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Ceramide Enhances Acrosomal Exocytosis Triggered by Calcium and the Calcium Ionophore A23187 in Boar Spermatozoa

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Abstract. Mammalian spermatozoa must undergo acrosomal exocytosis prior to penetration of the oocyte at fertilization. The mechanisms underlying acrosomal exocytosis have not yet been fully elucidated. This study explored the possible involvement of ceramide in exocytosis of the boar sperm acrosome. Ejaculated boar spermatozoa, stored with the Beltsville TS extender at 17°C for up to 3 days, were washed and preincubated for 10 min with C₂-ceramide, an analogue of endogenous ceramide, C₂-dihydroceramide (C₂-DH-ceramide), a negative control to C₂-ceramide, or with (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (D-erythro-MAPP), an inhibitor of alkaline ceramidase, followed by incubation and stimulation with 3 mM Ca²⁺ and 0.3 μM A23187 (Ca²⁺/A23187) at 37°C in air in a water bath. Spermatozoa fixed at specific intervals were examined, and the % of acrosomal exocytosis was monitored. Stimulation of spermatozoa with Ca²⁺/A23187 resulted in a time-dependent increase. There were no obvious changes at 5 min, but this was followed by a rapid increase at 10 min, reaching nearly a maximum level after 15 min or more of incubation. Preincubation with C₂-ceramide or D-erythro-MAPP enhanced acrosomal exocytosis triggered by Ca²⁺/A23187 in a dose-dependent manner, whereas C₂-DH-ceramide was without effect. These results suggest the possibility that ceramide may be involved in the mechanisms underlying acrosomal exocytosis.

Key words: Spermatozoa, Ceramide, Boar, A23187, Acrosomal exocytosis

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Mammalian spermatozoa undergo exocytosis of the acrosome, a single large granule, at fertilization in response to oocyte-derived stimuli, namely progesterone and zona pellucida glycoprotein [1, 2]. Acrosomal exocytosis can be induced *in vitro* by natural agonists but also by treatment with Ca²⁺ and the Ca²⁺ ionophore A23187

[3, 4].

Molecular mechanisms underlying acrosomal exocytosis, which ends in membrane fusion of the outer acrosomal membrane and the plasma membrane, have been extensively investigated. Studies have revealed that after Ca²⁺ entry, a variety of pathways are sequentially activated leading to membrane fusion. Polyphosphoinositide breakdown takes place very early following Ca²⁺ entry, thereby generating

diacylglycerol (DAG) [3, 5]. Two pathways have been suggested in the generation of DAG: one via the activation of phosphoinositide-specific phospholipase C (PIC) [3], and the other via phosphatidylcholine-specific phospholipase C [6]. DAG then stimulates phospholipase A₂ [7, 8], as such plays a second messenger role [9]. Fusigenic metabolites are generated by the action of PLA₂ to bring about membrane fusion. A product of PLA₂, lysophosphatidylcholine, mediates the final steps of membrane fusion in conjunction with rab3A protein, a small GTPase, via the cAMP/PKA pathway [10].

Another line of research has shown that a soluble NSF (N-ethyl-maleimide-sensitive fusion protein) attachment protein receptor (SNARE) may regulate membrane fusion [11]. v-SNARE is located on the vesicle membrane while t-SNARE is present on the plasma membrane [12]. Both combine to produce a complex for membrane fusion, and this mechanism has also been suggested as regulating membrane fusion in acrosomal exocytosis [13, 14].

Despite efforts to understand the molecular mechanisms underlying acrosomal exocytosis in mammalian spermatozoa, much remains to be clarified about the events leading to membrane fusion. The sphingomyelin cycle has been recognized as an important signalling pathway involved in many cell types and, upon activation, sphingomyelin is hydrolyzed into phosphorylcholine and ceramide, the latter playing an activator role [15, 16]. A messenger role for ceramide in cells activated by tumour necrosis factor (TNF)- α [17, 18], transforming growth factor- β [19], and 1 α ,25-dihydroxyvitamin D₃ [19] has been demonstrated. In human spermatozoa, the involvement of a sphingomyelin/ceramide pathway has been suggested as playing a role in the capacitating processes [21], and a neutral sphingomyelinase has been identified in ram spermatozoa [22]. However, the role of ceramide in the mechanism underlying acrosomal exocytosis has not yet been investigated. Improving our knowledge of the molecular mechanisms underlying acrosomal exocytosis in mammalian spermatozoa, which is an essential step to fertilization, would help to clarify the factors reducing reproductive competence or to solve problems associated with it.

