Journal of Andrology, Vol. 24, No. 1, January/February 2003
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Involvement of Cytoplasmic Free Calcium in Boar Sperm: Head-to-Head Agglutination Induced by a Cell-Permeable Cyclic Adenosine Monophosphate Analog

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ABSTRACT: When boar spermatozoa are incubated in a medium designed for in vitro fertilization, many of them become agglutinated at the acrosomes. We previously reported that bicarbonate and cyclic adenosine 3′,5′-monophosphate (cAMP) promote agglutination. The aim of the present study is to examine the role of cytoplasmic free Ca^{2+} in boar sperm agglutination induced by a cell-permeable cAMP analogue. Spermatozoa were collected from five mature boars, washed, and resuspended in a modified Krebs-Ringer-Hepes solution lacking calcium chloride. The sperm suspensions were incubated in a water bath (38.5°C) for 60 minutes and were then used to determine the percentages of head-to-head agglutinated spermatozoa. Percentages of head-to-head agglutinated spermatozoa in the samples rose significantly after incubation, from 28% to 61%–62%, after adding to the medium a cell-permeable, phosphodiesterase-resistant cAMP analogue (cBiMPS, 10 μM) or an adenylyl cyclase stimulator (sodium bicarbonate, 5 mM) plus a cell-permeable phosphodiesterase inhibitor (IBMX, 25 μM). However, the promoting effects of these reagents were blocked when spermatozoa were pretreated with a cell-permeable Ca^{2+} chelator (BAPTA-AM, 25 μM), whereas the same pretreatment with a cell-impermeable Ca^{2+} chelator (BAPTA, 25 μM) had almost no influence on sperm agglutination. Adding thapsigargin, a potential Ca^{2+}-ATPase inhibitor, to the medium raised the percentages of agglutinated spermatozoa in a concentration-dependent manner for concentrations up to 4 μM. When 4 μM thapsigargin and 10 μM cBiMPS were examined for their effects on free Ca^{2+} levels in sperm heads by using a cell-permeable Ca^{2+} indicator (fluo-3/AM), the samples incubated with both or either of these reagents contained many head-to-head agglutinated cells that exhibited intense fluorescence in the heads. In control samples incubated without these reagents by contrast, most spermatozoa were free (unagglutinated) cells and characterized by almost no or only slight fluorescence in the heads. Moreover, morphological observation of Giemsa-stained preparations revealed that most agglutinated spermatozoa possessed darkly stained acrosomes, which distinguished them from acrosome-reacted spermatozoa. This indicated that the sperm agglutination was not a result of the acrosome reaction. Furthermore, with indirect immunofluorescence of Ca^{2+}-ATPases, the mouse monoclonal antibody to this enzyme demonstrated high affinity to the acrosomes of permeabilized spermatozoa. Based on these results, we conclude that cytoplasmic free Ca^{2+} is involved in sperm head-to-head agglutination induced by a cAMP analogue.

Key words: Capacitation, BAPTA, thapsigargin, fluo-3, Ca^{2+}-ATPase.
J Androl 2003;24:91–99

Mammalian spermatozoa express their fertilizing ability during their stay in the female reproductive tract, a process known as capacitation (Austin, 1951; Chang, 1951). A number of sperm elements are altered when specific molecules are activated during this process (Visconti and Kopf, 1998; Visconti et al, 1998). The environment in the female reproductive tract can be simulated by incubating spermatozoa in a capacitation medium in vitro (Yanagimachi, 1994), and it has been observed that many spermatozoa become agglutinated at the acrosome during incubation (Bedford and Yanagimachi, 1991; Harayama et al, 1999; Tardif et al, 2001). This agglutination is promoted by capacitation-supporting factors such as extracellular Ca^{2+}, bicarbonate (Harayama et al, 1998), serum albumin, and a cell-permeable analogue of cyclic adenosine 3′,5′-monophosphate (cAMP), dibutyryl cAMP sodium salt (dbcAMP; Harayama et al, 2000). These findings suggest that head-to-head agglutination has biological significance in the fertilization process.

Several potential targets of the intracellular cyclic nucleotide have been proposed in mammalian spermatozoa such as protein kinase A (PKA), a hyperpolarization-activated cyclic nucleotide-gated channel, and guanine-nucleotide-exchange factors (Kaupp and Weyand, 2000). PKA seems to be involved in the regulation of sperm agglutination because the PKA inhibitor H89 reduces the promoting effects of dbcAMP (Harayama et al, 2000). To our knowledge, however, data do not exist on the downstream parts of the cAMP-PKA signaling system that lead to agglutination nor on the roles of other targets of the cyclic nucleotide in agglutination of mammalian spermatozoa, although it has been reported that PKA activation leads to changes in the lipid architecture in the sperm plasma membrane (Gadella and Harrison, 2000). Recent articles such as those by Wiesner et al (1998), Kobori et al (2000), and Ren et al (2001) have shown that an external Ca^{2+} influx is induced in the heads

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Received for publication April 5, 2002; accepted for publication July 10, 2002.

Table 1. *Composition of modified Krebs-Ringer Hepes**

Composition	Concentration
NaCl	94.60 mM
KCl	4.78 mM
$MgSO_4$	1.19 mM
KH_2PO_4	1.19 mM
Hepes	25.07 mM
Glucose	5.56 mM
Sodium pyruvate	0.50 mM
Sodium lactate	21.58 mM
Bovine serum albumin	4 mg/mL
Streptomycin sulphate	50 μg/mL
Potassium penicillin G	100 IU/mL
Phenol red	2 μg/mL

* pH = 7.4.

and tails of mouse and bull spermatozoa by treatment with cell-permeable cyclic nucleotide analogues. Moreover, it has been proposed in bull spermatozoa that internal Ca^{2+} in the putative acrosomal store moves into the cytoplasm through the cation channels of the outer acrosomal membrane that are opened by cAMP signaling (Spungin and Breitbart, 1996; Breitbart and Naor, 1999). Thus, it is likely that the cyclic nucleotide-mediated signaling induces mobilization of both external and internal Ca^{2+} into the cytoplasm of mammalian spermatozoa. The aims of the present study are to examine the role of cytoplasmic free Ca^{2+} in boar sperm agglutination induced by a cell-permeable cAMP analogue, and to assess the relationship between cAMP signaling and cytoplasmic free Ca^{2+} in this event.

