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Modulation of the Function of Boar Spermatozoa via Adenosine and Fertilization Promoting Peptide Receptors Reduce the Incidence of Polyspermic Penetration into Porcine Oocytes[1]

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ABSTRACT

Effects of adenosine and pGlu-Glu-ProNH_2 (FPP) on the function and in vitro penetration of boar spermatozoa were examined. First, the effects of dibutyryl cAMP or agonists and antagonists of adenosine receptors (inhibitory adenosine receptors, A1AdR; stimulatory adenosine receptors, A2AdR) on freshly ejaculated spermatozoa were determined by chlortetracycline fluorescence assessment. Capacitation of spermatozoa was stimulated when they were cultured in a medium with dibutyryl cAMP, adenosine, A2AdR agonist, and adenosine plus A1AdR antagonist (CPT). However, acrosome reaction was inhibited only by adenosine. A1AdR agonist did not affect intact spermatozoa. A2AdR antagonist (DMPX) neutralized all of the effects of adenosine. Second, interaction of adenosine and FPP was examined. Gln-FPP, a competitive inhibitor of FPP, and DMPX inhibited the effects of adenosine and FPP, and CPT neutralized the inhibitory effect of FPP on acrosome reaction. Last, the effects of adenosine, FPP, and caffeine on the rate of sperm penetration were examined using frozen-thawed spermatozoa. Adenosine, FPP, and caffeine significantly enhanced the rate of sperm penetration as compared with the case of no additions. Caffeine treatment resulted in a high rate of polyspermic fertilization. In contrast, adenosine and FPP treatments resulted in an increased proportion of normal fertilization in in vitro-matured oocytes. These results suggest that boar spermatozoa can be modulated by the adenylyl cyclase/cAMP pathway via A2AdR in intact cells to induce capacitation and A1AdR in capacitated cells to inhibit spontaneous acrosome loss and that FPP receptors interact with A2AdR in intact cells and with A1AdR in capacitated cells. Furthermore, adenosine and FPP seem to be useful in reducing the incidence of polyspermic penetration.

fertilization, IVF/ART, sperm capacitation/acrosome reaction

INTRODUCTION

A majority of current in vitro fertilization (IVF) systems for porcine oocytes contain caffeine, an inhibitor of cyclic nucleotide phosphodiesterase, in fertilization media. Caffeine is known to increase the intracellular cAMP level [1], to induce capacitation and/or acrosome reaction of boar spermatozoa [2], and to result in an increased rate of sperm penetration into porcine oocytes in vitro [3]. In these IVF systems, however, persistent abnormally high incidence of polyspermic penetration has been observed (see reviews [4–7]). Recently, we have demonstrated that the replacement of caffeine with adenosine in fertilization media increased the incidence of monospermic penetration of porcine oocytes by frozen-thawed spermatozoa. Because adenosine is known to induce capacitation but inhibit spontaneous acrosome loss of mouse [8] and boar spermatozoa [2], the inhibition of a spontaneous acrosome reaction of boar spermatozoa in fertilization media appears to be an effective means to reduce the incidence of polyspermy.

In mammalian spermatozoa, the adenylyl cyclase/cAMP pathway is known to be important in the modulation of sperm capacitation [9–11]. In mice, not only adenosine but also fertilization-promoting peptide (pGlu-Glu-ProNH_2) has been shown to promote sperm capacitation and to inhibit spontaneous acrosome loss via separate and specific receptors to modulate the adenylyl cyclase/cAMP pathway [12, 13]. Mouse spermatozoa have inhibitory (A1) and stimulatory (A2) adenosine receptors (AdRs) [14, 15] and a putative FPP receptor [16]. In our recent study [2], both adenosine and FPP simulated capacitation but inhibited the acrosome reaction in a caffeine-free fertilization medium. However, the stimulatory effect was masked in the same medium when caffeine was used to supplement, suggesting that boar spermatozoa also have two types of AdRs. In the same experimental series [2], Gln-FPP, a competitive inhibitor of FPP in mouse spermatozoa [17], interfered with adenosine binding to AdRs, suggesting that FPP receptors and AdRs of boar spermatozoa are located near each other and somehow interact. Therefore, FPP, as well as adenosine, reduces the number of spermatozoa partially acrosome reacted and consequently may reduce the incidence of polyspermic penetration of porcine oocytes. In porcine spermatozoa, however, there is little evidence to show how sperm function is regulated by stimulation of AdRs and FPP receptors.

