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Effect of Dimethyl Amiloride on the Acrosome Reaction in Mouse Epididymal Sperm *in vitro*

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생쥐 정자의 첨체반응에 미치는 Dimethyl Amiloride의 영향

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ABSTRACT : The possible role of Na^+/H^+ antiporter in both the capacitation and the acrosome reaction (AR) was examined in mouse epididymal spermatozoa. Spontaneous acrosome reaction was inhibited by dimethyl amiloride (DMA), a specific inhibitor of Na^+/H^+ antiporter, with dose dependent manner. Follicular fluid- or A23187-induced acrosome reaction was not inhibited by DMA. It suggests that change in pH_i by monovalent cation transport through the Na^+/H^+ antiporter is possibly engaged in the capacitation and that agonist- as well as A23187-induced AR in capacitated sperm might be independent from the Na^+/H^+ antiporter. Conclusively, changes in pH_i through the Na^+/H^+ antiporter might be important for sperm capacitation and it virtually occurs upstream of the Ca^{2+} influx which precedes the acrosome reaction in mouse epididymal spermatozoa.

Key words : Dimethyl amiloride, Na^+/H^+ antiporter, Capacitation, Acrosome reaction, Mouse sperm.

요 약 : 생쥐 정자의 수정능력획득과 첨체반응에 작용하는 Na^+/H^+ antiporter의 역할을 조사하고자 하였다. Na^+/H^+ antiporter를 특이적으로 억제하는 dimethyl amiloride는 정자의 자발적인 첨체반응을 농도 의존적으로 억제한 반면 난포액 및 calcium ionophore인 A23187에 의해 유도된 첨체반응은 억제하지 못하였다. 이러한 결과는 정자내 Na^+/H^+ antiporter에 의한 1가이온의 출입과 이에 따른 세포질내 pH 조절이 정자의 수정능력 획득과 자발적인 첨체반응에 조절요인으로 작용함을 암시한다. 수정능력을 획득한 정자에서 난포액 등의 agonist 또는 A23187에 의해 유도되는 첨체반응은 Na^+/H^+ antiporter와는 무관하게 진행되는 것으로 사료된다.

INTRODUCTION

Sperm activation prior to fertilization has many similarities to intercellular and intracellular signaling systems utilized by somatic cells (Ward & Kopf, 1993). During the transit through the female genital tracts, mammalian sperm obtain the ability to fertilize the egg, which is called sperm capacitation (Chang, 1951). Acrosome reaction (AR) of sperm, a Ca^{2+}-dependent exocytotic event is essential for sperm penetration of egg's extracellular matrix (Yanagimachi, 1994). There is an increase of intracellular Ca^{2+} at the end of capacitation, but large influx of extracellular Ca^{2+} is still required for AR (Fraser, 1987). Evidences obtained with a natural agonist for AR such as ZP3 indicate that several steps occur, with sperm-agonist interaction and activation of calcium channels not being linked directly but separated by intervenening interactions (Florman et al., 1992). Increase of pH_i by H^+ efflux is supposed to occur prior to Ca^{2+} influx by the ZP3 in mouse sperm (Lee & Storey, 1985; Florman et al., 1989). Calcium channels appear to play a fundamental role in triggering acrosomal exocytosis but the signalling linked to change in intracellular pH (pH_i), increase in $[Ca^{2+}]_i$, and AR have not been fully clarified in mammals.

pH_i is an important factor regulating sperm motility and protein phosphorylation (Tash & Means, 1983; Carr & Acott, 1989; Aitken et al., 1995), and control of acrosomal content stasis and release during AR of guinea pig sperm (Huang, 1985). pH_i rises during *in vitro* incubation of sperm (bovine, Parrish et al., 1989, 1994; mouse Lee & Storey, 1985). One of the prerequisites for the occurence of the AR is the alkalinization of the cytosol (Working & Meizel, 1983; Lee & Storey, 1989; Florman et al., 1989). Na^+/H^+

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antiporter is important for intracellular pH homeostasis and mediates various cellular responses dependent on intracellular pH (Moolenaar et al., 1983). So far, amiloride derivatives were widely used for elucidation of ion transport across the cell membrane (Kleyman & Cragoe, 1988). Previously it was reported that some analogues of amiloride increased spontaneous AR in mouse sperm (Fraser et al., 1993) and stimulated Ca^{2+} uptake into epididymal bull spermatozoa (Breitbart et al., 1990). But the amiloride used in the previous studies was known to inhibit other ion-transporting machineries such as Na^+/K^+-ATPase, Ca^{2+}-ATPase, and Na^+ channel as well Na^+/H^+ antiporter (Meng & Pierce, 1990; 1991). Therefore specific inhibitor of Na^+/H^+ antiporter is required to verify the precise role of Na^+/H^+ antiporter during capacitation and AR. Present study aimes to verify the involvement of Na^+/H^+ antiporter during the capacitation and AR of mouse sperm. We examined the effect of a dimethyl amiloride, a specific inhibitor of Na^+/H^+ antiporter (Meng & Pierce, 1990; 1991) on spontaneous AR. To examine the involvement of this ion transporter during the AR evoked by agonists human follicular fluid (hFF)-induced AR was monitored. In addition, A23187-induced AR was examined in the presence of DMA to examine whether the blocking of Na^+/H^+ antiporter can affect acrosomal exocytosis after the Ca^{2+} influx.

