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ORIGINAL ARTICLE

Progesterone-enhanced sperm hyperactivation through IP_3–PKC and PKA signals

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Abstract
Propose The present study examined whether regulation of progesterone-enhanced hyperactivation of spermatozoa is associated with the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by phospholipase C (PLC) and cyclic adenosine monophosphate (cAMP) by adenylate cyclase (AC), as well as activation of protein kinase C (PKC) and protein kinase A (PKA).
Methods Hamster spermatozoa were hyperactivated by incubation for 4 h in modified Tyrode's albumin lactate pyruvate (mTALP) medium. In order to examine the effects of IP_3 receptor (IP_3R), PKC and PKA on progesterone-enhanced hyperactivation, their inhibitors (xestospongin C, bisindolylmaleimide 1 and H-89) were used.
Results Progesterone-enhanced hyperactivation was significantly suppressed by the inhibitors of IP_3R, PKC and PKA.
Conclusions The results suggest that progesterone-enhanced sperm hyperactivation occurs through two signal pathways. One is an intracellular Ca^{2+} signal through production of IP_3 and DAG by PLC, binding of IP_3 to IP_3R and activation of PKC by DAG and Ca^{2+}. The other is a cAMP–PKA signal through production of cAMP by AC and activation of PKA by cAMP.

Keywords Capacitation · Hyperactivation · Non-genomic regulation · Progesterone · Spermatozoa

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Introduction

Hyperactivated spermatazoa exhibit a specialized flagellar movement with a high amplitude and asymmetrical beating pattern [1, 2]. During capacitation, spermatozoa are hyperactivated to create the propulsive force needed to penetrate the zona pellucida (ZP) [1, 3, 4]. After hyperactivation, capacitated spermatozoa undergo the acrosome reaction, which is a modified exocytosis that is required for penetration of the ZP and subsequent fusion of the plasma membranes of the sperm and egg [1, 2, 4, 5].

Spermatozoa can be capacitated in vitro in a culture medium containing albumin, HCO_3^-, and Ca^{2+}, of which albumin is an essential component [6–8] because it removes cholesterol from the plasma membrane and thus changes its fluidity [9]. HCO_3^- stimulates adenylate cyclase (AC) to increase cyclic adenosine monophosphate (cAMP) concentration [10]. After cAMP actives protein kinase A (PKA), PKA phosphorylates spermatic proteins at their serine/threonine residues and induces activation and capacitation [11–16]. In many cases, tyrosine phosphorylation also occurs in a cAMP-dependent manner during activation and capacitation [11, 12, 17, 18]. Ca^{2+} is involved in many intracellular signal transductions, including regulation of AC, phosphodiesterase and protein phosphorylation [11, 19–23].

Recent studies have demonstrated that hyperactivation is enhanced by ligands such as progesterone, melatonin and serotonin [2, 6–8, 24–26]. Moreover, it has been suggested that progesterone-enhanced hyperactivation is suppressed by 17β-estradiol [27]. Steroid hormones of these ligands regulate hyperactivation non-genomically in association with Ca^{2+} signals [2, 4, 6, 28, 29]. Non-genomic regulation of hyperactivation by progesterone is associated with phospholipase C (PLC) [6]. By activating PLC, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) are

produced from phosphatidylcholine (PC) and phosphatidylinositol (PI), respectively. Ho et al. [20, 22, 30, 31] reported that intracellular Ca^{2+}, which is released from an IP_3 receptor (IP_3R)-gated Ca^{2+}-store located at the base of the sperm flagellum, regulates hyperactivation. It has been also suggested that hyperactivation is regulated by calmodulin-dependent protein kinase II (CAMK2) [32]. Although DAG is an activator of protein kinase C (PKC), there is no other evidence for its involvement in sperm function. On the other hand, extracellular Ca^{2+} influx is also very important [6], and is induced by progesterone through the CatSper, which is a sperm-specific Ca^{2+} channel located in the principal piece of the flagellum [33, 34]. When progesterone enhances hyperactivation, tyrosine phosphorylations of spermatic proteins are also increased and/or enhanced [6]. In contrast, many tyrosine phosphorylations are inhibited when progesterone-enhanced hyperactivation is suppressed by 17β-estradiol [27]. In general, tyrosine phosphorylation is a very important event during hyperactivation and is regulated by Ca^{2+} signals [1, 2, 11, 19].