Materials and Methods

Sperm Collection and Washing

Sperm-rich fractions from ejaculates were collected from five mature boars by a manual method. A portion (2 mL) of each sperm-rich fraction was loaded on a discontinuous gradient of 2 mL of 90% and 5 mL of 60% isotonic Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) prepared with phosphate-buffered saline (PBS; 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, and 1.5 mM KH_2PO_4) in a 15-mL plastic centrifugation tube. For each experiment, 10 tubes were prepared and centrifuged at 700 × *g* for 10 minutes at room temperature. The spermatozoa were recovered and then washed twice in PBS containing 0.1% polyvinyl alcohol (molecular weight 30 000–70 000, Sigma Chemical Company, St Louis, Mo) by centrifugation at 700 × *g* for 5 minutes at room temperature.

Sperm Agglutination Assay

The sperm agglutination assay was performed as described previously with minor modifications (Harayama et al, 1994). Briefly, the washed spermatozoa were resuspended in a modified Krebs-Ringer-Hepes solution lacking calcium chloride (mKRH pH 7.4, Table 1) to give a final sperm concentration of 2.5×10^7 cells/mL. The spermatozoa were then incubated in a 38.5°C water bath for 60 minutes. After the incubation, an aliquot of each sample was gently smeared on a glass slide, dried, and stained in a phosphate-buffered Giemsa solution (Merck, Darmstadt, Germany). More than 300 spermatozoa were counted at random by light microscopy (400×) to determine the percentages of head-to-head agglutinated cells.

Calcium chloride (Wako Pure Chemical Industries, Ltd, Osaka Japan), ethylenediamine-N,N,N′,N′-tetraacetic acid, trisodium salt, trihydrate (EDTA·3Na, Dojindo Laboratories, Kumamoto, Japan), sodium bicarbonate (a stimulator of adenylyl cyclase; Nacalai Tesque, Kyoto, Japan; Okamura et al, 1985) and 3-isobutyl-1-methylxanthine (IBMX, a cell-permeable phosphodiesterase inhibitor; Sigma; Shafer et al, 1998) were dissolved in the mKRH and added to the sperm suspensions. Thapsigargin, a cell-permeable endoplasmic reticulum Ca^{2+}-ATPase inhibitor (Sigma; Thastrup et al, 1990) and Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole-3′,5′-monophosphorothioate (cBiMPS; a cell-permeable, phosphodiesterase-resistant cAMP analogue; Biomol Research Laboratories Inc, Plymouth Meeting, Penn; Schaap et al, 1993) were dissolved in dimethyl sulfoxide (DMSO, Nacalai Tesque) and added to the sperm suspensions. In each experiment, DMSO was added to equalize the final DMSO concentrations among all samples.

Pretreatment With Ca^{2+} Chelators

Ca^{2+} chelators including 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′,N′-tetrapotassium salt (BAPTA, Sigma) and 1,2-bis-(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid tetraacetoxy-methyl ester (BAPTA-AM, Biomol) were dissolved in DMSO as 25 mM stock solutions and added to the sperm suspensions. The washed spermatozoa were resuspended in mKRH containing either BAPTA or BAPTA-AM (final concentration 25 μM) to adjust the sperm concentration to 1.0×10^8 cells/mL, and were then incubated in a 25°C water bath for 90 minutes. In the control samples, DMSO instead of the stock solutions was added in order to equalize the final concentration of the solvent. After this pretreatment, the sperm suspensions were diluted with a threefold volume of mKRH containing cBiMPS (final concentration 10 μM) or sodium bicarbonate (final concentration 5 mM) plus IBMX (final concentration 25 μM), and then were incubated in a 38.5°C water bath for 60 minutes (see "Sperm Agglutination Assay").

Detection of Free Ca^{2+} in Spermatozoa

A cell-permeable Ca^{2+} indicator, fluo-3/AM (Calbiochem-Novabiochem Corporation, San Diego, Calif) was dissolved in DMSO containing 4% Pluronic F127 (Molecular Probes Inc, Eugene, Ore) to give a concentration of 1 mM. Washed spermatozoa suspended in mKRH (2.0×10^8 cells in 1.99 mL) were mixed with the fluo-3/AM solution (10 μL) and then loaded at 38.5°C in the dark for 30 minutes with fluo-3/AM (final concentration 5 μM) in the presence of 0.02% Pluronic F127. Subsequently, the sperm suspensions (2 mL) were diluted with mKRH (6 mL) and centrifuged at 700 × *g* for 5 minutes at room temperature. The spermatozoa were recovered, washed in mKRH (8 mL) by centrifugation at 700 × *g* for 5 minutes at room temperature, and then resuspended in mKRH containing thapsigargin (final concentration 4 μM), cBiMPS (final concentration 10 μM), or both to give a sperm concentration of 2.5×10^7 cells/mL. After incubation in a 38.5°C water bath for 60 minutes, an aliquot of each sample was placed on a glass slide, covered with a coverslip, and examined with a differential interference mi-

croscope equipped with epifluorescence (B2 set filter, excitation filter EX450-490, dichroic mirror DM510, and emission filter BA520, EFD2; Nikon Company, Tokyo, Japan) or with a confocal laser scanning microscope with a laser unit LSM-LU-100, excitation filter DM488, and emission filter BP535 (Olympus Optical Company Ltd, Tokyo, Japan).

