol/l, and 74 pmol/l FITC/BSA-17βE₂, although they did not affect the percentage of motile spermatozoa (Fig. 7C and D). The suppression of progesterone-enhanced hyperactivation by 7.4 nmol/l FITC/BSA-17βE₂ was significantly inhibited by 1 μmol/l tamoxifen, although tamoxifen did not affect the percentage of motile spermatozoa (Fig. 7E and F).

**Binding of 17βE₂ to hamster spermatozoa**

Progesterone binds to the acrosome region of the sperm head (Gadkar et al. 2002, Noguchi et al. 2008; Fig. 8Ac and Ad), although fluorescence of the middle piece of the sperm flagellum appears to be autofluorescence (Noguchi et al. 2008; Fig. 8Aa and Ab). As shown in Fig. 8Ac–h, it was observed that 7.4 nmol/l FITC/BSA-17βE₂ bound to the acrosome region of the sperm head. Interestingly, 1 μmol/l tamoxifen did not inhibit the binding of FITC/BSA-17βE₂ to the sperm head (Fig. 8B), although it did inhibit the effects of 17βE₂ and FITC/BSA-17βE₂ on progesterone-enhanced hyperactivation (Figs 6 and 7).

When spermatozoa were exposed to 20 ng/ml 17βE₂ before exposure to 20 ng/ml progesterone, progesterone-enhanced hyperactivation was suppressed by 17βE₂ (Fig. 2). However, binding of 7 nmol/l FITC/BSA-P, converted ~20 ng/ml progesterone, to the hamster sperm head was not inhibited by 20 ng/ml 17βE₂ when spermatozoa were exposed to 20 ng/ml 17βE₂ before exposure to 7 nmol/l FITC/BSA-P (Fig. 8Ca–d). On the other hand, progesterone-enhanced hyperactivation was not suppressed by 17βE₂ when spermatozoa were exposed to 20 ng/ml 17βE₂ after exposure to 20 ng/ml progesterone (Fig. 4). Binding of 7.4 nmol/l FITC/BSA-17βE₂ to the sperm head was not suppressed by 20 ng/ml progesterone when spermatozoa were exposed to 7.4 nmol/l FITC/BSA-17βE₂ after exposure to 20 ng/ml progesterone (Fig. 8Ce–h).
Effects of 17βE₂ on tyrosine phosphorylations

It is accepted that tyrosine phosphorylations are an important event in sperm hyperactivation (Yanagimachi 1994, Visconti et al. 1995, Visconti & Kopf 1998, Fujinoki et al. 2006, Fujinoki 2009). A previous study (Noguchi et al. 2008) suggested that tyrosine phosphorylations in hamster spermatozoa were enhanced by 20 ng/ml progesterone.

As shown in Fig. 9B, several tyrosine phosphorylations occurred during hyperactivation in a time-dependent manner. Four tyrosine phosphorylations, designated as 80u, 85u, 80ut, and 85ut (Fujinoki et al. 2006), were enhanced by 20 ng/ml progesterone (Noguchi et al. 2008; Fig. 9D). Phosphorylations of 80u and 85u were detected in the urea extract (Fujinoki et al. 2006; Fig. 9B lanes a–f). Phosphorylations of 80ut and 85ut were detected in the urea–thiourea extract (Fujinoki et al. 2006; Fig. 9B lanes g–l).

When hamster spermatozoa were exposed to 20 ng/ml 17βE₂, tyrosine phosphorylations including 80u and 85u were not detected in the urea extract (Fig. 9C lanes a–f), although 20 ng/ml 17βE₂ itself did not affect the percentage of motile spermatozoa and hyperactivation (Fig. 1). On the other hand, tyrosine phosphorylations were detected in the urea–thiourea extract when spermatozoa were exposed to 20 ng/ml 17βE₂ (Fig. 9C lanes g–l). When spermatozoa were exposed to 20 ng/ml progesterone, tyrosine phosphorylations of 80u, 85u, 80ut and 85ut were enhanced compared with vehicle (Fig. 9B and D). As shown in Fig. 9E lanes a–f, tyrosine phosphorylations were not detected in the urea extract when spermatozoa were exposed to 20 ng/ml 17βE₂ before they were exposed to 20 ng/ml progesterone. The tyrosine phosphorylations detected in the urea–thiourea extract, as shown in Fig. 9E lanes g–l, were the same as the tyrosine phosphorylations as shown in Fig. 9C lanes g–l. Under the same conditions, progesterone-enhanced hyperactivation was suppressed (Fig. 2). As shown in Fig. 6, 1 μmol/l tamoxifen canceled the effect of 20 ng/ml 17βE₂ on progesterone-enhanced hyperactivation. For tyrosine phosphorylations, 1 μmol/l tamoxifen inhibited the effect of 20 ng/ml 17βE₂ (Fig. 9F).