MATERIALS AND METHODS

1. Chemicals

All chemicals were of the highest purity available commercially. Dimethyl amiloride was purchased from Research Biochemical International (RBI). Others were obtained from Sigma.

2. Follicular fluids

Human follicular fluids (hFF) were collected from the aspirate of preovulating follicles from women during the IVF program. hFF showing red color were discarded to avoid blood contamination. AR inducing activities of hFF were pretested in mouse sperm and hFF that showed higher AR inducting activity were pooled and stored frozen at −20℃.

3. Sperm preparation

Cauda epididymis were removed from 3 months-old male mouse (ICR strain). Dissected epididymis were squeezed in modified Tyrode solution (Parrish et al., 1988) and sperm suspension was collected after 10 min. Sperm concentration was adjusted to 1×10^6 sperm/ml with fresh medium and 1 ml of aliqouts were incubated for 120 min under 5% CO_2, 95% air. To induce AR, human follicular fluid (10 %, v/v) or Ca^{2+} ionophore, A23187 (10 μM in 0.1 % DMSO) was added to sperm suspension and sperm suspension were incubated for further 60 min.

4. Treatment of dimethyl amiloride (DMA)

One hundred mM DMA stock solution was prepared in DW and serially diluted with modified Tyrode solution. DMA was added to sperm suspension with conc. of 0.1 ~100 μM for 90 min or primed to sperm suspension 5 min before the induction of AR by hFF or A23187.

5. Evaluation of acrosome reaction

To examine the occurrence of acrosome reaction, aliquotes of sperm suspension were collected every 60 min during the incubation. Acrosome stain was conducted according to Moller et al (1990) with some modification. Sperm were fixed with 5 % formaldehyde in PBS for 30 min and centrifuged at 2,000 g for 1 min. Sperm pellet was washed with phosphate buffered saline (PBS, pH 7.4) twice. One drop of sperm suspension was applied to slide and air dried. Sperm were stained with commercial protein assay reagent (Bio-rad) for 2 min. Slide were rinsed by dipping for 1 min twice with PBS and mounted with 50 % glycerol in PBS. Sperm showing no blue staining on the apical segment of sperm head were counted as acrosome-reacted sperm (Fig. 1). More than 200 sperm were counted per slide and AR rate was statistically analyzed using Student's *t*-test.

RESULTS

1. Effect of DMA on spontaneous acrosome reaction

Outer arch region of sperm head of acrosome-intact sperm was stained by Coomassie dye but acrosome-reacted

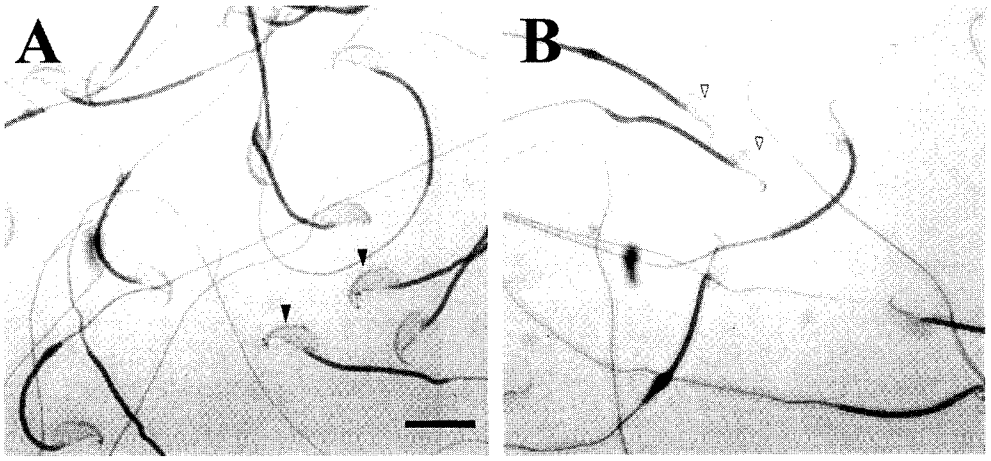

Fig. 1. Microphotographs of mouse spermatozoa stained by Coomassie brilliant blue. (A) Mouse sperm released from epididymis. Most of sperm have intact acrosome. Apical portion of sperm head is stained by dye (filled arrowhead). (B) Mouse epididydmal sperm after the A23187 treatment. Most of sperm lose their acrosome. Apical segment of sperm head is free from staining (open arrowhead). Bar=100 μm.