Assessment of Acrosome Morphology of Agglutinated Spermatozoa

Washed spermatozoa were incubated in mKRH containing thapsigargin (final concentration 4 μM), cBiMPS (final concentration 10 μM), or both in a 38.5°C water bath for 60 minutes (see ''Sperm Agglutination Assay''). An aliquot of each sample was smeared on a glass slide and air-dried on a hot plate (37°C). The slide was fixed for 45 minutes in the fixative (10% v/v formalin in 6.8% potassium dichromate solution), and then stained in a phosphate-buffered Giemsa solution for 90 minutes at room temperature (Kato et al, 1979). One hundred agglutinated cells were counted by light microscopy (1000×) to determine the percentages of agglutinated spermatozoa with the darkly stained acrosomes. Aspects of boar sperm acrosomes stained with Giemsa were described by Kovacs and Foote (1992).

Indirect Immunofluorescence of Ca^{2+}-ATPases

All procedures were undertaken at room temperature. Washed spermatozoa were resuspended in PBS (sperm concentration 4×10^8 cells/mL, 100 μL), placed onto polylysine-coated coverslips (Asahi Techno Glass, Tokyo, Japan), and left for 10 minutes. The coverslips on which the spermatozoa stuck were rinsed gently with PBS and then covered with methanol (permeabilized samples) or with PBS (nonpermeabilized samples) for 10 minutes. The samples were rinsed with PBS twice and blocked with 5% bovine serum albumin (BSA; Intergen Co, Purchase, NY) in PBS (blocking buffer) for 60 minutes, and were then given a 30-minute treatment with either the mouse monoclonal antibody to Ca^{2+}-ATPases (PL/IM430, 10 μg/mL immunoglobulin G1 [IgG1], Biogenesis Ltd, Poole, United Kingdom) or the mouse IgG1 negative control (10 μg/mL IgG1, DAKO A/S, Glostrup, Denmark) in the blocking buffer. The antibody PL/IM430 was raised against Ca^{2+}-ATPases that were present in the endoplasmic reticulum-like intracellular membranes of human blood platelets (Hack et al, 1988a,b). After being rinsed twice again with PBS, the coverslips were treated with the blocking buffer for 60 minutes and then with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (DAKO) diluted (1:50) with the blocking buffer for 30 minutes. After being rinsed twice, the coverslips were mounted on the glass slides with 1 mg/mL p-phenylenediamine (Sigma) dissolved in glycerol:PBS (9:1). The sperm preparations were examined with a differential interference microscope equipped with epifluorescence (B2 set filter, Nikon).

Statistical Analysis

Percentages of head-to-head agglutinated spermatozoa and percentages of agglutinated spermatozoa with the darkly stained acrosomes were subjected to one-way analysis of variance (ANOVA). When *F*-test results were significant in ANOVA, individual means were further tested with the Tukey multiple range test (Motulsky, 1995).

Table 2. *Effects of calcium chloride and cyclic adenosine 3′,5′-monophosphate analog on head-to-head agglutination of boar spermatozoa (n = 5)**

Incubation†	Concentrations of calcium chloride in the medium (mM)			
	0	1.71	0	1.71
	Concentrations of cBiMPS‡ in the medium (μM)			
	0	0	10	10
Before‡	4 ± 1	5 ± 1	4 ± 0	3 ± 0
After	17 ± 3¶	37 ± 6‖	52 ± 5‖	72 ± 4§

* Values (% of head-to-head agglutinated spermatozoa) are means ± SEM.

† Ejaculated spermatozoa were washed and incubated in mKRH containing calcium chloride, cBiMPS, or both, in a 38.5°C water bath for 60 minutes. Before or after the incubation, an aliquot of each sample was gently smeared on a glass slide, dried, and stained in a phosphate-buffered Giemsa solution. More than 300 spermatozoa were counted at random by light microscopy (400×) to determine the percentages of head-to-head agglutinated cells.

‡ Sp-5,6-dichloro-1-β-D-ribofuranosyl-benzimidazol-3′-5′-monophosphorothioate

§‖¶ Values within the same line with different superscripts differ significantly, $P < .05$ (Tukey multiple range test).

Results

Effects of External Ca^{2+} and cAMP Analogue on Sperm Agglutination

Table 2 shows the percentages of head-to-head agglutinated spermatozoa before and after incubation in mKRH containing calcium chloride (1.71 mM), cBiMPS (10 μM), or both. In all samples before incubation, most spermatozoa were free (unagglutinated). When the samples were incubated in the presence and absence of calcium chloride, the percentages of head-to-head agglutinated spermatozoa were 37% and 17%, respectively. Addition of cBiMPS significantly increased the percentages of agglutinated spermatozoa in mKRH and also dramatically showed the multiplier effects with external Ca^{2+} on sperm agglutination in mKRH containing calcium chloride. Moreover, addition of this analogue was effective at inducing agglutination in spermatozoa incubated in mKRH containing EDTA·3Na (2–3 mM, Table 3).

Effects of Pretreatment With Ca^{2+} Chelators on Sperm Agglutination

In the control samples pretreated in mKRH without the Ca^{2+} chelator, addition of 10 μM cBiMPS or 5 mM sodium bicarbonate plus 25 μM IBMX increased the percentages of head-to-head agglutinated spermatozoa significantly, from 28% to 61%–62% (Figure 1). However, pretreatment with 25 μM BAPTA-AM blocked the promoting effects of these reagents, whereas the same pretreatment with 25 μM BAPTA produced almost no influence on sperm agglutination (Figure 1).