In the present study, we conducted experiments to clarify 1) the relation between sperm function and AdRs, 2) the interaction between AdRs and FPP receptors, and 3) the effect of FPP on the penetration of frozen-thawed boar spermatozoa. Here we show that FPP binds to the FPP receptors and interacts with the A2AdRs to induce capacitation and also with the A1AdRs to inhibit spontaneous acrosome reaction. We also demonstrated that the inclusion of FPP in fertilization media increased normal penetration of oocytes by frozen-thawed boar spermatozoa.

MATERIALS AND METHODS

Culture Media and Reagents

The medium used for the collection of oocyte-cumulus complexes and washing was modified Tyrode lactate-Hepes-

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polyvinyl alcohol (TL-HEPES-PVA) medium [2]. The basic maturation medium (OMM37) used was BSA-free North Carolina State University 37 medium [18] supplemented with 0.6 mM cysteine, 5 μg/ml insulin, and 10% (v/v) porcine follicular fluid [19]. The basic medium used for chlortetracycline (CTC) fluorescence assessment of spermatozoa was modified Medium 199 with Earle salts (mM199) supplemented with 3.05 mM D-glucose, 2.92 mM calcium lactate, 0.91 mM sodium pyruvate, 12.00 mM sorbitol, 0.4% (w/v) BSA (Fraction V), 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate. The basic medium used for IVF was caffeine-free modified Brackett & Oliphant solution (mBO) [20, 21]. All media were equilibrated at 39°C in an atmosphere of 5% CO_2 in air overnight prior to incubation of oocytes. Porcine follicular fluid was prepared from antral follicles (3 to 6 mm in diameter) as described previously [22].

Lyophilized FPP was prepared as described previously [23], with 10 nmol of peptide in each tube. Stock solutions were prepared by dissolving the contents of one tube in 1 ml of protein-free mM199 or mBO which was then mixed well by vortexing. The FPP stocks were divided into aliquots, frozen, and kept for up to 4 wk. Stocks were thawed and diluted with medium as required for use. Adenosine was prepared in protein-free mM199 or mBO and diluted with media as required.

Preparation and Culture of Cumulus-Oocyte Complexes

Ovaries were collected from prepubertal gilts at a local abattoir. Transportation of ovaries to the laboratory was carried out at 23–27°C. Cumulus-oocyte complexes were aspirated into a disposable 10-ml syringe from antral follicles (3 to 6 mm in diameter) on the surface of ovaries through an 18-gauge needle, washed three times with modified TL-HEPES-PVA medium, and then collected in 3 ml of fresh modified TL-HEPES-PVA medium. Fifty cumulus-oocyte complexes with uniform ooplasm and a compact cumulus cell mass were washed three times with OMM37 supplemented with 1 mM dibutyryl cAMP, 10 IU/ml eCG, and 10 IU/ml hCG, and subsequently cultured in 500 μl of the same medium covered with paraffin oil for 20 h at 39°C in an atmosphere of 5% CO_2 in air. The complexes were then transferred to 500 μl of OMM37 (without dibutyryl cAMP, eCG, and hCG) after washing three times with the same medium. The complexes were cultured for an additional 24 h [22, 24]. After culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase and washed carefully three times with mBO.

Preparation of Boar Spermatozoa

The sperm-rich fraction of semen was collected from three boars by the gloved-hand method and used as freshly collected sperm. Frozen boar semen was produced according to Niwa [25] and thawed at 39°C. These semen samples were washed three times with Dulbecco PBS supplemented with 0.1% BSA (Fraction V) by centrifugation at 1000 × *g* for 4 min each time to remove seminal plasma. At the end of washing, the pellets containing fresh and frozen-thawed spermatozoa were resuspended at 2×10^8 cells/ml in mM199 and mBO media, respectively.

