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Calcium-Independent Acrosome Reaction by Methyl Beta Cyclodextrin in Mouse Epididymal Sperm *In Vitro*

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생쥐 부정소 정자의 첨체반응 유도의 Calcium 비의존성

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ABSTRACT : Sperm capacitation and acrosome reaction (AR) have been known to be Ca^{2+}-dependent events. Sperm capacitation accompanies with cholesterol efflux from plasma membrane, that eventually stimulates AR. However, whether the AR mediated by cholesterol efflux is Ca^{2+} dependent has not been verified yet. Recently, methyl beta cyclodextrin (MBCD) was found to evoke AR by stimulating the cholesterol efflux from sperm membrane. In the present study, we examined the requirement of Ca^{2+} in the MBCD-induced AR. During incubation of sperm in the bicarbonate buffered media, MBCD increased AR in a dose-dependent manner regardless of the Ca^{2+} presence. In the presence of low molar concentration of Ca^{2+} (100 μM), MBCD-induced AR was slightly increased compared to Ca^{2+}-free condition. In the absence of Ca^{2+} supplement, spontaneous AR was slightly increased during the incubation but inhibited by 100 μM EGTA. MBCD potentiated AR even the presence of EGTA. However, EGTA attenuated MBCD -induced AR, suggesting the functional involvement of intracellular Ca^{2+} in the MBCD-induced AR. Taken together, it was suggested that cholesterol efflux from the sperm plasma membrane was sufficient for induction of AR even in the absence of extracellular Ca^{2+} and that a condition permissive for mobilization of intracellular Ca^{2+} is important for MBCD-induced AR.

Key words : Cholesterol, Cyclodextrin, Ca^{2+}, Acrosome Reaction, Spermatozoa, Mouse.

요 약 : 정자의 수정능력획득과 첨체반응은 Ca^{2+}에 의존적으로 일어나며, 수정능력획득 과정에서는 원형질막으로부터 cholesterol이 방출되어 첨체반응이 일어나기 쉬운 상태로 전환된다. 최근 세포막으로부터 cholesterol 방출을 촉진하는 methyl beta cyclodextrin (MBCD)이 첨체반응을 유발함이 알려졌으나 정자 주변의 Ca^{2+} 농도와 관계없이 cholesterol 방출만으로 첨체반응이 유발되는지의 여부는 확인되지 않았다. 본 연구에서는 생쥐 정자에서 MBCD에 의한 첨체반응의 Ca^{2+} 의존성을 조사하였다. 첨가된 Ca^{2+}이 없는 경우에도 MBCD는 농도 의존적으로 첨체반응을 증가시켰다. 저농도 (100 μM)의 Ca^{2+}의 존재 시 MBCD에 의한 첨체반응이 유의하게 증가하였다. Ca^{2+}을 첨가하지 않은 배양액에 100 μM의 EGTA를 첨가한 경우 자발적 첨체반응을 유의하게 억제되었다. 같은 조건하에서 1 mM MBCD는 첨체반응을 유의하게 증가시켰으나 EGTA 비처리군보다 유의하게 낮아 MBCD에 의해 유발되는 첨체반응에 정자내부의 Ca^{2+}이 관여하는 것으로 사료된다. 이상의 결과에서 외부의 Ca^{2+} 이 존재하지 않더라도 MBCD를 이용하여 첨체반응을 유발할 수 있으며 정자 내부의 Ca^{2+}이 원형질막 cholesterol의 방출에 따른 첨체반응 조절에 관여함을 알 수 있다.

INTRODUCTION

In mammalian fertilization, sperm undergoes physiological changes called "capacitation" in the female genital tracts or *in vitro* (Austin, 1951; Chang, 1951). Acrosome reaction (AR), a Ca^{2+}-dependent exocytotic process, occurs just prior for capacitated sperm to penetrate zona pellucidae of the oocyte (Yanagimachi, 1994). Sperm capacitation and AR are regulated by numerous molecules present in female genital tracts, and these extracellular signals generate various intracellular chemical messengers (Ward and Kopf, 1993).