Effects of Thapsigargin on Sperm Agglutination

The level of cytoplasmic free Ca^{2+} is usually kept low because the cation is removed from the cytoplasm through the actions of Ca^{2+}-ATPases and Na^+/Ca^{2+} exchangers (Berridge

Table 3. *Effects of EDTA·3Na on head-to-head agglutination of boar spermatozoa (n = 4)**

	Concentrations of EDTA·3Na‡ in the medium (mM)					
	0	0	2	2	3	3
	Concentrations of cBiMPS in the medium (μM)					
Incubation†	0	10	0	10	0	10
Before	4 ± 1	6 ± 2	6 ± 1	7 ± 3	6 ± 1	6 ± 1
After	18 ± 2‖	54 ± 5§	16 ± 2‖	50 ± 2§	16 ± 3‖	44 ± 3§

* Values (% of head-to-head agglutinated spermatozoa) are means ± SEM.
† Ejaculated spermatozoa were washed and incubated in mKRH containing 3Na·EDTA, cBiMPS, or both, in a 38.5°C water bath for 60 minutes. An aliquot of each sample was observed to determine the percentages of head-to-head agglutinated cells, as described in Table 2.
‡ Ethylenediamine-N,N,N′,N′-tetraacetic acid, trisodium salt, trihydrate.
§‖ Values within the same line with different superscripts differ significantly, $P < .05$ (Tukey multiple range test).

et al, 2000). In this experiment we examined the effects of thapsigargin, a potential endoplasmic reticulum Ca^{2+}-ATPase inhibitor, on sperm agglutination. The addition of thapsigargin to mKRH raised the percentages of agglutinated spermatozoa in a concentration-dependent manner for concentrations up to 4 μM (Figure 2A). The promoting effects of thapsigargin (4 μM) were as high as those of cBiMPS (10 μM) and were further enhanced by the addition of cBiMPS (10 μM) (Figure 2B).

Detection of Free Ca^{2+} in Spermatozoa

Effects of thapsigargin (4 μM) and cBiMPS (10 μM) were examined on the levels of free Ca^{2+} in sperm heads by using fluo-3/AM, a cell-permeable Ca^{2+} indicator (Figure 3). In the samples after incubation with both or either of these reagents (highly agglutinating condition; Figure 3, A–D), many spermatozoa exhibited head-to-head agglutination with intense fluorescence in the heads, although the remaining free spermatozoa (ie, unagglutinated) exhibited almost no or only slight fluorescence in the heads. However, in the control samples before incubation (nonagglutinating condition; Figure 3F) or after incubation without these reagents (slightly agglutinating condition; Figure 3E), most spermatozoa were free and exhibited almost no fluorescence in the heads. In addition, most spermatozoa exhibited intense fluorescence in their middle pieces, regardless of agglutination in any sample. When a cell-impermeable Ca^{2+} indicator, fluo-3 (pentapotassium salt, Molecular Probes) instead of the fluo-3/AM was used in order to eliminate the possibility that agglutinated spermatozoa had trapped the fluo-3/AM between the surface of the cells rather than within cells, no fluorescence was detected in either agglutinated or free spermatozoa after incubation with (Figure 3G) or without cBiMPS (data not shown). This strongly supported the

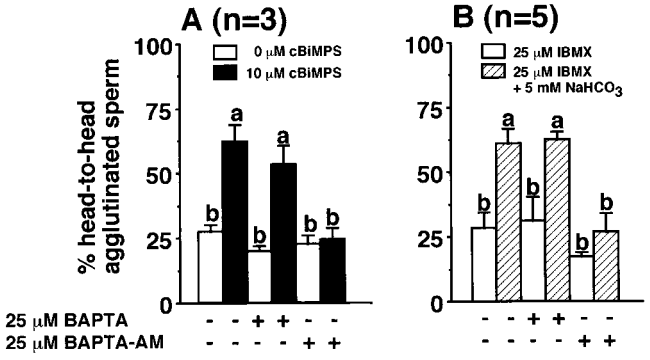

Figure 1. Effects of pretreatment with Ca^{2+} chelators on head-to-head agglutination of boar spermatozoa. Ejaculated boar spermatozoa were washed and incubated in mKRH containing a cell-impermeable Ca^{2+} chelator BAPTA (25 μM) or a cell-permeable Ca^{2+} chelator, BAPTA-AM (25 μM) in a 25°C water bath for 90 minutes. After pretreatment, each sperm suspension was diluted with a three-fold volume of mKRH containing cBiMPS, a cell-permeable analogue of cAMP (final concentration: 10 μM) or sodium bicarbonate, an adenylyl cyclase stimulator (final concentration, 5 mM) plus IBMX, a cell-permeable phosphodiesterase inhibitor (final concentration, 25 μM), and were then incubated in a 38.5°C water bath for 60 minutes. After the incubation, an aliquot of each sample was gently smeared on a glass slide, dried, and stained in a phosphate-buffered Giemsa solution. More than 300 spermatozoa were counted at random by light microscopy (400×) to determine the percentages of head-to-head agglutinated cells. Values are means ± SEM. [a,b] Values within the same panels with different letters differ significantly, $P < .05$ (Tukey multiple range test).