Chlortetracycline Fluorescence Assessment of Spermatozoa

The suspensions of fresh spermatozoa were diluted to a concentration of 1×10^6 cells/ml in mM199 containing or not containing various supplements described below (in *Experimental Design*). Two-milliliter aliquots of sperm suspension were incubated in 5% CO_2 in air at 39°C for 2 h and then assessed as detailed below. The methods used for CTC analysis were essentially those described previously [2] with minor modification. Briefly, 8 μl of 100 μg/ml bisbenzimide (Hoechst 33258; Sigma Chemical Co., St. Louis, MO) in mM199 was added to 792 μl sperm suspension. After gentle mixing, each suspension was incubated for 3 min at room temperature in the dark, then layered onto 4 ml of 3% (w/v) polyvinylpyrolidone (average molecular weight: 40 000) in Dulbecco PBS (dPBS) and centrifuged at 1400 × *g* for 5 min. The pelleted spermatozoa were resuspended in 45 μl of BSA-saline solution, and 45 μl of this suspension was added to 45 μl of CTC solution, containing 750 μM CTC, 5 mM cysteine, 130 mM NaCl, and 20 mM Tris (pH 7.8). Sperm cells were fixed by adding 8 μl of 12.5% (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.4). The CTC solution was prepared daily. Ten microliters of the fixed sperm suspension was placed on a slide, and then one drop of 0.22 M 1,4-diazabicyclo[2.2.2]octane dissolved in glycerol:dPBS (9:1) was carefully mixed with the sperm suspension in order to retard the fading of fluorescence. The sperm suspension was covered with a coverslip and sealed with colorless nail varnish. Chlortetracycline staining patterns of spermatozoa were assessed under a phase-contrast microscope, equipped with epifluorescent optics, on the same day. Each cell was first observed under ultraviolet illumination (excitation at 330–380 nm; emission at 420 nm) to determine the live/dead status; the sperm cells showing bright blue staining of the nucleus were considered as dead and were not counted. One hundred live sperm were then examined under blue-violet illumination (excitation at 400–440 nm; emission at 470 nm) and classified according to CTC staining patterns. The three fluorescent staining patterns identified were as follows: F, with uniform fluorescence over the whole sperm head; B, with a fluorescence-free band in the postacrosome region; and AR, with almost no fluorescence over the sperm head except for a thin band of fluorescence in the equatorial segment.

In Vitro Fertilization and Assessment of Sperm Penetration

The sperm suspensions were diluted to various concentrations in mBO containing or not containing adenosine, FPP, or caffeine. Thirty denuded oocytes were cocultured with spermatozoa at various concentrations in 100-μl droplet of mBO under paraffin oil for 9 h at 39°C in an atmosphere of 5% CO_2 in air. At 9 h after insemination, the cultured eggs were fixed, stained with 1% (w/v) orcein, and examined under the phase-contrast microscope at 200× and 400× magnification. Oocytes were considered penetrated when they had at least one sperm head, a decondensed sperm nucleus, or a male pronucleus and corresponding sperm tail in the vitellus. Only oocytes containing male and female pronuclei with intact nuclear membrane were defined as having formed pronuclei.

Experimental Design

Experiment 1. To determine the relation between sperm characteristics and adenosine receptors, sperm responses to dibutyryl cAMP, adenosine, and A1 or A2 adenosine receptor (AdR) agonists were examined by analysis of CTC fluorescence patterns in freshly ejaculated boar spermatozoa. The suspensions of spermatozoa were cultured at a

concentration of 1×10^6 cells/ml in mM199 containing or not containing 1 mM dibutyryl cAMP, 100 nM adenosine, 100 nM 2-chloro-N^6-cyclopentyladenosine (CCPA, a highly selective A1AdR agonist), or 100 nM N^6-[2-(3,5-dimethoxyphenyl)-2-(2-methylphenyl)-ethyl]-adenosine (DPMA, a selective A2AdR agonist) in 5% CO_2 in air at 39°C for 2 h. After culture, spermatozoa were assessed for their functional status by using CTC fluorescence assessment. Effects of 100 nM 8-cyclopentyl-1,3-dimethylxanthine (CPT, a selective A1AdR antagonist) or 100 nM 3,7-dimethyl-1-propargylxanthine (DMPX, a selective A2AdR antagonist) in the presence of 100 nM adenosine in mM199 on sperm characteristics were also determined following culture in 5% CO_2 in air at 39°C for 2 h.

Experiment 2. To examine the interaction between FPP receptors and adenosine receptors, fresh boar spermatozoa were cultured in the absence and presence of 100 nM adenosine or 100 nM FPP alone or plus 100 nM Gln-FPP (pGlu-Gln-ProNH_2), a competitive inhibitor of FPP, in mM199 in 5% CO_2 in air at 39°C for 2 h. After culture, spermatozoa were assessed for their functional status by using CTC fluorescence assessment. Effects of 100 nM FPP on sperm response to absence and presence of 100 nM CPT (A1AdR antagonist) or 100 nM DMPX (A2AdR antagonist) in mM199 were also examined after culture in 5% CO_2 in air at 39°C for 2h.