Cholesterol has widespread effects on the behavior of lipid molecules in cell membranes. Among the membrane lipid, chole-

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sterol increases rigidity of the plasma membrane, and reversibly inhibits sperm fertilizing ability (Davis, 1980; Fayrer-Hosken et al., 1987; Visconti et al., 1999). In spermatozoa, changes in the distribution of filipin-sterol complex were reported during maturation and capacitataion (Seki et al., 1992; Lopez and de Souza, 1991; Lin & Kan, 1996; James et al., 1999). Sperm capacitation accompanies with cholesterol efflux and the changes in the phospholipid components in the sperm plasma membrane. Following capacitation, sperm plasma membrane become to be prone to fuse with outer acrosomal membrane (Langlais and Roberts, 1985; Suzuki and Yanagimachi, 1989). Recently, methylbetacyclodextrin (MBCD) which stimulates cholesterol efflux from cell membrane was found to induce capacitation of mouse spermatozoa without loss in the phospholipid content and increase AR (Choi & Toyoda, 1998; Iborra et al., 2000). However, whether the manipulation of AR by stimulating the cholesterol efflux from the plasma membrane was dependent on the extra- or intracellular Ca^{2+} has not been clarified yet. In the present study, we examined the requirement of Ca^{2+} in the MBCD-induced AR of mouse epididymal spermatozoa.

MATERIALS AND METHODS

1. Chemicals

All chemicals used in this study were purchased from Sigma.

2. Sperm preparation and incubation

Cauda epididymis was removed from 8-wk-old ICR mice and placed in Ca^{2+} -free human tubal fluid medium (HTF, Quinn et al., 1985). Luminal content was released by gentle squeezing and incubated for 10 min at room temperature. Motile spermatozoa were collected and sperm concentration was adjusted to 1×10^6 sperm/ml with fresh medium. Aliquots of sperm suspension were placed in 4-well culture dish. Treatment of MBCD (final concentration 0, 0.1, 1, and 10 mM) and $CaCl_2$ (final concentration 0, 100 μM, and 1,71 mM) was followed by incubation under 5% CO_2 in air. To chealate the intracellular Ca^{2+} in the Ca^{2+}-free condition, ethylene glycol-bis (B aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA, 100 μM) treatment was done.

3. Evaluation of acrosome reaction

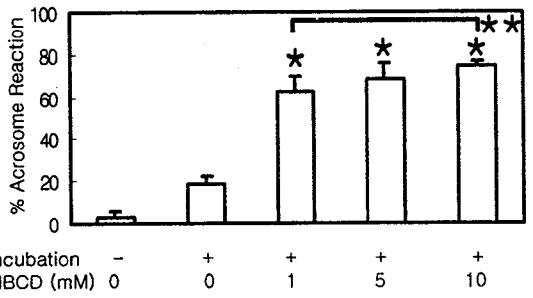

Fig. 1. Microphotographs of mouse spermatozoa stained with Coomassie brilliant blue G250. A, Freshly prepared cauda epididymal spermatozoa. Most of spermatozoa had intact acrosome stained by dye (arrowheads); B, Spermatozoa following the MBCD treatment. Most of spermatozoa lost their acrosome. Acrosome region of sperm head was free from staining (arrows). Bar = 50 μm.

For the staining of acrosome, previously reported method of Moller et al. (1990) was partially modified. After incubation, sperm suspension was directly fixed with 10 volumes of 5% formaldehyde in phosphate buffered saline (PBS) for 30min. After centrifugation at 1,000g for 10min, sperm pellet was washed with PBS. A drop of sperm suspension was smeared on the slide and air-dried. After drying, the slide was stained with protein assay reagent (Bio-rad) for 2 min. After washing twice in PBS, the slide was dehydrated with graded ethanol and permanently mounted. Absence of blue stain on the acrosome was regarded as sign of AR (Fig. 1). The AR rate ((acrosome -reacted sperm/ total sperm counted) × 100) was obtained by counting more than 200 sperm per slide and statistical significance was analyzed by Student's t-test.

RESULTS

1. Effect of extracellular Ca^{2+} on the AR by MBCD

During incubation of sperm in the bicarbonate buffered media containing physiological concentration of Ca^{2+} (1.71 mM), MBCD increased AR in dose-dependent manner (Fig. 2). In the absence of Ca^{2+}supplement, spontaneous AR was slightly increased during the incubation and MBCD increased AR in dose-dependent manner (Fig. 3).