In non-genomic regulation, progesterone also activates AC to increase cAMP concentration [4, 28, 29, 35–37]. Cyclic AMP is an essential molecule for hyperactivation, and regulates tyrosine phosphorylation of spermatic proteins through PKA signals [1, 11, 12, 17]. However, it is unclear if progesterone enhances hyperactivation through cAMP–PKA signals.

Therefore, the present study examined whether progesterone-enhanced hyperactivation is regulated through IP_3–PKC signals and/or cAMP–PKA signals.

Materials and methods

Chemicals

Progesterone was purchased from Sigma Chemical Company (St. Louis, MO, USA). 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indole-3-yl)-maleimide (bisindolylmaleimide 1) and bovine serum albumin (BSA) fraction V were purchased from Merck KGaA (Darmstadt, Germany). *N*-[2-(*N*-formyl-*p*-chlorocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-85) and *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) were purchased from Seikagaku Corporation (Tokyo, Japan). Xestospongin C and other reagent-grade chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of hyperactivated spermatozoa

Spermatozoa were obtained from the caudal epididymis of sexually mature male golden hamsters (*Mesocricetus auratus*), which were housed in accordance with the guidelines of the Dokkyo Medical University and the Laboratory Animal Research Center in Dokkyo Medical University for the care and use of laboratory animals.

Hyperactivated spermatozoa were prepared according to the method described previously [16], using a modified Tyrode's albumin lactate pyruvate (mTALP) medium containing 101.02 mM NaCl, 2.68 mM KCl, 2 mM $CaCl_2$, 1.5 mM $MgCl_2$-$6H_2O$, 0.36 mM NaH_2PO_4-$2H_2O$, 35.70 mM $NaHCO_3$, 4.5 mM D-glucose, 0.09 mM sodium pyruvate, 9 mM sodium lactate, 0.5 mM hypotaurine, 0.05 mM (-)epinephrine, 0.2 mM sodium taurochoric acid, 5.26 μM sodium metabisulfite, 0.05 % (w/v) streptomycine sulfate, 0.05 % (w/v) potassium penicillin G and 15 mg/ml BSA (pH 7.4 at 37 °C under 5 % (v/v) CO_2 in air). An aliquot of caudal epididymal spermatozoa was placed at a culture plate (35-mm dish), and 3 ml of the mTALP medium were carefully added before incubation for 5 min to allow the spermatozoa to swim up. The supernatant containing motile spermatozoa was collected, placed on a culture plate and incubated for 4 h at 37 °C under 5 % CO_2 in air to accomplish hyperactivation. Progesterone and inhibitors were added to the medium after placing motile spermatozoa on the culture plate. For examination of the effects of inhibitors, spermatozoa were exposed to progesterone after exposure to each inhibitor for 5 min. Progesterone was dissolved in methanol (MeOH). Bisindolylmaleimide 1, H-85, H-89 and xestospongin C were dissolved in dimethyl sulfoxide (DMSO). In all experiments, the maximal concentration of vehicle was 0.2 % by volume.

Measurement of the motility and hyperactivation of spermatozoa

Motility and hyperactivation measurements were performed according to the method described previously [16], with some modifications. Motility and hyperactivation were recorded on VHS via 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 CO_2 incubator (MI-IBC, Olympus). Each observation was performed at 37 °C, recorded for 2 min, and analyzed by manually counting the numbers of total spermatozoa, motile spermatozoa and hyperactivated spermatozoa in 10 different fields. Motile spermatozoa that exhibited asymmetric and whiplash flagellar movement and a circular and/or octagonal swimming locus were defined as hyperactivated [38]. The percentages of motile and hyperactivated spermatozoa were respectively defined as the number of motile spermatozoa/number of total spermatozoa × 100, and the number of hyperactivated spermatozoa/number of total spermatozoa × 100. Experiments were performed four times using four

