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Effect of Extracellular Ca^{2+} and Ca^{2+}-ATPase on the Acrosome Reaction of Spermatozoa

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Abstract: This study has been designed in order to examine a physiological role of Ca^{2+} which has been known as an essential factor for capacitation, to confirm whether the enzyme activity of Ca^{2+}-ATPase on capacitation is important or not, and to clarify relationship between various levels of the Ca^{2+} concentration and Ca^{2+}-ATPase which has been known to be an important factor of the plasma membranes.

In the present study applying quercetin, a Ca^{2+}-ATPase inhibitor, the enzymatic effect of Ca^{2+}-ATPase on capacitation was found to be remarkable: a significant increase of the transition from the original type (type A) to the type B and the type AR of the spermatozoa. This finding suggests that Ca^{2+}-ATPase plays an important role in the efflux and the influx of the Ca^{2+} which has been known to be an essential factor the capacitation and acrosome reaction, and that the inhibitory action of the Ca^{2+}-ATPase might be a prerequsite step toward the acrosome reaction.

The conclusion reached can be deduced as follows: increment of the intracelluar Ca^{2+} concentration occurred by controlling the slope of Ca^{2+} concentration through Ca^{2+}-ATPase activities in both the intra- and extracelluar fluid may be an important procedure for capacitation and acrosome reaction, and ultimately for fertilization of the spermatozoa and the ova.

Key Words: Acrosome reaction, Ca^{2+}, Ca^{2+}-ATPase, Quercetin, Spermatozoa

INTRODUCTION

It has been known by Yanagimachi[14] that capacitation is an essential procedure for spermatozoa during fertilization, and a number of physiological factors function in the procedure. Among these factors, the influx of Ca^{2+} into the spermatozoan cytoplasm may play an important role during capacitation: the higher Ca^{2+} concentration of the extracellular fluid, the higher Ca^{2+} concentration of the intracelllar fluid was kept to induce the capacitation, then finally to enter acrosome reaction. It is generally accepted that internalization of extracellular Ca^{2+}, causing a rise in the intracellular Ca^{2+} concentration, is required for both capacitation and acrosomal exocytosis.

It has been known that during acrosome reaction the higher Ca^{2+} concentration is necessary for spermatozoa and the enzymatic and the membranous activities should be augmented in the plasma membranes. Based on the ex-

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perimental results obtained by Yanagimachi[14], dynamic motility of spermatozoa is in need of passing through the female reproductive tract, the lumen of which is full of the mucous materials, and also essential for penetrating the thick zona pellucida. Another experimental results indicate that the spermatozoa incubated in the media with Ca^{2+} ions show more vigorous capacitation than those in Ca^{2+}-deficient medium[13]: Fraser[5] reported that the extracellular concentration of Ca^{2+} in the culture media for obtaining capacitation has been known to be about 1.8 mM.

A Ca^{2+}-ATPase located in the plasma membrane, in somatic cell, helps to maintain low $[Ca^{2+}]i$ by pumping Ca^{2+} out of the cell. Such an Ca^{2+}-ATPase has been identified in mammalian spermatozoa. Recent experiments have been suggested that mouse spermatozoa in the presence of compounds known to inhibit Ca^{2+}-ATPase activity in the somatic cells shortents acrosome reaction, indicating that the enzyme activity may play a role in regulating the events of acrosome reaction[6].

Based on the former experimental reports, the present study was designed in order to examine physiological role of Ca^{2+} which has been known as an essential factor capacitation, to confirm functional relation between the control of Ca^{2+} and the Ca^{2+}-ATPase in the plasma membrane by means of chlortetracycline fluorescence technique applying quercetin which has been known as an ATPase inhibitor[11].

MATERIALS AND METHODS

1. Preparation of Incubation Media

The modified Tyrode's solution was used according to Fraser[3]: 1.8 mM $CaCl_2$ solution was added into the Tyrode's solution. $CaCl_2$ was not added into the Tyrode's solution for the Calcium-deficient medium and the tiny amount of Ca^{2+} in the media was neglected for measurement. The $CaCl_2$ stock solution was 22.5 mM. For preparation of the 1.8 mM Ca^{2+} solution, 230 μl of the calcium-deficient medium and 20 μl of the $CaCl_2$ stock solution were mixed together.