In the low-Ca^{2+} (100 μM) media, AR slightly but significantly increased according to incubation (Fig. 4). MBCD (1mM) potentiated AR regardless of the presence of Ca^{2+} but MBCD with Ca^{2+} (100 μM) was more effective than MBCD alone (Fig. 4).

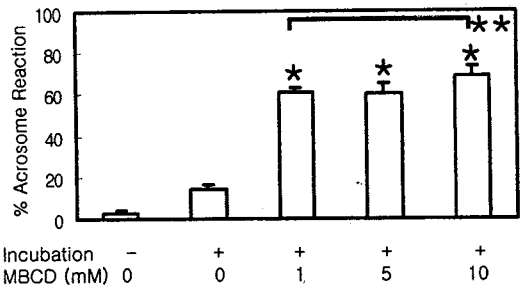

Fig. 2. Effect of MBCD on the acrosome reaction (AR) of mouse epididymal spermatozoa in the presence of physiological concentration of calcium. Metyl beta cyclodextrin (MBCD) was added to sperm suspension at 0, 0.1, 1, and 10 μM in the presence of 1.71 mM $CaCl_2$. AR was evaluated after incubation for 60 min. The difference in AR rate was analyzed by Student's t-test. * and **, significantly different from MBCD-free control and 1mM MBCD, respectively ($p< 0.05$). Error bar = SD (n=4).

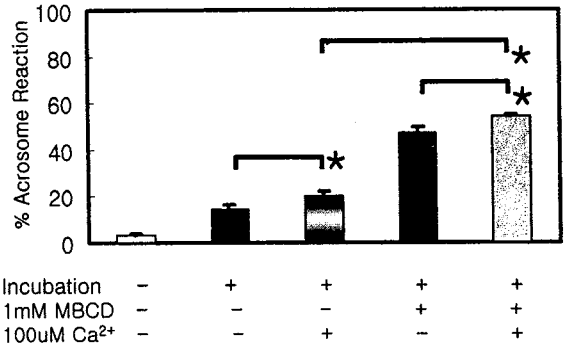

Fig. 3. Effect of MBCD on the acrosome reaction (AR) of mouse epididymal spermatozoa in the absence of calcium. Metyl beta cyclodextrin (MBCD) was added to sperm suspension at 0, 0.1, 1, and 10 μM in the absence of $CaCl_2$. AR was evaluated after incubation for 60 min. The difference in AR rate was analyzed by Student's t-test. * and **, significantly different from MBCD-free control and 1mM MBCD, respectively ($p<0.05$). Error bar = SD (n=4).

2. Effect of EGTA on the AR by MBCD

In the Ca^{2+}-free media, AR was monitored in the presence or absence of EGTA (100 μM) to chealate minute contamination of Ca^{2+}. EGTA significantly inhibited the spontaneous AR in the absence of Ca^{2+} supplement, and EGTA attenuated MBCD -induced AR (Fig. 5).

DISCUSSION

Sperm capacitation and AR have been known to be highly

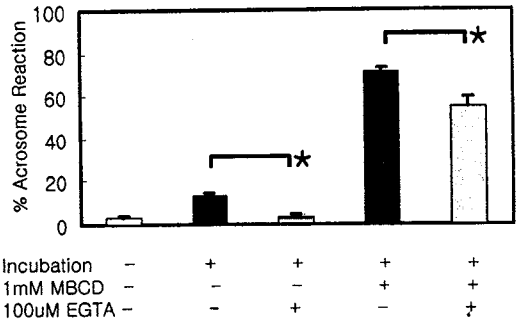

Fig. 4. Effect of MBCD on the acrosome reaction (AR) of mouse epididymal spermatozoa in the low molar concentration of calcium. Metyl beta cyclodextrin (MBCD) was added to sperm suspension at 1mM in the presence or absence of 100 μM $CaCl_2$ for 60 min. The difference in AR rate was analyzed by Student's t-test. Asterisks indicate mean values significantly different from MBCD-free control ($p< 0.05$). Error bar = SD (n=4).