Experiment 3. Sperm penetration in IVM oocytes in the presence of 100 nM FPP was compared with those in the absence or presence of 10 μM adenosine or 1 mM caffeine. The suspensions of frozen-thawed spermatozoa were diluted to a concentration of 2×10^6 cells/ml in mBO containing or not containing 1 mM caffeine or 10 μM adenosine. Fifty microliters of diluted sperm suspension was added to a 50-μl droplet of the same medium containing 30 denuded oocytes to adjust to a final sperm concentration of 1×10^6 cells/ml. Oocytes were cocultured with spermatozoa for 9 h at 39°C in an atmosphere of 5% CO_2 in air and then fixed for observation. Second, to determine the effect of sperm concentrations on sperm penetration into oocytes, the sperm suspensions were diluted to various concentrations (2×10^6, 1×10^7, 2×10^7, and 1×10^8 cells/ml) in mBO containing 100 nM FPP. Fifty microliters of diluted sperm suspension was added to a 50-μl droplet of the same medium containing 30 denuded oocytes to adjust final sperm concentrations to 1×10^6, 5×10^6, 1×10^7, and 5×10^7 cells/ml. Oocytes were cocultured with spermatozoa for 9 h at 39°C in an atmosphere of 5% CO_2 in air and then fixed for observation.

Chemicals

Medium 199 with Earle's salts was purchased from Gibco Laboratories, Life Technologies Inc. (Grand Island, NY). Agonists (CCPA and DPMA) and antagonists (CPT and DMPX) of AdR were purchased from Research Biochemicals International (Natick, MA). All other chemicals described here were purchased from Sigma Chemical Co.

Statistical Analysis

Statistical analyses of data from three to five replicate trials were made by analysis of variance (ANOVA) and Fisher's protected least significant difference test using the STATVIEW (Abacus Concepts, Inc., Berkeley, CA) program to determine treatment differences. All percentage data were subjected to arcsine transformation before statistical analysis. Data were expressed as mean ± SEM. Probability results of $P < 0.05$ were considered to be statistically significant.

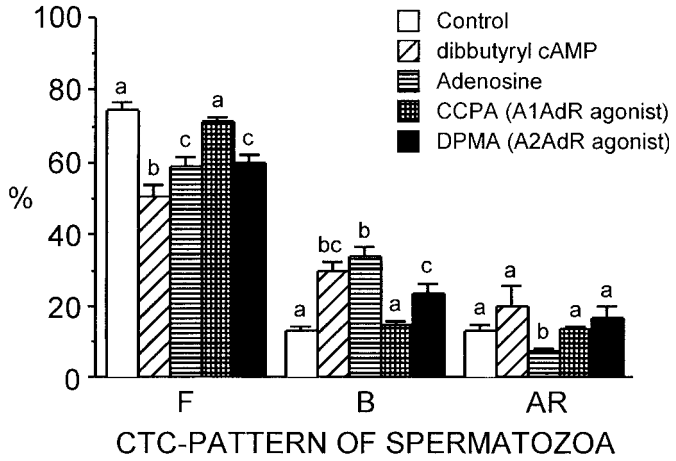

FIG. 1. Effects of dibutyryl cAMP, adenosine and A1 or A2 adenosine receptor (AdR) agonists on sperm characteristics as determined by analysis of chlortetracycline fluorescence patterns in fresh boar spermatozoa: F, intact (uncapacitated) cells; B, capacitated cells; AR, acrosome-reacted cells. Spermatozoa were cultured in the absence or presence of 1 mM dibutyryl cAMP, 100 nM adenosine, 100 nM CCPA (A1AdR agonist), or 100 nM DPMA (A2AdR agonist) in mM199 for 2 h. Different letters above the bars denote statistically significant differences (at least $P < 0.05$).

RESULTS

Response of Fresh Boar Spermatozoa to dbcAMP and Adenosine

Responses of fresh boar spermatozoa to dbcAMP, adenosine, CCPA (A1AdR agonist), and DPMA (A2AdR agonist) are shown in Figure 1. In the presence of dbcAMP, adenosine, and DPMA, capacitation of spermatozoa was induced, and the incidence of uncapacitated spermatozoa was reduced significantly 2 h after the start of culture. However, an acrosome reaction was inhibited in the presence of adenosine but not in the presence of dbcAMP or DPMA. CCPA did not affect the appearance of spermatozoa. Secondarily, sperm response to CPT (A1AdR antagonist) and DMPX (A2AdR antagonist) was examined in the presence of adenosine (Fig. 2). In the presence of adenosine alone or adenosine plus CPT, sperm capacitation was induced, and capacitated cells were spontaneously acrosome-reacted in the presence of CPT. Therefore, CPT neutralized the inhibitory effect of adenosine on spontaneous acrosome reaction but did not prevent the stimulatory effect on capacitation (Fig. 2). DMPX neutralized all of the effects of adenosine (Fig. 2).