sperm was not (Fig. 1). DMA inhibited spontaneous acrosome reaction with concentration dependent (0~100 μM) manner (Fig. 2). Half of maximum inhibition was observed at 0.1~1 μM DMA. In the absence of DMA spontaneous AR rapidly increased during 120 min of incubation but significant increase in AR was not observed during further 60 min (Fig. 2). In the presence of 1 μM DMA, AR increased during incubation for 120 min but AR rate was significantly lower than that of the drug-free sperm. Interestingly, even after the 120 min of incubation AR increased during further 60 min of incubation (Fig. 2).

2. Effect of DMA on human follicular fluid-induced acrosome reaction

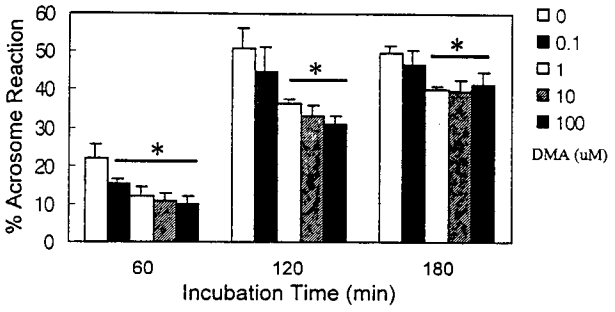

Fig. 2. Effect of DMA on spontaneous acrosome reaction at different concentration. DMA inhibited spontaneous acrosome reaction with concentration dependent (0~100 μM) manner. Error bars are SD (n=4). *, significantly ($p<0.05$) different from control.

Human follicular fluid has been known to contain several ligands responsible for AR (Tesarik et al., 1993; Miska et al., 1994). To examine the effect of DMA on ligand induced AR, hFF was challenged to sperm to induce AR at the end of 120 min of incubation. As expected, addition of the hFF increased AR. When DMA (1 μM) was primed to hFF (10 %, v/v), AR was slightly inhibited but not significantly when compared to DMA-free sperm exposed to hFF (Fig. 3).

3. Effect of DMA on Ca^{2+} ionophore induced acrosome reaction

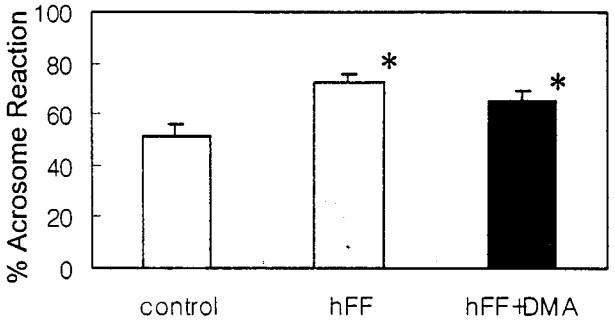

Fig. 3. Effect of DMA on human follicular fluid induced acrosome reaction in capacitated sperm. Epididymal sperm was preincubated for 120 min and treated with human follicular fluid (10%, v/v) in the presence of 1 μM DMA. Addition of DMA did not reduced the hFF-induced AR significantly. Error bars are SD (n=4). *, significantly ($p<0.05$) different from control.

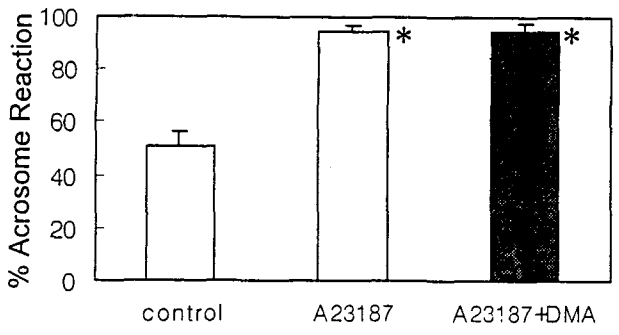

Fig. 4. Effect of DMA on acrosome reaction induced by A23187. Epididymal sperm was preincubated for 120 min and treated with Ca^{2+}-ionophore, A23187 (10 μM) in the presence of 1 μM DMA. Addition of DMA did not reduced the hFF-induced AR significantly. Error bars are SD (n=4). *, significantly ($p<0.05$) different from control.

Undoubtedly, Ca^{2+} influx is an irreversible trigger for acrosomal exocytosis of capacitated sperm in response to various AR-inducing ligands. So Ca^{2+} ionophore A23187 was challenged to capacitated sperm to induce AR. When DMA (1 μM) was primed to A23187 (10 μM), AR was not different from DMA-free sperm exposed to A23187 (Fig. 4).