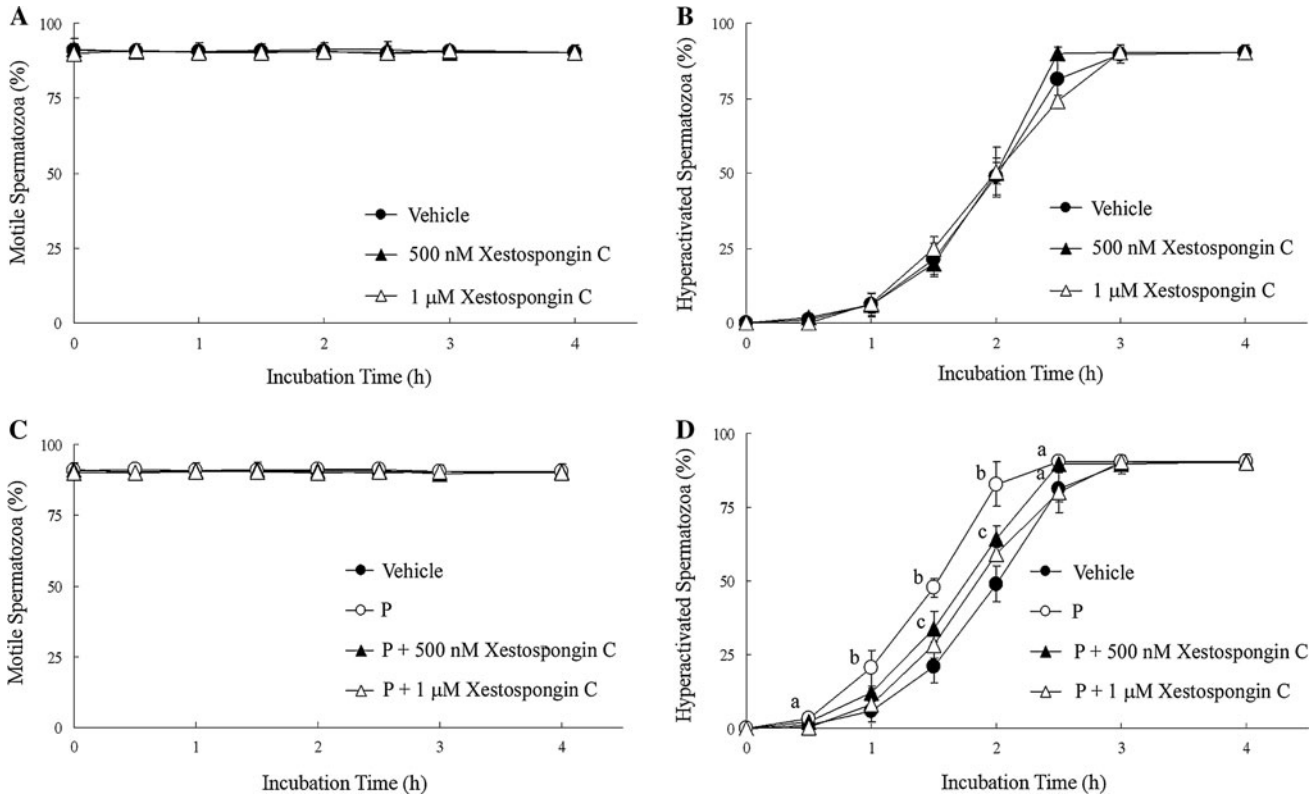

Fig. 1 Effects of xestospongin C on progesterone-enhanced hyperactivation. After exposure to xestospongin C for 5 min, spermatozoa were exposed to progesterone. The percentages of motile spermatozoa (**a**) and hyperactivated spermatozoa (**b**) are shown when 500 nM or 1 μM xestospongin C were added to the mTALP medium. The percentages of motile spermatozoa (**c**) and hyperactivated spermatozoa (**d**) are shown when 500 nM or 1 μM xestospongin C and 20 ng/ml progesterone were added to the mTALP medium. Data are expressed as mean ± SD. In **a** and **b** (Vehicle), mTALP + 0.1 % (v/v) DMSO; (500 nM Xestospongin C), mTALP + 500 nM xestospongin C + 0.1 % (v/v) DMSO; (1 μM xestospongin C), mTALP + 1 μM xestospongin C + 0.1 % (v/v) DMSO. In **c** and **d** (Vehicle), mTALP + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (P), mTALP + 20 ng/ml progesterone + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (P + 500 nM Xestospongin C), mTALP + 20 ng/ml progesterone + 500 nM xestospongin C + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (P + 1 μM Xestospongin C), mTALP + 20 ng/ml progesterone + 1 μM xestospongin C + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO. [a]Significant difference compared with "Vehicle" and "P + 1 μM Xestospongin C" ($P < 0.05$); [b]Significant difference compared with "Vehicle", "P + 500 nM Xestospongin C" and "P + 1 μM Xestospongin C" ($P < 0.05$); [c]Significant difference compared with "Vehicle" ($P < 0.05$)