Interaction Between Adenosine Receptors and FPP Receptors

First, the effect of Gln-FPP, which competitively inhibits responses of spermatozoa to FPP [17], on sperm response was determined in the presence of adenosine. Gln-FPP completely inhibited both the stimulatory effect of adenosine and FPP on capacitation and the inhibitory effect on spontaneous acrosome reaction (Fig. 3). Second, sperm response to CPT (A1AdR antagonist) and DMPX (A2AdR antagonist) was examined in the presence of FPP (Fig. 4). In the presence of FPP alone or FPP plus CPT, sperm capacitation was induced. However, the acrosome reaction was not prevented when CPT was supplemented. Therefore,

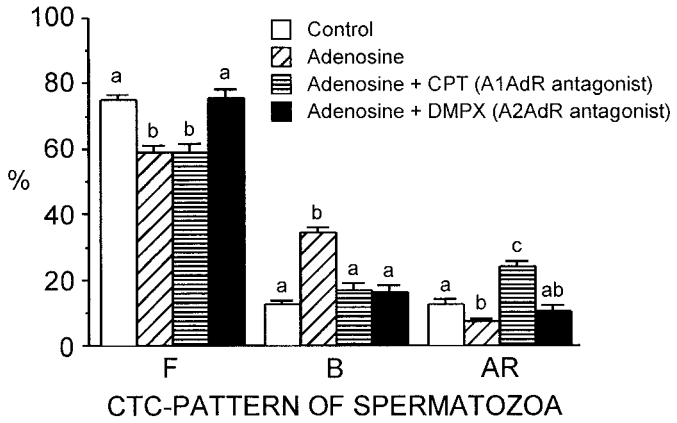

FIG. 2. Effects of A1 and A2 adenosine receptor (AdR) antagonists on sperm responses in the presence of adenosine, as determined by analysis of chlortetracycline fluorescence patterns in fresh boar spermatozoa: F, intact (uncapacitated) cells; B, capacitated cells; AR, acrosome-reacted cells. Suspensions were cultured in the absence or presence of 100 nM adenosine alone or plus 100 nM CPT (A1AdR antagonist) or 100 nM DMPX (A2AdR antagonist) in mM199 for 2 h. Different letters above the bars denote statistically significant differences (at least $P < 0.05$).

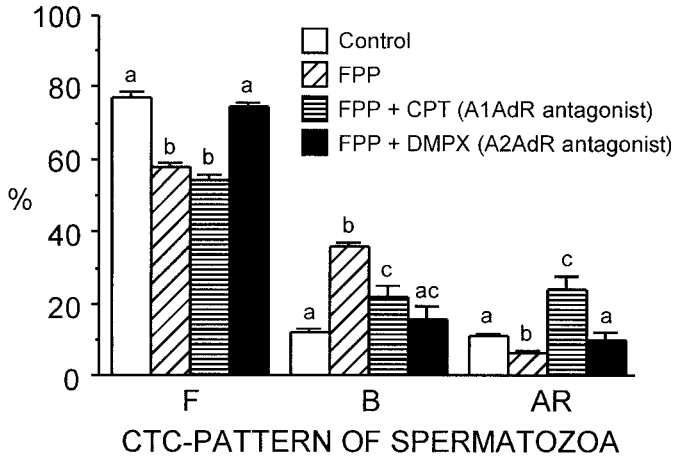

FIG. 4. Effects of A1 and A2 adenosine receptor (AdR) antagonists on sperm responses in the presence of FPP, as determined by analysis of chlortetracycline fluorescence patterns in fresh boar spermatozoa: F, intact (uncapacitated) cells; B, capacitated cells; AR, acrosome-reacted cells. Suspensions were cultured in the absence or presence of 100 nM FPP alone or plus 100 nM CPT (A1AdR antagonist) or 100 nM DMPX (A2AdR antagonist) in mM199 for 2 h. Different letters above the bars denote statistically significant differences (at least $P < 0.05$).

CPT neutralized the inhibitory effect of FPP on spontaneous acrosome reaction but not the stimulatory effect on capacitation (Fig. 4). DMPX neutralized all of the effects of FPP (Fig. 2).

Sperm Penetration in the Presence of FPP

Supplementation of the fertilization medium with adenosine, FPP, or caffeine significantly enhanced the sperm penetration rate of IVM oocytes (Table 1). In the presence of caffeine, both the rate of polyspermy and the mean number of spermatozoa in a penetrated oocyte were higher than other groups. However, adenosine and FPP did not affect those parameters as compared with controls. Consequently, the proportion of oocytes penetrated normally (percentage of monospermic oocytes in matured oocytes) was high when adenosine or FPP were supplemented (Fig. 5). In contrast, caffeine drastically reduced the proportion.