Discussion

The AR is induced in a ligand-dependent manner. Progesterone is a major ligand of the AR (Osman et al. 1989, Baldi et al. 1998, 2009, Lösel & Wehling 2003, Luconi et al. 2004). On the other hand, progesterone also enhances sperm hyperactivation (Suelo et al. 1993, Noguchi et al. 2008). Recently, melatonin was also shown to be a ligand that enhances sperm hyperactivation in hamster (Fujinoki 2008). It would therefore appear that hyperactivation is enhanced by ligands (Fujinoki 2009).

Progesterone-induced AR is suppressed by 17βE₂ through non-genomic regulation in human spermatozoa (Luconi et al. 1999, 2004, Baldi et al. 2000, 2009). In the present study, I showed that 17βE₂ suppresses progesterone-enhanced hyperactivation through...
non-genomic regulation in hamster spermatozoa (see Figs 2, 3, 6 and 7). Since 17βE₂ binds to the acrosome region of the sperm head (see Fig. 8), it appears that signals of 17βE₂ start at the sperm head and are transmitted to the sperm flagellum. Calcium is a key molecule in the regulation of AR and hyperactivation by steroid hormones (Luconi et al. 1999, Baldi et al. 2000, 2009). After progesterone binds to the sperm head, it stimulates calcium influx and activation of PLC (Fukami et al. 2003, Lösel & Wehling 2003, Harper et al. 2004, Luconi et al. 2004, Noguchi et al. 2008, Baldi et al. 2009, Fujinoki 2009). Moreover, spermatozoa are not hyperactivated in the mTALP medium without calcium (Fujinoki 2008, Noguchi et al. 2008). In human spermatozoa, 17βE₂ also stimulates calcium influx and suppresses progesterone-induced AR (Luconi et al. 1999, Baldi et al. 2000, 2009). Although the 17βE₂ signal is unknown, it is likely that the 17βE₂ signal differs from progesterone signals and suppresses progesterone signals via calcium influx (Luconi et al. 1999, 2004, Baldi et al. 2000, 2009). In the present study, I did not examine calcium influx induced by 17βE₂ in hamster spermatozoa. Based on the assumption that 17βE₂ stimulates calcium influx, in a future study, I will investigate the relationship between 17βE₂ and calcium influx using hamster spermatozoa. When spermatozoa are hyperactivated, a major intracellular signaling event that occurs is tyrosine phosphorylation (Yanagimachi 1994, Visconti & Kopf 1998, Visconti et al. 1998, Ho & Suarez 2001, Ho et al. 2002, Fujinoki et al. 2006, Fujinoki 2009). As part of progesterone-enhanced hyperactivation, progesterone enhances tyrosine phosphorylations of 80 and 85 kDa proteins such as 80u, 85u, 80ut, and 85ut (Noguchi et al. 2008; see Figs 2 and 9). Interestingly, 17βE₂ partially suppressed tyrosine phosphorylations (see Fig. 9C), and 17βE₂ suppressed progesterone-enhanced tyrosine phosphorylations (see Fig. 9E). Tamoxifen inhibited the suppression of progesterone-enhanced tyrosine phosphorylations by 17βE₂ (see Fig. 9F), although it did not
inhibit the binding of 17βE₂ at the sperm head (see Fig. 8B). Tyrosine phosphorylation of proteins detected in the urea extract was suppressed by 17βE₂ (see Fig. 9C). The urea extract contained proteins from many sperm structures, except the fibrous sheath and head (Fujinoki et al. 2006). On the other hand, tyrosine phosphorylation of proteins detected in the urea–thiourea extract was unaffected (see Fig. 9C). The urea–thiourea extract contained proteins of fibrous sheath (Fujinoki et al. 2006). One of the tyrosine phosphorylations...
associated with hyperactivation is that of AKAP, 80 and/or 85 kDa components of the fibrous sheath (Carrera et al. 1996, Jha & Shivaji 2002). Therefore, tyrosine phosphorylations of 80 and/or 85 kDa proteins detected in the urea–thiourea extract (80ut and 85ut) corresponded to AKAP and were not affected by 17βE2, although they were affected by progesterone (Noguchi et al. 2008; see Fig. 9C and D). Because hamster spermatozoa were hyperactivated in the presence of 17βE2, it is likely that tyrosine phosphorylations of 80ut and 85ut are associated with the essential regulatory mechanisms of hyperactivation. Because tyrosine phosphorylation of AKAP is regulated by calcium signals associated with calcium/calmodulin-dependent protein kinase (Carrera et al. 1996), it seems that tyrosine phosphorylations of 80ut and 85ut are regulated by calcium signals associated with calcium/calmodulin-dependent kinase. At least, tyrosine phosphorylations of 80ut and 85ut were enhanced by progesterone through calcium signals associated with PLC (Noguchi et al. 2008). On the other hand, tyrosine phosphorylations of 80 and 85 kDa proteins detected in the urea extract (80u and 85u) were suppressed by 17βE2 and enhanced by progesterone (Noguchi et al. 2008; see Fig. 9C and D). Even if 80u, 85u, and other proteins detected in the urea extract were not phosphorylated at tyrosine residues by 17βE2, spermatozoa were hyperactivated (see Figs 2 and 9C). These results suggest that tyrosine phosphorylations detected in the urea extract are not associated with the essential regulatory mechanisms of hyperactivation. Because tyrosine phosphorylations detected in the urea extract responded to the effects of steroid hormones, however, it is likely that they are associated with modulatory mechanisms of hyperactivation. Although tyrosine phosphorylations of 80u and 85u are enhanced by progesterone (Noguchi et al. 2008; see Fig. 9D), their regulatory mechanisms are not known at all and have not been identified yet. In a future study, I will investigate the regulatory mechanism of tyrosine phosphorylation of 80u and 85u and their identification.