hamsters. Statistical analysis was carried out using post hoc analysis of the variance (ANOVA) test. A value of $P < 0.05$ was considered significant.

Results

Effects of IP_3R and PKC inhibitors on progesterone-enhanced hyperactivation

In order to examine whether progesterone-enhanced hyperactivation is regulated through IP_3R, hyperactivated hamster spermatozoa were exposed to xestospongin C (IP_3R inhibitor) in mTALP medium or mTALP medium with 20 ng/ml progesterone (Fig. 1). The percentage of motile spermatozoa was not inhibited by xestospongin C under either condition (Fig. 1a, c). Although neither 500 nM nor 1 μM xestospongin C suppressed hyperactivation, they significantly inhibited progesterone-enhanced hyperactivation (Fig. 1b, d). As shown in Fig. 1d, enhancement of hyperactivation by progesterone was significantly inhibited by 500 nM xestospongin C after incubation for 1, 1.5 and 2 h. However, progesterone weakly but significantly enhanced hyperactivation under exposure to 500 nM xestospongin C after incubation for 1.5 and 2 h. On the other hand, enhancement of sperm hyperactivation by progesterone was strongly inhibited by 1 μM xestospongin C (Fig. 1d).

The next step in examining whether progesterone-enhanced hyperactivation is associated with PKC used bisindolylmaleimide 1 as a non-specific PKC inhibitor (Fig. 2). The percentage of motile spermatozoa was not inhibited by bisindolylmaleimide 1 under any conditions (Fig. 2a, c). Although 10 nM bisindolylmaleimide 1 did