When sperm concentration was increased to 5×10^7 cells/ml in the presence of FPP, penetration rate significantly decreased to 11.1% (Table 2) compared with other groups (63.4–70.3%). Furthermore, both monospermic penetration rate and number of spermatozoa in a penetrated oocyte did not change with an increase in the sperm concentration (Table 2). Consequently, the proportion of normal fertilization in matured oocytes did not differ in the presence of FPP among sperm concentrations at 1×10^6 to 1×10^7 cells/ml, whereas the sperm penetration rate decreased significantly at 5×10^7 cells/ml (Fig. 6).

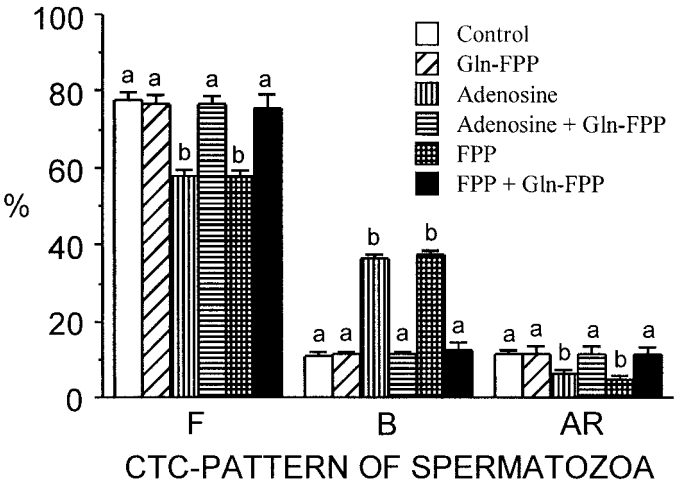

FIG. 3. Inhibitory effects of Gln-FPP on responses to adenosine and FPP as determined by analysis of chlortetracycline fluorescence patterns in fresh boar spermatozoa: F, intact (uncapacitated) cells; B, capacitated cells; AR, acrosome-reacted cells. Suspensions were cultured in the absence or presence of 100 nM adenosine and/or 100 nM Gln-FPP or of 100 nM FPP and/or 100 nM Gln-FPP in mM199 for 2 h. Different letters above the bars denote statistically significant differences (at least $P < 0.05$).

DISCUSSION

In the present study, dbcAMP stimulated capacitation of boar spermatozoa. This result shows that capacitation of

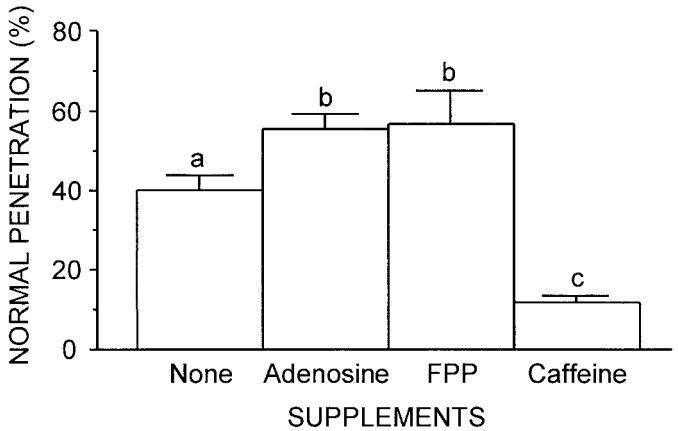

FIG. 5. Effect of adenosine, FPP and caffeine on the proportion of normal penetration. Porcine oocytes were cocultured with frozen-thawed boar spermatozoa at 5×10^5 cells/ml in mBO containing or not containing 10 μM adenosine, 100 nM FPP, or 1 mM caffeine for 9 h. Data are presented as mean % SEM of monospermic penetration in matured oocytes. Different letters above the bars denote statistically significant differences ($P < 0.05$).

TABLE 1. Effect of 10 μM adenosine, 100 nM FPP, or 1 mM caffeine on in vitro fertilization of porcine IVM oocytes in modified BO medium.[a]

Supplements	No. of oocytes examined	% (mean ± SEM) of oocytes			No. (mean ± SEM) of sperm in penetrated eggs
		Matured	Penetrated[b]	Monospermy[c]	
None	87	92.8 ± 1.8[d]	50.0 ± 1.1[d]	81.3 ± 7.5[d]	1.19 ± 0.08[d]
Adenosine	87	92.9 ± 1.0[d]	70.9 ± 3.3[e]	79.3 ± 5.6[d]	1.22 ± 0.07[d]
FPP	80	91.6 ± 2.7[d]	75.3 ± 8.2[e]	75.2 ± 3.3[d]	1.38 ± 0.06[d]
Caffeine	83	98.8 ± 1.2[e]	97.7 ± 1.2[f]	12.6 ± 1.5[e]	3.26 ± 0.06[e]