Using boar spermatozoa as a model, this study examined the possible involvement of ceramide in

exocytosis of the sperm acrosome induced by Ca²⁺ and the Ca²⁺ ionophore A23187.

Materials and Methods

Collection of spermatozoa

The sperm-rich fraction of semen was collected by the gloved-hand method from a mature fertile Large White boar kept at the Swine Research Department of the Gifu Prefectural Livestock Research Institute, Gifu, Japan. Immediately after collection, sperm motility was assessed subjectively under a phase-contrast microscope. The % of motile spermatozoa was also subjectively evaluated, and the gross appearance of sperm motion (motility grade) was classified into 5 categories: – (all immotile), $\pm$ (motile without progression), + (progressively motile at low speed), ++ (progressively motile at moderate speed), +++ (progressively motile at the highest speed). Samples showing >95% motile spermatozoa and with a category of motility grade +++ were used. Sperm concentration was also estimated by photometry (Celltac; model MEK-5254, Nihon Kohden, Tokyo, Japan). Semen was then diluted with the Beltsville TS extender [23] to give a concentration of 1.5×10^8 /ml at room temperature. After dilution, the semen was protected from cold air, transported to the laboratory within 1.5 h, then gradually cooled in a refrigerator, and stored at 17°C for up to 72 h until use.

Media

Saline medium used for the washing and incubation of spermatozoa consisted of 142 mM NaCl, 2.5 mM KOH, 10 mM glucose, and 20 mM Hepes, adjusted to pH 7.55 at 20°C with NaOH [3]. Saline medium consisting of 222 mM sucrose in place of the NaCl was used for washing spermatozoa (sucrose medium; [3]). Both media contained 0.1 w/v% polyvinyl alcohol (average molecular weight 9,000–10,000; Aldrich Chemical Co., MO, USA), and 0.1 w/v% polyethylene glycol (Sigma Chemical Co., MO, USA). A stock solution of the calcium ionophore A23187 (Free acid; Calbiochem-Novabiochem Corp., CA, USA) was prepared at 30 μ M in dimethyl sulfoxide (DMSO) and diluted in saline medium to give a final concentration of 0.3 μ M. N-acetyl-D-sphingosine (C₂-ceramide; Sigma A7191), C₂-dihydro-ceramide

(C₂-DH-ceramide; Sigma C7980), and (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (D-erythro-MAPP; Biomol, SL-221) were dissolved in ethanol at 10 mM and stored at -30 C. After the solvent had been evaporated under a nitrogen gas stream at 40 C, they were dissolved in DMSO at 1 or 2.5 mM for use. C₂-ceramide, C₂-DH-ceramide or D-erythro-MAPP in DMSO was added to the saline medium with 3 mM CaCl₂ to give a final concentration of 10 or 25 μ M for sperm preincubation.

Preparation and incubation of spermatozoa

A portion of the stored semen (2.5 ml) was placed in a test tube and left standing for about 5 min to allow the sedimentation of large cell clumps. The supernatant containing spermatozoa was overlaid onto a sucrose medium and centrifuged at 125 g for 5 min, followed by 275 g for 7 min at room temperature. The supernatant was aspirated, and 200 μ l of the loose pellets were mixed with 2 ml of saline medium. Spermatozoa were then centrifuged at 275 g for 5 min, and resuspended in saline medium with 3 mM CaCl₂ for preincubation.

The suspended spermatozoa were pre-incubated at 37 C in air in a water bath for 10 min in the presence or absence of C₂-ceramide, C₂-DH-ceramide or D-erythro-MAPP, and further incubated with 0.3 μ M A23187 for stimulation under the same conditions for 0, 5, 10, 15 and 30 min, except for the experiments using D-erythro-MAPP, in which cells were incubated, for stimulation, for up to 15 min.

Sperm concentration during preincubation and incubation was $2-5 \times 10^7$ /ml.

Examination of acrosomal exocytosis, sperm motility and viability

At various intervals, subsamples were taken and fixed by mixing with an equal volume of 2% glutaraldehyde/0.165 M cacodylate buffer (0.165 M sodium cacodylate, pH 7.3). Spermatozoa were examined under a light microscope (400 $\times$), and acrosomal exocytosis was monitored as previously described [24]: spermatozoa showing a dense apical ridge of the head were considered as being acrosome-intact, and the others as having undergone acrosomal exocytosis.

Experiments were carried out 3 times each for C₂-ceramide, C₂-DH-ceramide and D-erythro-MAPP.

In order to check a non-specific effect of the

additives on spermatozoa, sperm samples incubated without A23187 were examined for the % of motile spermatozoa as described above and viable spermatozoa (% viability) by the eosin-nigrosin method immediately after the end of the incubation period. Sperm motility and viability were examined in each experiment except for D-erythro-MAPP, where the number of replicates was 2.