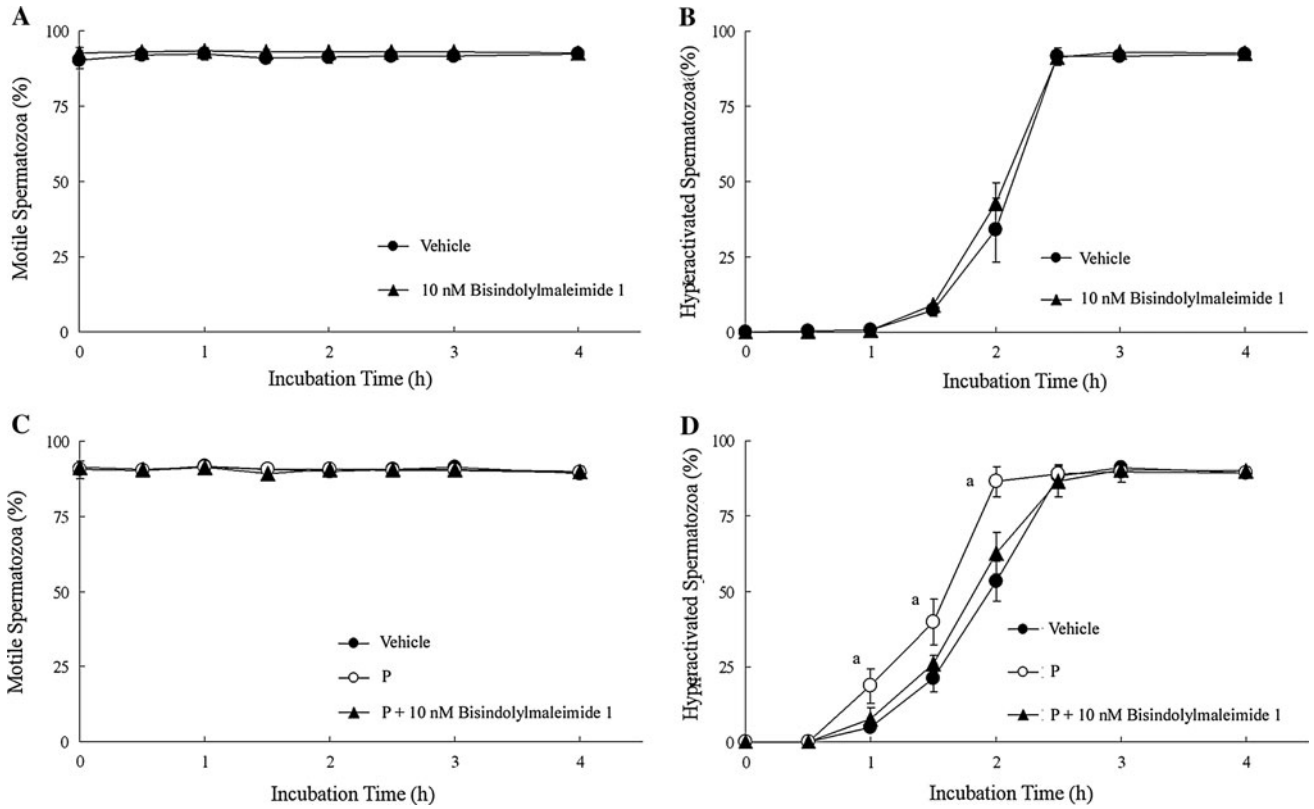

Fig. 2 Effects of bisindolylmaleimide 1 on progesterone-enhanced hyperactivation. After exposure to bisindolylmaleimide 1 for 5 min, spermatozoa were exposed to progesterone. The percentages of motile spermatozoa (**a**) and hyperactivated spermatozoa (**b**) are shown when 10 nM bisindolylmaleimide 1 was added to mTALP medium. The percentages of motile spermatozoa (**c**) and hyperactivated spermatozoa (**d**) are shown when 10 nM bisindolylmaleimide 1 and 20 ng/ml progesterone were added to mTALP medium. Data are expressed as mean ± SD. In **a** and **b** (Vehicle), mTALP + 0.1 % (v/v) DMSO; (10 nM bisindolylmaleimide 1), mTALP + 10 nM bisindolylmaleimide 1 + 0.1 % (v/v) DMSO. In **c** and **d** (Vehicle), mTALP + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (P), mTALP + 20 ng/ml progesterone + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (*P* + 10 nM bisindolylmaleimide 1), mTALP + 20 ng/ml progesterone + 10 nM bisindolylmaleimide 1 + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO. [a]Significant difference compared with "Vehicle" and "*P* + 10 nM bisindolylmaleimide 1" ($P < 0.05$)

not inhibit hyperactivation at all (Fig. 2b), it significantly inhibited progesterone-enhanced hyperactivation (Fig. 2d).

Effects of PKA inhibitors on progesterone-enhanced hyperactivation

Neither H-89 nor H-85 at 1 μM affected the percentage of motile spermatozoa in both the mTALP medium and mTALP medium with 20 ng/ml progesterone (Fig. 3a, c). As for hyperactivation, they did not affect the percentage of hyperactivated spermatozoa in the mTALP medium (Fig. 3b). As for progesterone-enhanced hyperactivation, 1 μM H-89 significantly inhibited it, but 1 μM H-85 did not affect progesterone-enhanced hyperactivation (Fig. 3d).

Discussion

Hyperactivation is a special flagellar movement exhibited by capacitated spermatozoa [1, 2]. Hyperactivation is spontaneously regulated by albumin, cAMP–PKA signals and Ca^{2+} signals [1, 2, 11, 12, 19–22], but recent studies have demonstrated that hyperactivation can be modulated by hormones such as progesterone, 17β-estradiol, melatonin and serotonin [6–8, 24, 27]. Progesterone enhances hyperactivation through extracellular Ca^{2+}, the membrane progesterone receptor and PLC [6], whereas 17β-estradiol suppresses progesterone-enhanced hyperactivation through the membrane estrogen receptor [27]. Serotonin enhances hyperactivation through extracellular Ca^{2+} and two types of serotonin receptor ($5HT_2$ and $5HT_4$) [8], whereby hyperactivation is enhanced through PLC–IP_3 signals when serotonin stimulates the $5HT_2$ receptor, but is enhanced through AC–cAMP–PKA signals when the $5HT_4$ receptor is stimulated. Melatonin also enhances hyperactivation by stimulating melatonin receptor type 1 [7], which suppresses nitric oxide synthase (NOS) and leads to a low concentration of NO. Low concentrations of NO stimulate a MAP kinase cascade and tyrosine phosphorylations, which are associated with sperm capacitation [39].