[a] Data from three replicated experiments.
[b] Percentage in oocytes matured.
[c] Percentage in oocytes penetrated.
[d,e,f] Values with different superscripts within each column are significantly different ($P < 0.05$).

boar spermatozoa is induced by an increased level of intracellular cAMP. In mouse spermatozoa, stimulation of the adenylyl cyclase/cAMP pathway results in an initiation of sperm capacitation [9–11]. Our current evidence is consistent with previous results reported for different species. Furthermore, adenosine has been shown to control the ability of mouse spermatozoa to penetrate oocytes via specific receptors that modulate the adenylyl cyclase/cAMP pathway [12, 13]. Recently, we have confirmed in boar spermatozoa that adenosine promoted capacitation but inhibited spontaneous acrosome loss [2]. In the present study, both A1AdR agonist and adenosine plus A2AdR antagonist did not affect the characteristics of spermatozoa. This evidence indicates that A1AdR does not function in intact boar spermatozoa. On the other hand, both A2AdR agonist and adenosine plus A1AdR antagonist stimulated sperm capacitation but did not inhibit the spontaneous acrosome reaction. Therefore, it was suggested that A2AdR functions in intact boar spermatozoa to stimulate the shift to capacitated status. Furthermore, considering the fact that adenosine alone inhibited spontaneous acrosome loss in capacitated cells but adenosine plus A1AdR antagonist did not, it also suggests that A1AdR functions in capacitated boar spermatozoa to inhibit spontaneous acrosome reaction. In our preliminary experiment, A3AdR (another adenosine receptor) antagonist (N^6-2-(4-aminophenyl)ethyladenosine) did not affect either intact or capacitated spermatozoa (unpublished results), suggesting that A3AdR does not appear to exist or function in boar spermatozoa. Thus, the current results demonstrate that capacitation of boar spermatozoa is modulated via at least two types of adenosine receptors, A1AdR and A2AdR. Adenosine appears to induce capacitation via A2AdR of intact cells and to inhibit spontaneous acrosome reaction via A1AdR in capacitated cells. Recently, Fraser and Adeoya-Osiguwa [13] demonstrated similar functional patterns of A1AdR and A2AdR in mouse spermatozoa. These regulatory mechanisms may be common to at least several mammalian species.

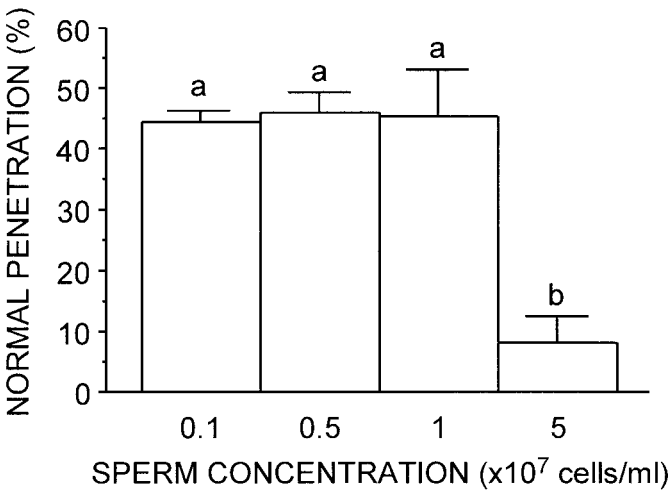

FIG. 6. Effect of sperm concentration on the proportion of oocytes penetrated normally in the presence of FPP. The incidence of monospermy in matured oocytes co-cultured with frozen-thawed boar spermatozoa at various concentrations in mBO containing 100 nM FPP for 9 h. Data are presented as mean % SEM of monospermic penetration in matured oocytes. Different letters above the bars denote statistically significant differences ($P < 0.05$).

FPP is a tripeptide found in the prostate gland and seminal plasma of several mammalian species, including rabbits [26], marmosets [27], humans, and various rodents [28, 29], as well as in the pituitary glands of birds and mammals [30–32]. In the pig, FPP has been found in the pituitary [30], but only very low picomolar levels could be detected in seminal plasma (see Funahashi et al. [2]). However, not only in mouse [23] and human [33] spermatozoa but also in boar spermatozoa [2], FPP, as well as adenosine, has been shown to stimulate capacitation but to inhibit acrosome reaction. Recently, a putative FPP receptor, the protein TCP-11 coded by a *t*-complex gene, was identified in mouse spermatozoa [16].