Statistical analyses

Results are presented as average $\pm$ standard error of the mean (SEM). Differences in the % of acrosomal exocytosis and that of viability were tested by Student's *t*-test, F-test or 2-factor analysis of variance (ANOVA). After the F-test, data between the two groups were compared by Duncan's multiple range test. Differences with $P < 0.05$ were regarded as statistically significant.

Results

Stimulation of spermatozoa with 3 mM Ca²⁺ and 0.3 μ M A23187 (Ca²⁺/A23187) resulted in a time-dependent increase in the % of acrosomal exocytosis. There were no obvious changes at 5 min, but this was followed by a rapid increase at 10 min, reaching nearly a maximum level at 15 min or more of incubation (Fig. 1). No major changes in this profile of response were noted after storage with BTS for different periods of time up to 3 days (data not shown). Preincubation of cells with C₂-ceramide enhanced the acrosomal exocytosis induced by Ca²⁺/A23187 in a dose-dependent manner (2-Factor ANOVA; time, $P < 0.001$, treatment, $P < 0.001$ among Ca²⁺/A23187, +10 μ M C₂-ceramide, and +25 μ M C₂-ceramide; time $\times$ treatment, $P = 1$). The % of acrosomal exocytosis was increased slightly but significantly by incubation without A23187 although it did not vary significantly among the different concentrations of C₂-ceramide (2-Factor ANOVA; time, $P < 0.006$; treatment, $P > 0.08$ among Ca²⁺ alone, Ca²⁺/10 μ M C₂-ceramide, and Ca²⁺/25 μ M C₂-ceramide; time $\times$ treatment, $P > 0.84$; Fig. 1). Sperm motility after the end of incubation was $95.0 \pm 0.0\%$ in each of Ca²⁺ alone, Ca²⁺/10 μ M ceramide, and Ca²⁺/25 μ M ceramide, and was not significantly different among those samples (One-way ANOVA; $P = 1$). Although sperm viability after the end of

Fig. 1. Changes in % of acrosomal exocytosis of boar spermatozoa stimulated with Ca^{2+} (3 mM) and A23187 (0.3 μM) after preincubation in the presence or absence of C_2 -ceramide (10 or 25 μM) for 10 min. Open circle, Ca^{2+} alone; open triangle, Ca^{2+} /10 μM C_2 -ceramide; open square, Ca^{2+} /25 μM C_2 -ceramide; closed circle, Ca^{2+} /A23187; closed triangle, +10 μM C_2 -ceramide; closed square, +25 μM C_2 -ceramide.

Fig. 2. Changes in % acrosomal exocytosis of boar spermatozoa stimulated with Ca^{2+} (3 mM) and A23187 (0.3 μM) after preincubation in the presence or absence of 10 μM C_2 -ceramide or C_2 -DH-ceramide for 10 min. Open circle, Ca^{2+} alone; open triangle, Ca^{2+} /10 μM C_2 -ceramide; open square, Ca^{2+} /10 μM C_2 -DH-ceramide; closed circle, Ca^{2+} /A23187; closed triangle, +10 μM C_2 -ceramide; closed square, +10 μM C_2 -DH-ceramide.

incubation did not differ between 0 μM ($85.3 \pm 2.2\%$) and 10 μM ($87.7 \pm 1.3\%$) C_2 -ceramide, the inclusion of 25 μM C_2 -ceramide significantly reduced sperm viability to $58.0 \pm 3.5\%$ (Duncan's multiple range test, $P < 0.01$). Thus the next experiment used C_2 -ceramide at 10 μM .

That experiment examined whether the effects of exogenous ceramide were specific. When spermatozoa were preincubated with 10 μM C_2 -DH-ceramide (a negative control to C_2 -ceramide) at the same concentration as C_2 -ceramide, no enhancement of acrosomal exocytosis induced by Ca^{2+} /A23187 was observed (2-Factor ANOVA; time, $P < 0.001$; treatment, $P > 0.09$ between Ca^{2+} /A23187 and +10 μM C_2 -DH-ceramide; time $\times$ treatment, $P > 0.35$), compared to that observed with 10 μM C_2 -ceramide (2-Factor ANOVA; time, $P < 0.001$; treatment, $P < 0.001$ between Ca^{2+} /A23187 and +10 μM C_2 -ceramide; time $\times$ treatment, $P < 0.001$). Incubation without A23187 in the presence of 10 μM C_2 -ceramide (2-Factor ANOVA; time, $P > 0.25$, treatment, $P > 0.34$, between Ca^{2+} alone and Ca^{2+} /10 μM C_2 -ceramide; time $\times$ treatment,