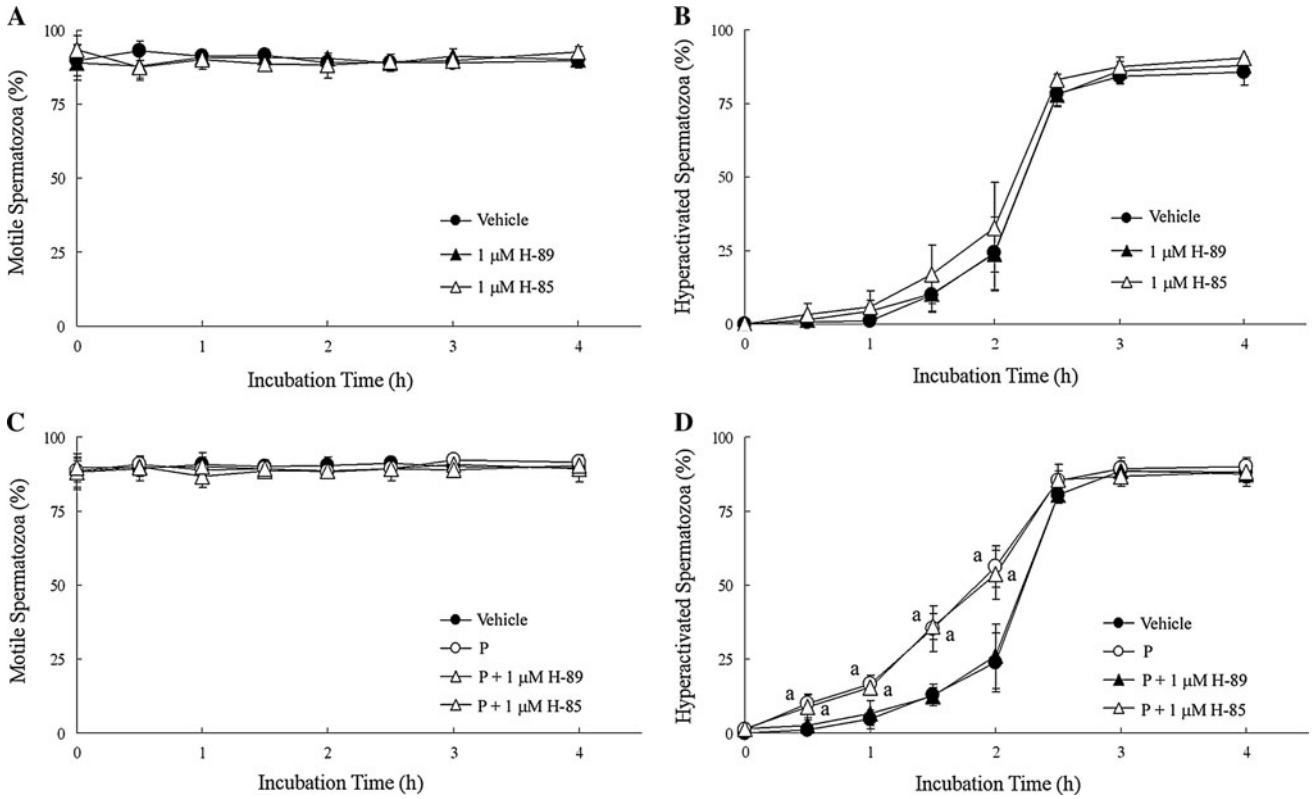

Fig. 3 Effects of H-89 and H-85 on progesterone-enhanced hyperactivation. After exposure to H-89 or H-85 for 5 min, spermatozoa were exposed to progesterone. The percentages of motile spermatozoa (**a**) and hyperactivated spermatozoa (**b**) are shown when 1 μM H-89 or 1 μM H-85 were added to mTALP medium. The percentages of motile spermatozoa (**c**) and hyperactivated spermatozoa (**d**) are shown when 1 μM H-89 or 1 μM H-85 and 20 ng/ml progesterone were added to mTALP medium. Data are expressed as mean ± SD. In **a** and **b** (Vehicle), mTALP + 0.1 % (v/v) DMSO; (1 μM H-89), mTALP + 1 μM H-89 + 0.1 % (v/v) DMSO; (1 μM H-85), mTALP + 1 μM H-85 + 0.1 % (v/v) DMSO. In **c** and **d** (Vehicle), mTALP + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (P), mTALP + 20 ng/ml progesterone + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (*P* + 1 μM H-89), mTALP + 20 ng/ml progesterone + 1 μM H-89 + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO; (*P* + 1 μM H-85), mTALP + 20 ng/ml progesterone + 1 μM H-85 + 0.1 % (v/v) MeOH + 0.1 % (v/v) DMSO. [a]Significant difference compared with "Vehicle" and "*P* + 1 μM H-89" ($P < 0.05$)

The intracellular signal transductions associated with the regulation of progesterone-enhanced hyperactivation were examined in the present study. Because progesterone enhances hyperactivation through PLC [6], it was examined whether IP_3 and DAG are associated with the enhancement of hyperactivation by progesterone, using both IP_3R and PKC inhibitors (Figs. 1, 2). Progesterone-enhanced hyperactivation was significantly inhibited by both inhibitors, although hyperactivation itself was not inhibited by them. On the other hand, it is reported that intracellular Ca^{2+} is released from an IP_3R-gated Ca^{2+}-store and regulates hyperactivation through CAMK2 [20, 22, 30–32], so it is likely that progesterone enhances hyperactivation through activation of PLC, production of IP_3, binding of IP_3 to the IP_3R-gated Ca^{2+}-store, release of intracellular Ca^{2+} and activation of CAMK2. Because it is reported that CAMK2 regulates tyrosine phosphorylations [40], it seems that non-genomic regulation by progesterone includes the spontaneous regulatory mechanism of hyperactivation. In the previous study, progesterone increased and enhanced tyrosine phosphorylations when it enhanced hyperactivation [6]. Because progesterone stimulates activation of PLC, moreover, it is likely that progesterone induces the production of DAG and activates PKC. After activation of PKC, however, the regulatory mechanisms are not clear.