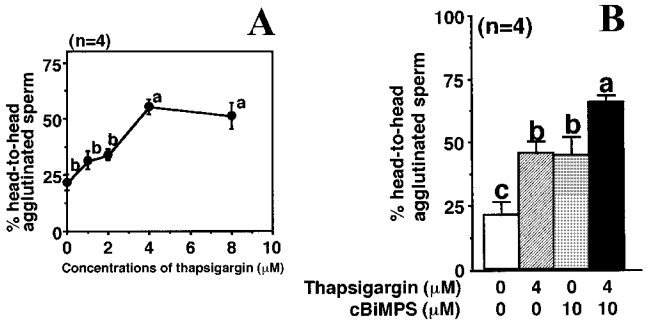

Figure 2. Effects of thapsigargin and cBiMPS on head-to-head agglutination of boar spermatozoa. Ejaculated boar spermatozoa were washed and incubated in mKRH in a 38.5°C water bath for 60 minutes. **(A)** Thapsigargin, a potential Ca^{2+}-ATPase inhibitor (1–8 μM), was added to the medium. **(B)** Thapsigargin (4 μM), cBiMPS (10 μM), or both were added to the medium. In all experiments, the sperm samples after incubation were observed in order to determine the percentages of head-to-head agglutinated cells, as described in Figure 1. Values are means ± SEM. [a–c] Values within the same panels with different letters differ significantly, $P < .05$ (Tukey multiple range test).