In the present study, performed with a caffeine-free medium, it was demonstrated that Gln-FPP, a competitive inhibitor of FPP in mouse spermatozoa [17], inhibited the stimulatory effect of adenosine, the FPP effect on capacitation, and the inhibitory effect of FPP on the spontaneous acrosome reaction. These results confirm that FPP receptors and AdRs of boar spermatozoa are located near each other and somehow interact. Furthermore, current results also demonstrated that A2AdR and A1AdR antagonists prevented the stimulatory effect of FPP on capacitation and inhibitory effect of FPP on spontaneous acrosome reaction, respectively. These results supply strong evidence suggesting that FPP receptors located near A2AdR interact with the adenosine receptor to induce capacitation in intact cells and that the FPP receptors near A1AdR interact to inhibit spontaneous acrosome loss in capacitated cells.

Adenosine and FPP have been known to stimulate in vitro fertilizing ability of spermatozoa in a few species (adenosine, mouse [14]; FPP, mouse [23]; and human [33]). The current results confirm our recent observation that adenosine in a fertilization medium not only increased the penetrability of frozen-thawed boar spermatozoa in vitro but also decreased the incidence of polyspermic penetration into porcine oocytes. Furthermore, we have shown here that supplementation of the fertilization medium with FPP also increased both the proportion of sperm penetration and the

TABLE 2. Effect of sperm concentration in fertilization media containing 100 nM FPP on in vitro fertilization of porcine oocytes.[a]

Sperm concentration ($\times 10^7$ cells/ml)	No. of oocytes examined	% (mean ± SEM) of oocytes			No. (mean ± SEM) of sperm in penetrated eggs
		Matured	Penetrated[b]	Monospermy[c]	
0.1	90	95.5 ± 2.9	63.4 ± 6.1[d]	72.9 ± 10.7[d]	1.34 ± 0.14
0.5	91	95.6 ± 2.2	68.8 ± 4.1[d]	68.2 ± 4.1[d]	1.41 ± 0.09
1.0	85	90.6 ± 4.3	70.3 ± 7.7[d]	75.3 ± 5.9[d]	1.29 ± 0.09
5.0	89	86.5 ± 3.9	11.1 ± 7.0[e]	95.2 ± 4.8[e]	1.05 ± 0.05

[a] Data from three replicated experiments.
[b] Percentage in oocytes matured.
[c] Percentage in oocytes penetrated.
[d,e] Values with different superscripts within each column are significantly different ($P < 0.05$).

proportion of oocytes penetrated normally. On the other hand, the caffeine that has been used in a majority of porcine IVF systems [3, 34–38] induced both capacitation and spontaneous acrosome reaction and increased not only the incidence of penetrated oocytes but also that of polyspermic penetration. Because in boar spermatozoa the sticky acrosomal matrix, proacrosine, has been considered to be a secondary ligand molecule that binds acrosome-reacted cells tightly to the matrix of the zona pellucida [39], not only capacitated cells but also recently or partially acrosome-reacted sperm appear to contribute to successful penetration. Results in the current study therefore support our hypothesis that a major cause of a high incidence of polyspermic penetration in porcine IVF systems, a persistent problem [7], is likely due to simultaneous penetration by a number of spermatozoa with induced acrosome reaction caused by the caffeine in fertilization medium. In the presence of not only adenosine but also FPP, the incidence of polyspermic penetration appears to be drastically reduced and allows for the acrosome reaction to be induced on the surface of the zone pellucida. Furthermore, in the presence of FPP, the proportion of normal fertilization in matured oocytes did not differ among relatively high sperm concentrations (1×10^6 to 1×10^7 cells/ml). Mechanisms to inhibit polyspermic penetration in the pig appear to require full induction of the acrosome reaction in capacitated spermatozoa at the zona pellucida level, even when a relatively large number of capacitated spermatozoa are bound to the surface. In the present study, however, the incidence of sperm penetration was drastically decreased when spermatozoa were inseminated at 5×10^7 cells/ml in the presence of FPP. In the present study, we could not clarify the reason. The presence of too many spermatozoa in the current IVF system may induce severe sperm agglutination that appears to be associated with capacitation [40], consequently resulting in failure of sperm penetration.

In conclusion, our present study demonstrates that capacitation of boar spermatozoa is modulated by the adenyl cyclase/cAMP pathway through at least two types of adenosine receptors, A2AdR in intact cell to induce capacitation and A1AdR in capacitated cells to inhibit spontaneous acrosome reaction. Furthermore, FPP receptors locating near A2AdR appear to interact with the adenosine receptor to induce capacitation in intact cells, and FPP receptors near A1AdR appear to interact to inhibit spontaneous acrosome loss in capacitated cells. Adenosine and FPP in fertilization medium increased the rate of sperm penetration and decreased the proportion of polyspermic oocytes. Use of adenosine and FPP instead of caffeine in fertilization media is recommended to reduce polyspermic penetration in porcine IVF systems.

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