2. Preparation of Spermatozoa

The human semen obtained from healthy men was used in accordance with physiological standards established by WHO (world health organization) in the present study.

The spermatozoa were prepared by means of mini-Percoll gradients[9]: The semen was centrifuged at 600 g for 5 minutes and the supernatant was removed and the new medium was added to centrifuge again. The specimen was swim-uped under the 5% CO_2, in 37℃ in accordance with the experimental methods. The sperm concentration was controlled using a haemocytometer and adjusted to 5×10^6 cells/ml. The motility showed 80% and more generally.

3. Chlortetracycline (CTC) Assessment

The modified Fraser & McDermott method[6] was applied for chlortetracycline measurement. CTC solution was prepared as follows: the Tris-buffer (130 mM NaCl, 5 mM cysteine, 20 mM Tris-HCl, pH 7.8) solution was mixed with 750 μm CTC and kept under the dark cold room (5℃). 100 μl of the sperm suspension and the CTC solution were well mixed and the 10 μl of the mixture was dropped on the slideglass, and then covered with the coverglass. The Olympus BHS microscope provided with phase contrast and epicfluorescence was used, and its exitation beam adjusted to pass the filter at 405 nm band, and the CTC fluorescence emission was observed through the DM 455 dichroic mirror.

4. Fluorescein Isothiocyanate-Conjugated Peanut Agglutinin (FITC-PNA) Method

The acrosome reaction was discriminated by means of fluorescein isothiocyanate-conjugated Peanut (Arachis hypogea) agglutinin using fluorescent vital stain (H33258)[7,8]. The stainability of the acrosome was divided into the following

4 classes: class 1 was represented by whole acrosome (intact acrosomal cap and equatorial segment, class 2 by patchy acrosome (fused acrosomal membranes and vesicle formation, class 3 by only equatorial segment (absence of acrosomal cap and intact equatorial segment), and class 4 by no fluorescence (absence of acrosomal cap and equatorial segment).

Three CTC fluorescence patterns of the acrosome were classified as follows: 200 spermatozoa were observed in accordance with regional fluorescence, 1) type F (fluorescence)- a certain degree of fluorescence, no capacitation, and intact acrosome, 2) type B (Fluorescence band in the post-acrosomal region)- capacitated but intact acrosome, and 3) type AR (acrosome reaction)- hardly fluorescence in the head, capacitated, and acrosome reaction[2].

The experimental data was analyzed by Cochran's test and student's t-test.

RESULTS

1. Capacitation and Acrosome Reaction in Accordance with Extracellular Ca^{2+} Concentration

The acrosome reaction status was analyzed after incubation for 30, 60, 120 and 180 mins in the media containing either 1.8 mM or 3.6 mM Ca^{2+} (Table 1). The type B spermatozoa incubated in the 1.8 mM Tyrode's solution were found in the maximum percentage at 60 mins but thereafter decreased but the type AR spermatozoa incubated in 1.8 mM Tyrode's solution increased in percentage gradually since from 60 mins (21.5) to 180 mins (35.5). This finding seems to be general characteristics which can be induced from routine procedures. The type B spermatozoa incubated in 3.6 mM Tyrode's solution increased in number gradually but the type AR spermatozoa increased abruptly in number at 60 mins (44.3) and were kept in high level of percentage. These findings indicate that the intracellular concentration of Ca^{2+} may play an important role during capacitation.