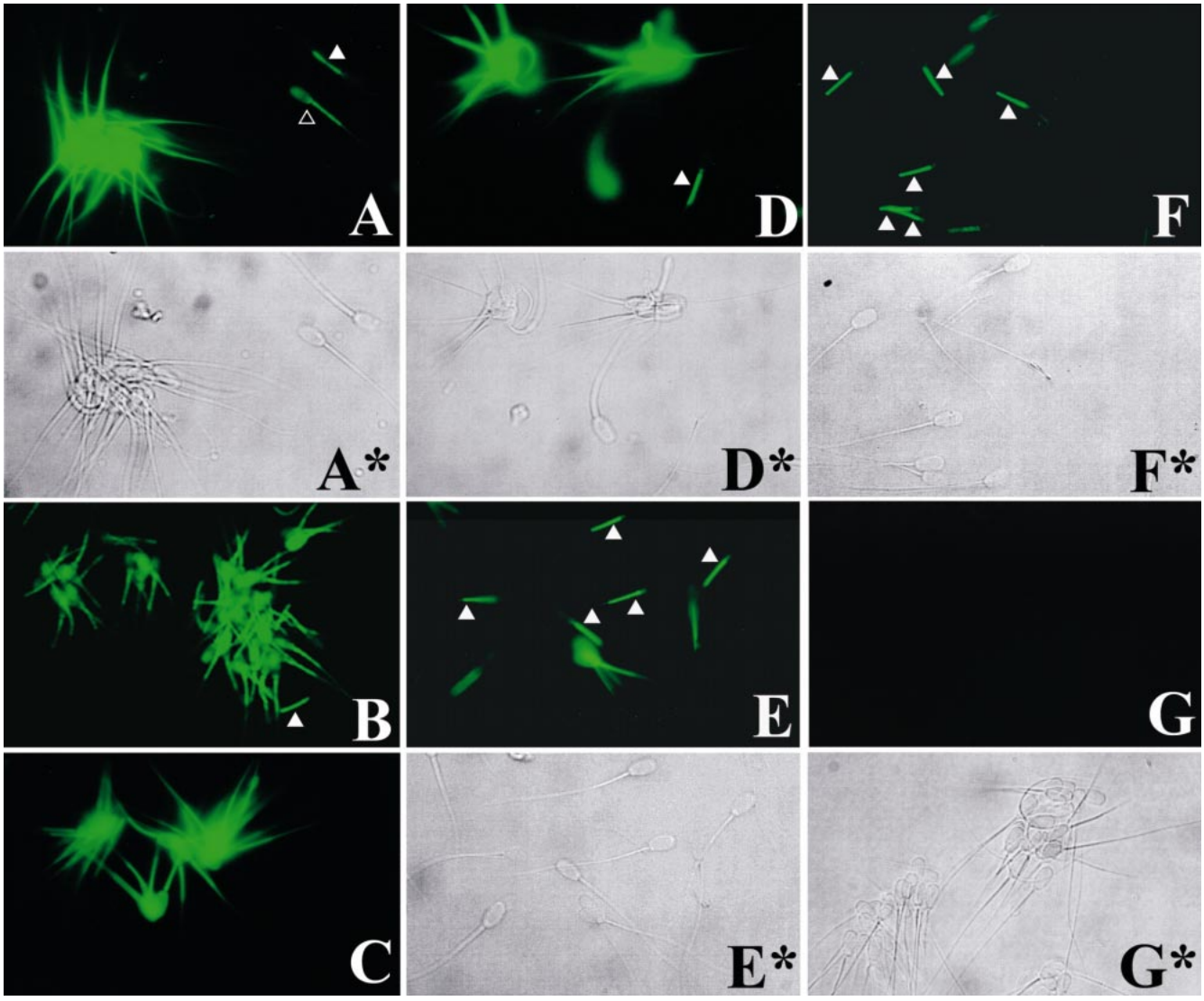

Figure 3. Detection of free Ca^{2+} in agglutinated and free (unagglutinated) boar spermatozoa. Ejaculated boar spermatozoa were washed and loaded at 38.5°C in the dark for 30 minutes with fluo-3/AM, a cell-permeable Ca^{2+} indicator (5 μM in the presence of 0.02% Pluronic F127) in mKRH. In some experiments to confirm whether or not the Ca^{2+} indicator was trapped on the sperm surface, a cell-impermeable fluo-3 (pentapotassium salt) was used instead of fluo-3/AM. Subsequently, spermatozoa were washed in mKRH twice and then incubated with thapsigargin (4 μM), cBiMPS (10 μM), or both in a 38.5°C water bath for 60 minutes. Before or after incubation, sperm samples were observed with a differential interference microscope equipped with epifluorescence **(A** and **C–G)** or with a confocal laser scanning microscope **(B)**. Data are representative of three replicates. **(A** and **B)** Spermatozoa loaded with fluo-3/AM, washed, and incubated in mKRH containing both thapsigargin and cBiMPS; **(C)** spermatozoa loaded with fluo-3/AM, washed, and incubated in mKRH containing thapsigargin; **(D)** spermatozoa loaded with fluo-3/AM, washed, and incubated in mKRH containing cBiMPS; **(E)** spermatozoa loaded with fluo-3/AM, washed, and incubated in mKRH; **(F)** spermatozoa loaded with fluo-3/AM and washed before incubation; **(G)** spermatozoa treated with fluo-3, washed, and incubated in mKRH containing cBiMPS. Sperm clusters show head-to-head agglutinated spermatozoa exhibiting intense fluorescence in the heads and middle pieces. Filled triangles show free spermatozoa exhibiting intense fluorescence only in the middle pieces; open triangle shows a free spermatozoon exhibiting relatively bright fluorescence in the head and intense fluorescence in the middle piece. **A*, D*, E*, F*,** and **G*** show differential interference corresponding to **A, D, E, F,** and **G,** respectively.

notion that fluo-3/AM could be introduced into spermatozoa without being trapped on their surfaces.

Acrosome Morphology of Agglutinated Spermatozoa

The acrosomes of the agglutinated spermatozoa were morphologically examined in the samples that were incubated in mKRH, mKRH containing 10 μM cBiMPS, mKRH containing 4 μM thapsigargin, or mKRH containing both 10 μM cBiMPS and 4 μM thapsigargin. In all these samples after incubation and Giemsa staining, most of the agglutinated spermatozoa (means ± SEM; 87% ± 6% to 95% ± 3%) possessed darkly stained acrosomes, and a slight swell was observed at the apical portion of the acrosomes of some agglutinated cells (Figure 4, A and B). The spermatozoa with the acrosomes could be easily distinguished from those without acrosomes (Figure 4C).

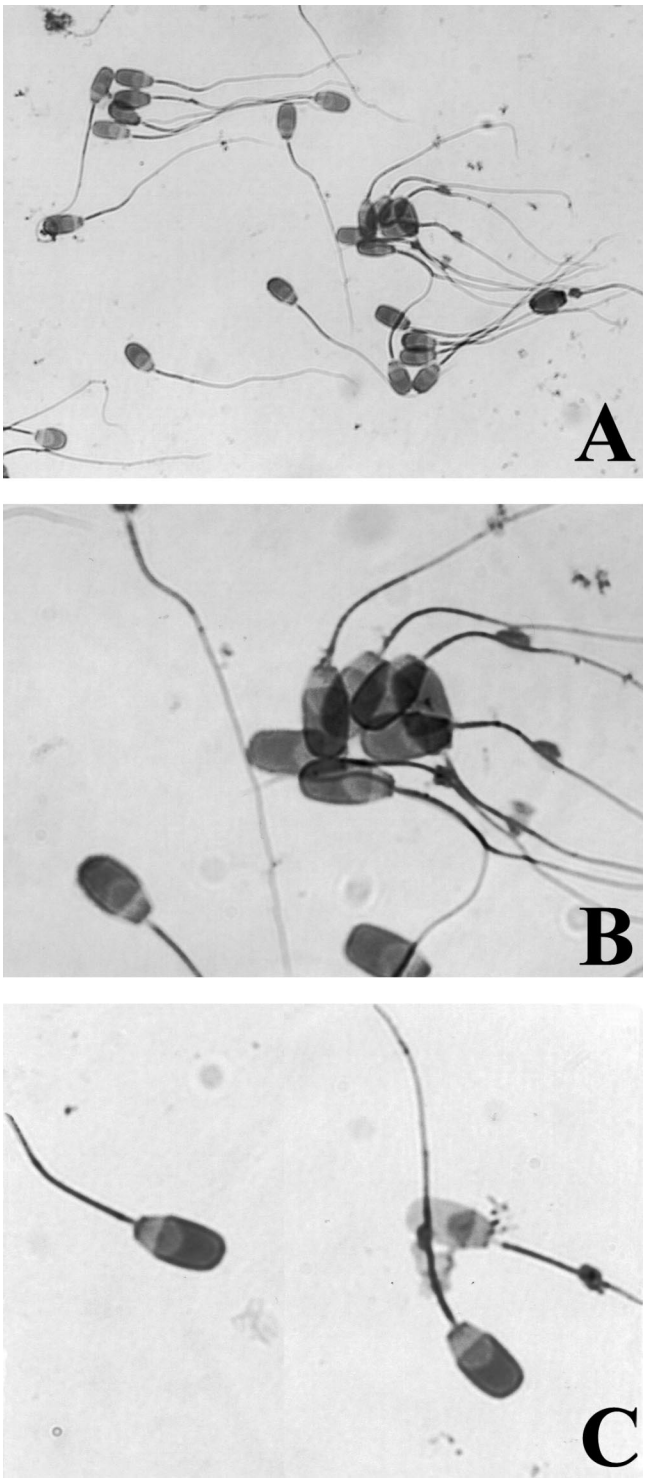

Figure 4. Boar spermatozoa stained via the Giemsa-staining technique. **(A-C)** Ejaculated boar spermatozoa were washed and incubated in mKRH containing thapsigargin (4 μM) and cBiMPS (10 μM) in a 38.5°C water bath for 60 minutes. After incubation spermatozoa were smeared on a glass slide, fixed, and stained in a phosphate-buffered Giemsa solution. Data are representative of three replicates. **(B)** Is an enlargement of **(A)**. Most agglutinated spermatozoa had darkly stained acrosomes, and a slight swell was observed at the apical portion of the acrosome in some agglutinated cells. **(C)** Free spermatozoa with or without acrosomes.