$P > 0.71$) or 10 μM C_2 -DH-ceramide (2-Factor ANOVA; time, $P > 0.19$; treatment, $P > 0.09$, between Ca^{2+} alone and Ca^{2+} /10 μM C_2 -DH-ceramide; time $\times$ treatment, $P > 0.70$) did not affect acrosomal exocytosis (Fig. 2). The % of sperm motility (F-test; $P = 1$) and viability (F-test; $P > 0.31$) after the end of incubation without A23187 was not significantly different among Ca^{2+} alone (motility, 93.3 ± 1.0 ; viability, $87.3 \pm 2.0\%$), Ca^{2+} /10 μM C_2 -ceramide (motility, 93.3 ± 1.0 ; viability, $82.0 \pm 2.5\%$), and Ca^{2+} /10 μM C_2 -DH-ceramide (motility, 93.3 ± 1.0 ; viability, $89.7 \pm 0.8\%$).

In order to examine whether ceramide itself or its metabolites is involved in the possible pathways, D-erythro-MAPP (an inhibitor of alkaline ceramidase which breaks down ceramide into sphingosine and fatty acid) was used to accumulate endogenous ceramide in spermatozoa after the beginning of stimulation. When spermatozoa were preincubated in the presence of D-erythro-MAPP, acrosomal exocytosis triggered by Ca^{2+} /A23187 was enhanced in a dose-dependent manner (Fig. 3; F-test; time, $P < 0.0002$; treatment, $P < 0.0002$ among

Fig. 3. Changes in % of acrosomal exocytosis of boar spermatozoa stimulated with Ca^{2+} (3 mM) and A23187 (0.3 μM) after preincubation in the presence or absence of D-erythro-MAPP (10 or 25 μM) for 10 min. Open circle, Ca^{2+} alone; open triangle, Ca^{2+} /10 μM D-erythro-MAPP; open square, Ca^{2+} /25 μM D-erythro-MAPP; closed circle, Ca^{2+} /A23187; closed triangle, +10 μM D-erythro-MAPP; closed square, +25 μM D-erythro-MAPP.

Ca^{2+} /A23187 and +10 μM and +25 μM D-erythro-MAPP; time $\times$ treatment, $P < 0.008$). Incubation with D-erythro-MAPP alone did not affect acrosomal exocytosis (2-Factor ANOVA; time, $P > 0.09$; treatment, $P > 0.32$; time $\times$ treatment $P > 0.47$). Sperm motility (F-test; $P > 0.46$) or viability (F-test; $P > 0.32$) immediately after the end of incubation without A23187 did not differ among the samples preincubated with Ca^{2+} alone (motility, $90.0 \pm 0.0\%$; viability, $83.0 \pm 1.4\%$), Ca^{2+} /10 μM D-erythro-MAPP (motility, $90.0 \pm 0.0\%$; viability, $83.0 \pm 1.4\%$) or Ca^{2+} /25 μM D-erythro-MAPP (motility, $87.5 \pm 1.2\%$; viability, $78.5 \pm 0.7\%$).

Discussion

The response of spermatozoa to the ionophore A23187 was similar after different times of storage up to 3 days (data not shown), therefore it appears that capacitation did not occur during storage. Protein tyrosine phosphorylation is an important mechanism related to boar sperm capacitation [25,

26], but it was considered that chelating extracellular calcium by the EDTA contained in BTS may have prevented tyrosine phosphorylation [27]. Recently, it has been reported that short-term storage with BTS keeps boar spermatozoa in a dormant state [28]. In this study the experiment was designed so that capacitation followed by acrosomal exocytosis was induced by the treatment with Ca and A23187 after storage.

The enhancement of acrosomal exocytosis by an exogenous ceramide analogue suggests that ceramide probably plays a role in acrosomal exocytosis. The specific action of exogenous ceramide on acrosomal exocytosis was supported by the observation that preincubation with C_2 -ceramide alone did not affect the % of acrosomal exocytosis during incubation without A23187. No significant changes in sperm motility by 10 or 25 μM C_2 -ceramide alone were observed, while viability (measured based on the eosin incorporation) was reduced at the higher concentration (25 μM) of C_2 -ceramide. The latter results were not in agreement with those of motility, which was not reduced at 25 μM . This discrepancy might have been due not to the influence on viability itself or to cytotoxicity as shown in HL-60 cells ($10 \mu\text{M} \leq [15]$), but rather to possible changes in membrane fluidity or the permeability of spermatozoa by the effect of 25 μM C_2 -ceramide [29]. The capacitating effect identified in human spermatozoa [21] might also explain this possible permeabilizing effect. The permeability of spermatozoa might have been increased at 25 μM of C_2 -ceramide, resulting in changes in the dye incorporation of plasma membrane without altering sperm motility.