In the present study, I demonstrated that enhancement of sperm hyperactivation was competitively regulated by steroid hormones (see Figs 2 and 3). It is suggested that the AR is also competitively regulated by steroid hormones (Luconi et al. 1999, 2004, Baldi et al. 2000, 2009). For both the AR and the hyperactivation, progesterone enhances the functions and 17βE2 suppresses the effects of progesterone (Luconi et al. 1999, 2004, Baldi et al. 2000, 2009, Noguchi et al. 2008, Fujinoki 2009; see Figs 2 and 3). The hyperactivation threshold concentration of 17βE2 was 20 pg/ml when spermatozoa were exposed to 17βE2 before exposure to 20 ng/ml progesterone (see Fig. 2). When spermatozoa were exposed to both steroid hormones at the same time, the threshold concentration of 17βE2 was 200 pg/ml (see Fig. 3). Female mammals have an estrous or menstrual
cycle, causing concentrations of 17βE2 and progesterone in the blood and tissues to fluctuate. The balance between the concentrations of 17βE2 and progesterone accordingly appears to regulate sperm AR and hyperactivation. When 17βE2 is more dominant over progesterone, enhancement of AR and hyperactivation is suppressed. Conversely, AR and hyperactivation are enhanced when progesterone is dominant over 17βE2. In a future study, I will examine the changes in sperm function under different patterns of 17βE2 and progesterone concentrations.

Materials and Methods

Chemicals
Anti-phosphotyrosine MAB (clone, PT-66), 17zE2, 17βE2, progesterone, FITC-conjugated and BSA-conjugated 17βE2 (FITC/BSA-17βE2), FITC-conjugated and BSA-conjugated progesterone (FITC/BSA-P), and tamoxifen were purchased from Sigma Chemical Company. BSA fraction V and Nonidet P-40 (NP-40) were purchased from Merck KGaA. ECL Plus kits and films for ECL were purchased from Amersham Biosciences. Other reagent grade chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals and preparation of hyperactivated spermatozoa
Spermatozoa were obtained from the caudal epididymis of sexually mature (12–20 weeks old) male golden hamsters (Mesocricetus auratus). 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.