Similar morphological aspects have been reported in boar spermatozoa stained by the triple-staining techniques, and acrosomes with a sight swell are apparently distinct from those of acrosome-reacted spermatozoa (Harayama et al, 1993). Thus, in this study, sperm agglutination is rarely due to the acrosome reaction.

Immunolocalization of Antigens Recognized by the Monoclonal Antibody to Ca^{2+}-ATPases

As shown in Figure 5, the mouse monoclonal antibody to the Ca^{2+}-ATPases had a high affinity to the acrosomes of permeabilized spermatozoa, but it had almost no affinity to the acrosomes of nonpermeabilized spermatozoa. In addition, when negative control mouse IgG1 was used instead of the primary antibody at the same concentration, no reaction was observed in either permeabilized or nonpermeabilized spermatozoa (data not shown).

Discussion

In mammalian spermatozoa, calcium plays a pivotal role in the expression of fertilizing ability, including capacitation, hyperactivation, and the acrosome reaction (eg, Florman et al, 1989; Fraser and McDermott, 1992; Storey et al, 1992; Ho and Suarez, 2001). We also showed that external Ca^{2+} (1.71 mM calcium chloride) promoted sperm head-to-head agglutination (Table 2; Harayama et al, 1999, 2000). However, addition of cBiMPS (10 μM, a cell-permeable, phosphodiesterase-resistant cAMP analogue) was effective in inducing sperm agglutination when an external Ca^{2+} deficiency existed (Tables 2 and 3). This suggests that external Ca^{2+} is not essential for sperm agglutination, which is induced by the actions of cAMP, although this external cation apparently promotes agglutination.

An increase in levels of cytoplasmic free Ca^{2+} is generally modulated by selective cation channels that control the entry of external Ca^{2+} through the plasma membrane. The several families of Ca^{2+} entry channels are defined by the way in which they are activated: voltage-operated channels, receptor-operated channels, cyclic nucleotide-gated channels, and store-operated channels. The increase in cytoplasmic free Ca^{2+} is also derived from mobilization of this cation from internal stores through the channels, including via the inositol 1,4,5-triphosphate receptor and ryanodine receptor (Berridge et al, 2000). In the present study, cBiMPS (10 μM) or sodium bicarbonate (5 mM, a stimulator of adenylyl cyclase) plus IBMX (25 μM, a cell-permeable phosphodiesterase inhibitor) promoted head-to-head agglutination in boar spermatozoa in conditions of an external Ca^{2+} deficiency (Table 2 and Figure 1). However, the promoting effect of cBiMPS or sodium bicarbonate plus IBMX was greatly reduced by pretreat-