C_2 -ceramide has been extensively used as an analogue to endogenous ceramide to examine signalling pathways, and C_2 -DH-ceramide serves as a negative control identical to C_2 -ceramide except in structures other than those lacking a trans 4–5 double bond in a sphingoid base. The specific action of exogenous C_2 -ceramide was confirmed in this study by the lack of enhancement after preincubation in the presence of C_2 -DH-ceramide, a finding in agreement with the report by Bielawska *et al.* [30].

The origin of ceramide after stimulation and its target were not determined in this study. Cross [21] showed that sphingomyelin may be degenerated with the concomitant generation of

ceramide during the capacitating processes of human spermatozoa, and suggested the role of ceramide in the capacitation of spermatozoa. Further studies will be required to determine ceramide formation and sphingomyelin degradation during sperm acrosomal exocytosis.

The observation that acrosomal exocytosis was enhanced by *D-erythro*-MAPP suggests that ceramide might be accumulated after stimulation and be further metabolized into sphingosine and free fatty acid by alkaline ceramidase in boar spermatozoa. Ceramides themselves, rather than their metabolites, might play a role in the sequence of events leading to membrane fusion, since, if the metabolites of ceramide were to have worked as signals, the reaction should have been inhibited by the action of *D-erythro*-MAPP [30]. Another explanation might be that the reduced generation of sphingosine by *D-erythro*-MAPP might have affected acrosomal exocytosis via a mechanism mediated by sphingosine, which has been recognized as a signaling molecule [30, 31]. The enhancement of acrosomal exocytosis by exogenous C_2 -ceramide together with endogenous C_2 -ceramide accumulated by the action of *D-erythro*-MAPP indicates that ceramide may be formed and may work as a signal during acrosomal exocytosis after stimulation with Ca^{2+} and A23187. Since no significant reduction in sperm motility and viability by C_2 -ceramide or *D-erythro*-MAPP were observed at the concentrations used (C_2 -ceramide, 10 μ M; *D-erythro*-MAPP, ≤ 25 μ M), it was considered that the enhancement of acrosomal exocytosis was not due to degenerative changes by cell death.

De novo synthesis and the hydrolysis of sphingomyelin by sphingomyelinase are two possible pathways leading to ceramide generation. In $TNF-\alpha$ signaling, *de novo* synthesis via serine palmitoyltransferase action has been demonstrated [32].

Another route to generate ceramide after the stimulation of cells is the hydrolysis of sphingomyelin, which is considered to be a major pathway in the sphingomyelin cycle to generate ceramide [16]. Arachidonic acid generated by PLA_2 action has been shown to be a mediator of sphingomyelin hydrolysis induced by $TNF-\alpha$ in

human leukemia-derived HL-60 cells [33, 34]. It has also been demonstrated that, in L929 cells stimulated with $TNF-\alpha$, arachidonic acid generated by the action of cytosolic PLA_2 causes glutathione depletion followed by sphingomyelinase activation [18]. Although in boar spermatozoa, no decrease in the levels of sphingomyelin during capacitating processes was found [35], further studies will be required to examine the hydrolysis of sphingomyelin after stimulation for acrosomal exocytosis.

In ram spermatozoa stimulated with Ca^{2+} /A23187 for acrosomal exocytosis, diacylglycerol generated via PIC and PLC activation activates PLA_2 to form lysophosphatidylcholine and arachidonic acid [7, 8], though the link between arachidonic acid generation and sphingomyelin breakdown in spermatozoa has yet to be established.

On the other hand, the effectors of ceramide have been investigated in somatic cells. Some of the suggested effectors include ceramide-activated protein kinases such as p42/p44 mitogen-activated protein kinase and c-Jun N-terminal-directed protein kinase [36] as well as ceramide-activated protein phosphatases [16, 37, 38] and caspases [16]. Sphingomyelin degradation by sphingomyelinase action is followed by secretory PLA_2 activation in MV1Lu cells [39], and PLA_2 has also been suggested as a target of ceramide [37, 40]. The ceramide effectors mediating acrosomal exocytosis have not yet been investigated.

The present study suggests the possibility that ceramide may mediate acrosomal exocytosis in boar spermatozoa stimulated with Ca^{2+} /A23187, and that ceramide itself, rather than its metabolites, may play a role in the sequence of events leading to membrane fusion. Further investigations are necessary to identify the formation of ceramides upon stimulation of spermatozoa.

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