In the non-genomic regulation of hyperactivation, progesterone also activates AC to increase cAMP concentration [4, 28, 29, 35]. Because cAMP activates PKA, which regulates sperm hyperactivation and tyrosine phosphorylations [1, 11, 12, 17], the present study examined the effects of PKA inhibitors on progesterone-enhanced hyperactivation (Fig. 3). H-89 at 1 μM significantly inhibited progesterone-enhanced hyperactivation but H-85 at 1 μM did not have effect (Fig. 3d). Therefore, it is likely that PKA also regulates progesterone-enhanced hyperactivation through AC. Because AC and PKA are essentially related to the spontaneous regulatory mechanism of hyperactivation [1, 2,

11, 12], it seems that stimulation by progesterone includes the spontaneous regulatory mechanism of hyperactivation through the activation of AC. Does the same PKA regulate all sperm functions such activation, hyperactivation and progesterone-enhanced hyperactivation? The responses of spermatozoa to PKA inhibitors were multiple in the present study. Although 1 μM H-89 did not affect the percentage of motile spermatozoa and hyperactivated spermatozoa at all, it suppressed progesterone-enhanced hyperactivation (Fig. 3). Thus, it seems that PKA regulation of progesterone-enhanced hyperactivation differs from PKA regulation of motility or hyperactivation.

It is known that non-genomic progesterone-regulated hyperactivation is associated with extra- and/or intra-cellular Ca^{2+} and PLC [2, 4, 28, 29]. The results of the present study suggest that hyperactivation is enhanced through IP_3R and PKC signaling after spermatozoa are exposed to progesterone. Moreover, it is also suggested that progesterone activates PKA during progesterone-enhanced hyperactivation. Because progesterone increases and enhances tyrosine phosphorylations [6], it is proposed that aforementioned signals also increase and enhance tyrosine phosphorylations.

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