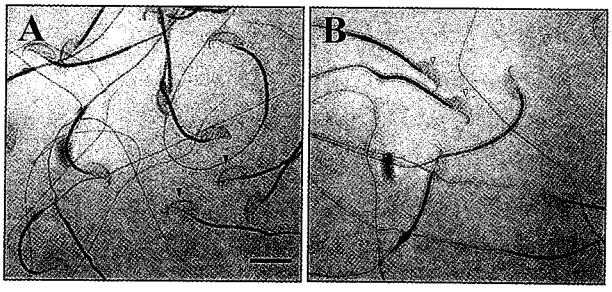

Fig. 5. Effect of MBCD on the acrosome reaction (AR) of mouse epididymal spermatozoa in the presence of EGTA. Mouse epididymal spermatozoa were incubated in the presence or absence of 100 μM $CaCl_2$ for 60 min. The difference in AR rate was analyzed by Student's t-test.. Asterisks indicate mean values significantly different from drug-free control ($p<0.05$). Error bar = SD (n=4).

dependent on extracellular Ca^{2+} (Fraser, 1987). In our experiment, even the absence of added Ca^{2+}, some population of spermatozoa underwent spontaneous AR (Fig. 3). In the presence of low concentration of Ca^{2+}, some population of spermatozoa also underwent spontaneous AR (Fig. 4). Previously, it was reported that minimal concentration of Ca^{2+} required for capacitation was not higher than 100 μM and furthermore some of the capacitated sperm spontaneously underwent AR *in vitro* (Fraser, 1987). So Ca^{2+} requirement of spontaneous AR might be different from those of AR induced by physiological ligands such as zona pellucida glycoprotein 3 (ZP3) and progesterone. The inclusion of 100 μM EGTA which is sufficient to chealate both intracellular Ca^{2+} and minute amount of Ca^{2+} possibly

derived from epididymal luminal fluid, could be effective for inhibition of spontaneous AR (Fig. 5). It suggested that Ca^{2+} is indispensible for the capacitation and spontaneous AR. Recently Kim et al. (2001) demonstrated that the process of AR consists of transitional intermediates may have significant functions in the fertilization process. Therefore it is tempting to speculate that Ca^{2+} requirement necessary for initial event of acrosomal exocytosis would be relatively lower than that for the complete AR.

MBCD increased AR in dose-dependent manner regardless of the Ca^{2+} supplement (Figs. 2 and 3). It suggested that cholesterol efflux was sufficient for spontaneous AR. However, in the presence of low molar concentration of Ca^{2+}, AR evoked by MBCD was potentiated (Fig. 4). EGTA (100 μM) significantly inhibited the spontaneous AR and attenuated MBCD-induced AR in the absence of Ca^{2+} supplement, suggesting that the might be permissive to intracellular Ca^{2+} mobilization and was positive to MBCD-induced AR. It suggested that Ca^{2+} concentration sufficient to support sperm capacitation was beneficial but not the absolute requirement for MBCD-induced AR. It also suggested that physiochemical changes in lipid composition result from cholesterol efflux in the sperm plasma membrane should have changed the cell signaling culminating to acrosomal exocytosis. Recently, the membrane domain rich in cholesterol called "rafts" was found in plasma membrane and cholesterol efflux changes cell signaling through the protein complexes in rafts (Janes et al., 2000; Kabouridis et al., 2000). In mouse spermatozoa, cholesterol efflux increases protein tyrosine phosphorylation during capacitation (Visconti et al., 1999). The changes in cell signaling by cholesterol efflux should be verified to understand MBCD induced AR. Taken together, it was suggested that cholesterol efflux from sperm plasma membrane was sufficient for induction of spontaneous AR even the absence of Ca^{2+} and that both extra- and intracellular Ca^{2+} was stimulatory but not absolute for MBCD-induced AR.

In summary, it was found that cholesterol efflux was sufficient for AR and that the Ca^{2+} requirement of MBCD -induced AR was quite smaller than those of the AR by physiological ligands in mouse spermatozoa. We suggested that AR driven by cholesterol efflux occurred by means of mechanism somewhat different from physiological AR and that MBCD supplementation would be helpful for induction of sperm capacitation and AR *in vitro*.

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