DISCUSSION

It has been known that several anions such as Cl^- (hamster, Yoshimatsu and Yanagimachi, 1988; Shi and Roldan, 1995), bicarbonate (mouse, Lee & Storey, 1986; hamster, Yoshimatsu & Yanagimachi, 1988; porcine spermatozoa, Okamura et al., 1991) have functional importance in AR of spermatozoa. In addition, monovalent cations such as Na^+ is closely linked with capacitation and AR (guinea pig, Hyne et al., 1984, 1985; mouse, Fraser et al., 1993). Increase in $[Na^+]_i$ and depolarization of plasma membrane culminated to the activation of L-type Ca^{2+} channel (Fraser, 1993). Nifedifin was reported to inhibit the monovalent cation ionophore monensin-induced AR in mouse sperm (Fraser, 1993). It has been known that the elevation of cytosolic $[Ca^{2+}]$ and pH_i are modulated by voltage-dependent and pH-sensitive mechanisms in mammalian sperm (Babcock & Pfeiffer, 1987). These bodies of evidences talk the possible link between the rise of pH_i and monovalent ion transport system. During the preincubation in the physiological medium, the permeability of the plasma membrane to Ca^{2+} is enhanced (Ben-Av et al., 1988) and the proportion of capacitated and spontaneously acrosome-reacted sperm increase with time (Lee & Storey, 1985). In this experiment, steady increase in the spontaneous AR during the first 120 min of incubation (Fig. 2) implies that sperm capacitation preceded during this period. It was further confirmed by occurrence of AR in most of sperm challenged to hFF (Fig. 3) or A23187 (Fig. 4) after incubation for 120 min. In the presence of DMA (0.1~100 μM) spontaneous AR was significantly ($p<0.05$) inhibited (Fig. 2) and it suggested possible involvement of Na^+/H^+ antiporter in capacitation of mouse epididymal sperm. Previously, it was reported that the Na^+/H^+ exchange induced by monensin causes an increase in intracellular Na^+, which is the driving force for the Ca^{2+} entry via a Ca^{2+}/Na^+ antiporter during capacitation (Ben-Av et al., 1988). In this experiment, spontaneous AR hardly increased in drug-free sperm after 120 min. But during the further 60 min of incubation acrosome-reacted sperm steadily increase in the presence of higher concentration DMA (10~100 μM). It suggested that DMA did not block but retarded capacitation and that prolongation of incubation under physiological condition could offset the inhibitory effect of DMA on capacitation.

In next series we examined the possible involvemenet of Na^+/H^+ antiporter in capaitation to AR transition. After 120 min of incubation sperm was treated with two different AR-inducing substances; hFF and A23187. Follicular fluid has a complex composition and several substances in this body fluid were reported to promote capacitation and acrosome reaction with no species specificity (Suarez et al., 1986; Gye et al., 1996). Follicular fluid is rich in AR-inducing ligands such as progesterone (P_4) (Osman et al., 1989; Blackmore et al., 1991; Tesarik et al., 1993; Shi and Roldan, 1995), and complex of P_4-serpin CBG (corticoid binding protein), an acrosome reaction inducing substance (ARIS) (Miska et al., 1994; Baltes et al., 1997). There was a significant ($p<0.05$) increase in AR in the 10 % (v/v) hFF-treated sperm, indicative of the presence of functional AR-inducing ligand(s) in hFF. When DMA (1 μM) was

primed the AR induction by hFF, AR was slightly inhibited but not significantly (Fig. 3). It suggested that agonistic effect of hFF on AR was not affected by DMA. Two kinds of Ca^{2+} channel were reported to operate in mammalian spermatozoa; L- (O'Toole et al., 1996; Cooper et al., 1998) and T-type (Arnoult et al., 1996) Ca^{2+} channel. It was reported that calcium influx induced by hFF occurs via T-type voltage-independent Ca^{2+} channels (Shiomi et al., 1996). Similarly, A23187 treatment to capacitated sperm in the presence of DMA did not inhibit AR (Fig. 4). Taken togather, it suggests that ions movement through the Na^+/H^+ antiporter is an upstream event of Ca^{2+} influx which occurs before the initiation of AR. In general, Na^+/H^+ antiporter has been supposed to influence to Ca^{2+} influx by raising pHi (Walensky & Snyder, 1995). However, absence of inhibitory effect of DMA on both hFF- and A23187-induced AR of preincubated epididymal sperm suggested that change in pH_i by H^+ efflux through the Na^+/H^+ antiporter might have little or no influence in the transition from capacitation to acrosome reaction.

Result from this experiment suggested that Na^+/H^+ antiporter do operate in the upstream of Ca^{2+} influx leading to AR and that DMA affects transition of sperm from uncapacitated to capacitated state but not transition from capacitation to acrosome reaction of mouse spermatozoa.

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