Hyperactivated spermatozoa were prepared according to the method described previously (Fujinoki et al. 2006), using modified TALP (mTALP) medium containing 101.02 mmol/l NaCl, 2.68 mmol/l KCl, 2 mmol/l CaCl2, 1.5 mmol/l MgCl2·6H2O, 0.36 mmol/l NaH2PO4·2H2O, 35.70 mmol/l NaHCO3, 4.5 mmol/l D-glucose, 0.09 mmol/l sodium pyruvate, 9 mmol/l sodium lactate, 0.5 mmol/l hypotaurine, 0.05 mmol/l (-)-epinephrine, 0.2 mmol/l sodium taurocholic acid, 5.26 mmol/l sodium metabisulfite, 0.05% (w/v) streptomycin sulfate, 0.05% (w/v) potassium penicillin G, and 15 mg/ml BSA (pH 7.4 at 37 °C under 5% (v/v) CO2 in air). An aliquot of caudal epididymal spermatozoa was placed on the bottom of a test tube, and several milliliters of mTALP medium with FITC/BSA-17βE2 or FITC/BSA-P were carefully added, followed by incubation for 5 min to allow spermatozoa to swim up. The supernatant containing motile spermatozoa was collected, placed on a culture dish, and incubated for 5 min at 37 °C under 5% (v/v) CO2 in air. After incubation, several microliters of the supernatant were placed on a glass slide without fluorescence and observed using a light microscope (IX70, Olympus) with phase-contrast illumination and a fluorescence unit.

Preparation of sperm protein extracts
Sperm proteins were extracted according to the method described previously (Fujinoki et al. 2006). In brief, spermatozoa were suspended at 30 mg spermatozoa/ml in the urea solution containing 7 mol/l urea and 10% (v/v) 2-mercaptoethanol. After pipetting, the suspension was incubated on ice for 10 min. Following centrifugation at 15 000 g for 10 min at 4 °C, the supernatant was used as the urea extract. The precipitate was resuspended in the same volume of urea–thiourea solution containing 5 mol/l urea, 1 mol/l thiourea, 10% (v/v) 2-mercaptoethanol, and 2% (v/v) NP-40 as the urea solution. After pipetting, the suspension was incubated on ice for 10 min, centrifuged at 15 000 g for 10 min at 4 °C, and the supernatant was used as the urea–thiourea extract.

SDS-PAGE
SDS-PAGE was performed using the Laemmli protocol (Laemmli 1970) using a separating gel of 10% (w/v) polyacrylamide containing 0.1% (w/v) SDS.

Measurement of the percentage of motile spermatozoa and hyperactivation
The percentage of motile spermatozoa and hyperactivation measurements were performed according to the method described in previous studies (Fujinoki et al. 2001b, 2006). Hamster spermatozoa suspended in the mTALP medium were diluted 10-fold and placed on the culture plate (35 mm dish). Motile and hyperactivated spermatozoa were recorded on a DVD using a CCD camera (Progressive 3CCD, Sony Corp., Tokyo, Japan) attached to a microscope (IX70, Olympus Corp., Tokyo, Japan) with phase-contrast illumination and a small CO2 incubator (MI-IBC, Olympus). Each observation was performed at 37 °C and recorded for 2 min, and the analyses comprised counting the numbers of total spermatozoa, motile spermatozoa, and hyperactivated spermatozoa in 20 different fields. The percentage of motile spermatozoa and hyperactivation were respectively defined as (the number of motile spermatozoa/the number of total spermatozoa)×100 and (the number of hyperactivated spermatozoa/numbers of total spermatozoa)×100. Statistical analyses were performed using the post hoc test of ANOVA.

Ligand assays of 17βE2 and progesterone
Ligand assays of 17βE2 and progesterone were performed according to the method described previously (Noguchi et al. 2008). An aliquot of caudal epididymal spermatozoa was placed on the bottom of a test tube, and several milliliters of mTALP medium with FITC/BSA-17βE2 or FITC/BSA-P were carefully added, followed by incubation for 5 min to allow spermatozoa to swim up. The supernatant containing motile spermatozoa was collected, placed on a culture dish, and incubated for 5 min at 37 °C under 5% (v/v) CO2 in air. After incubation, several microliters of the supernatant were placed on a glass slide without fluorescence and observed using a light microscope (IX70, Olympus) with phase-contrast illumination and a fluorescence unit.
Western blotting
Western blotting was performed according to the method described previously (Fujinoki et al. 2001b, 2006). The blotted membrane was blocked with 5% (w/v) skim milk in Tris-buffered saline (TBS) containing 0.15 mol/l NaCl and 20 mmol/l Tris–HCl (pH 7.5) for 1 h at 20 °C, and incubated with anti-phosphotyrosine MAB (clone, PT-66) (1:1000 dilution with 5% (w/v) skim milk in TBS) for 1 h at 20 °C. Following a TBS wash, the membrane was incubated with anti-mouse IgG antibody-conjugated peroxidase (1:5000 dilution with 5% (w/v) skim milk in TBS). Color reactions were conducted using the ECL Plus kit.

Declaration of interest
The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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