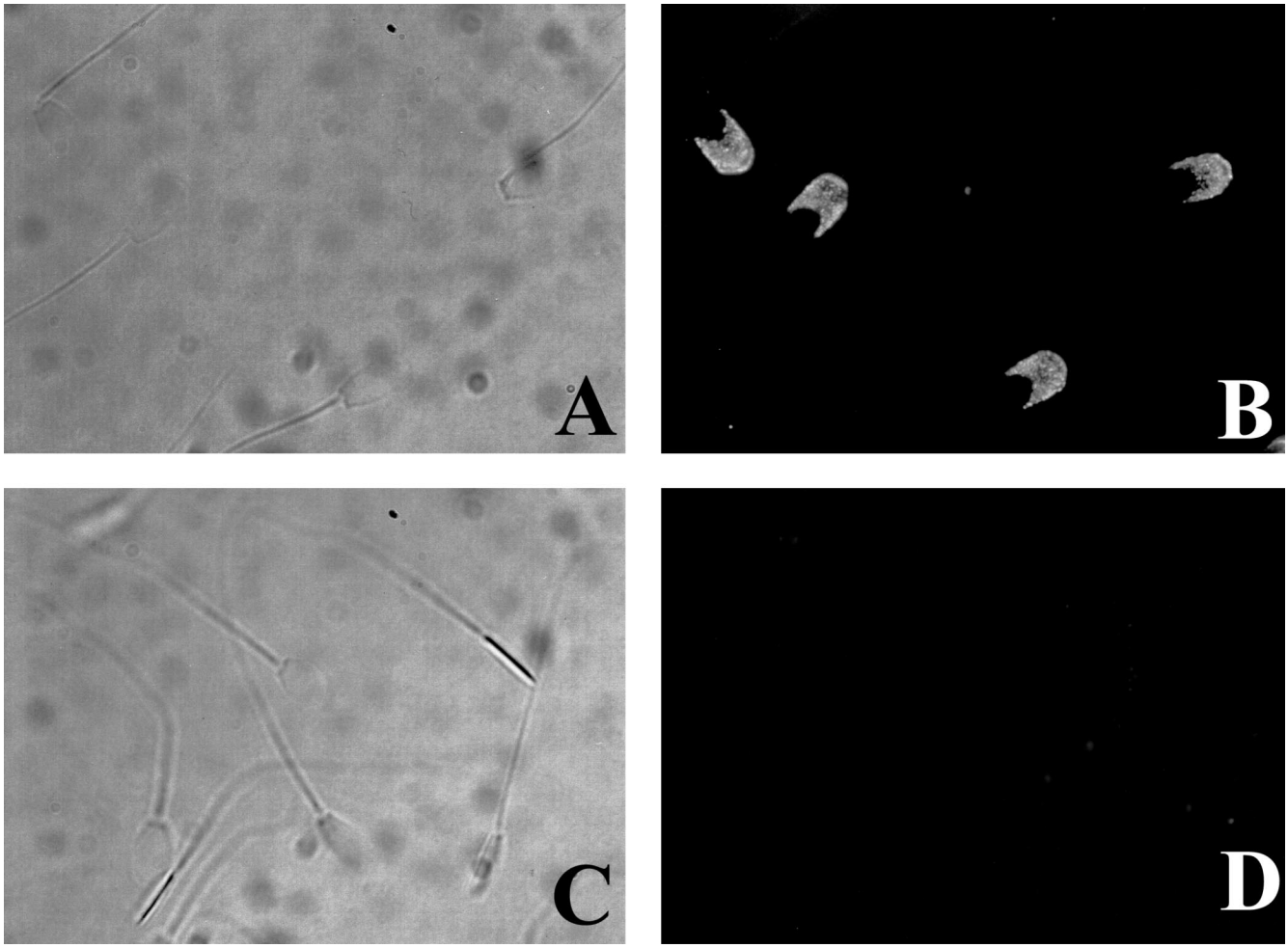

Figure 5. Indirect immunofluorescence of boar spermatozoa for Ca^{2+}-ATPases. Ejaculated boar spermatozoa were washed, attached to polylysine-coated coverslips, and then treated with methanol (permeabilized samples, **A** and **B**) or with PBS (nonpermeabilized samples, **C** and **D**). After the blocking treatment, they were immunostained with the mouse monoclonal antibody to Ca^{2+}-ATPases (10 μg/mL IgG1) of endoplasmic reticulum-like intracellular membranes of human blood platelets. Corresponding differential interference **(A** and **C)** and immunofluorescence **(B** and **D)** are shown. Data are representative of three replicates.

ing spermatozoa with BAPTA-AM (25 μM, a cell-permeable Ca^{2+} chelator), but not by pretreatment with BAPTA (25 μM, a cell-impermeable Ca^{2+} chelator; Figure 1). These findings can be interpreted as showing that cytoplasmic free Ca^{2+} is essential for sperm agglutination induced by the actions of cAMP. Moreover, the detection of free Ca^{2+} with fluo-3/AM (a cell-permeable Ca^{2+} indicator) revealed that cBiMPS-agglutinated spermatozoa exhibited more intense fluorescence in the heads than control spermatozoa (ie, free spermatozoa) did (Figure 3), demonstrating the higher level of free Ca^{2+} in the heads of cBiMPS-agglutinated spermatozoa. These findings strongly indicate that cAMP signaling is connected to cytoplasmic free Ca^{2+}. Because the spermatozoa were incubated in a Ca^{2+}-deficient medium, this increase in free Ca^{2+} in agglutinated spermatozoa by cBiMPS might result from Ca^{2+} mobilization from the putative acrosomal store through the cation channels of the outer acrosomal membrane that are opened by cAMP signaling, as indicated in bull spermatozoa (Spungin and Breitbart, 1996; Breitbart and Naor, 1999). In addition, it still remains unclear whether or not another Ca^{2+} channel (inositol 1,4,5-triphosphate receptor; Walensky and Snyder, 1995) on the outer acrosomal membrane could be involved in this process before the acrosome reaction occurs.

There are two main mechanisms for removing Ca^{2+} from the cytoplasm: both Ca^{2+}-ATPases and Na^{+}/Ca^{2+} exchangers pump cytoplasmic free Ca^{2+} to the external space or into the internal stores, including the endoplasmic reticulum and mitochondria (Berridge et al, 2000). Thapsigargin was reported as a specific inhibitor of endoplasmic reticulum Ca^{2+} pumps (Thastrup et al, 1990). For mammalian spermatozoa, this cell-permeable inhibitor raises the level of cytoplasmic free Ca^{2+} and promotes

the expression of fertilizing ability, including capacitation and the subsequent acrosome reaction (eg, Blackmore, 1993; Meizel and Turner, 1993; Parrish et al, 1999). In the present study, thapsigargin promoted head-to-head agglutination of boar spermatozoa in a concentration-dependent manner for concentrations up to 4 μM (Figure 2A). Thapsigargin (4 μM) was as effective at promoting sperm agglutination as cBiMPS (10 μM) was (Figure 2B). Moreover, the agglutination-promoting effect of thapsigargin (4 μM) was significantly enhanced by adding cBiMPS (10 μM; Figure 2B). As shown in Figure 3, the cytoplasmic free Ca^{2+} level was higher in the heads of thapsigargin-agglutinated spermatozoa. This increase was not likely to result from the entry of external Ca^{2+} because our mKRH was a Ca^{2+}-deficient medium (see ''Materials and Methods''). Moreover, indirect immunofluorescence revealed that acrosomal antigens were recognized by the PL/IM430 monoclonal antibody to Ca^{2+}-ATPases of endoplasmic reticulum-like intracellular membranes in human blood platelets (Figure 5). These results are consistent with the suggestion that thapsigargin-sensitive Ca^{2+}-ATPases suppress agglutination by removing cytoplasmic free Ca^{2+} and maintaining it at a low level in the cytoplasm. Spungin and Breitbart (1996) reported that the acrosomal membrane of bull spermatozoa possesses Ca^{2+} pumps that are inhibited by thapsigargin.

In conclusion, this report represents the first evidence that cytoplasmic free Ca^{2+} is involved in the head-to-head agglutination of mammalian spermatozoa. It also suggests that cytoplasmic free Ca^{2+} is released from the putative acrosomal store by the actions of cAMP signaling and is removed from the cytoplasm by the thapsigargin-sensitive Ca^{2+}-ATPases. Because agglutination seems to be associated with capacitation (Harayama et al, 1999, 2000), our present data could contribute to a disclosure of the unknown signaling cascades that lead to sperm capacitation.

Acknowledgment

We thank the staff of Hyogo Prefectural Agricultural Institute for their cooperation in sample collection.

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