2. Capacitation and Acrosome Reaction in the Calcium-deficient Medium

The acrosome reaction stastus was analyzed after incubation for 3 hrs in the media without Ca^{2+} and with Ca^{2+} (Table 2). The type B spermatozoa incubated in the media without Ca^{2+} and with Ca^{2+} were found to be no change in

Table 1. CTC fluorescence patterns of spermatozoa for acrosome reaction (%) (Incubation in the media containing 1.8 mM and 3.6 mM Ca^{2+})

Incubation Time (min)	Type B Spermatozoa		Type AR Spermatozoa	
	1.8 mM	3.6 mM	1.8 mM	3.6 mM
30	13.5±1.3	16.5±1.0	14.0±2.2	23.3±3.0
60	18.8±1.0	20.5±1.3	21.5±1.3	44.3±2.5
120	10.8±1.3	11.5±2.6	32.8±2.1	47.0±3.6
180	8.5±1.3	7.3±1.3	35.5±2.4	47.0±1.8

Table 2. CTC fluorescence patterns of spermatozoa for acrosome reaction (%) (Incubation in the media without and with Ca^{2+})

Incubation Time (min)	Type B Spermatozoa		Type AR Spermatozoa	
	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}
30	12.0±1.2	12.5±2.0	14.5±2.1	14.0±2.2
60	10.0±0.8	15.5±4.1	16.3±2.2	21.8±1.0
120	8.5±2.4	11.3±1.7	13.0±3.2	32.0±1.4
180	7.5±1.0	11.0±0.8	13.8±2.5	36.3±1.0

Table 3. Acrosome reaction rate in accordance with incubation time and media with Ca^{2+} or without Ca^{2+}

Incubation Time (hrs)	No. of Samples	Absence of Acrosome (%)	Intact Acrosome (%)
5 With Ca^{2+}	10	6.7±0.6	88.7±1.5
5 Without Ca^{2+}	10	7.2±2.6	87.6±1.8
20 With Ca^{2+}	10	13.5±3.8	70.9±3.4
20 Without Ca^{2+}	10	9.1±1.4	85.2±2.1

Table 4. CTC fluorescence patterns of the type B spermatozoa (%) (Incubation for 5 hrs in various concentrations of quercetin)

Quercetin (μl)	Incubation Time (hrs)				
	1	2	3	4	5
Control	4.5±1.5	10.3±1.0	12.0±1.4	18.5±4.4	22.0±3.4
50	11.0±0.8	17.8±1.0	21.0±0.8	25.0±1.4	27.3±1.3
100	13.5±0.6	20.5±2.9	26.8±2.1	29.3±1.7	33.8±1.3
200	16.3±1.0	24.3±1.9	31.8±1.5	33.8±2.5	36.5±4.2

Table 5. CTC fluorescence patterns of the type AR spermatozoa (%) (Incubation for 5 hrs in various concentrations of quercetin)

Quercetin (μl)	Incubation Time (hrs)				
	1	2	3	4	5
Control	3.8±1.3	12.0±1.4	19.5±1.3	22.3±3.5	25.0±1.8
50	6.8±0.5	14.0±2.2	21.8±1.0	26.8±2.1	29.3±1.7
100	7.0±1.6	17.3±1.3	23.8±2.5	31.3±1.7	29.3±1.0
200	9.3±1.7	17.8±1.0	30.0±2.2	33.3±1.7	30.8±3.3

percentage but the type AR spermatozoa increased in number gradually to reach up to maximum 36.3 % at 180 mins ($p<0.05$).

In the measurements using FITC-PNA and H33258, the spermatozoa motility in the media with Ca^{2+} seem to be increased in number but the acrosome reaction rate was found to be no change (Table 3).

3. Effect of Quercetin on Acrosome Reaction of Spermatozoa In Accordance with the Type B and Type AR

In examining the effect of quercetin (Ca^{2+}-ATPase inhibitor) on acrosome reaction, the CTC fluorescence patterns of the spermatozoa were analyzed by means of chlortetracycline fluoresence technique. The type B spermatozoa (Table 4) abruptly increased in number after 1 hr and slowly decreased in number thereafter. The type AR spermatozoa (Table 5) also increased significantly after 3hrs. These significant increases in both the type B and the type AR spermatozoa after 3 hrs incubation suggest that the Ca^{2+} may function as an important for capacitation and acrosome reaction in the fertilization.

DISCUSSION

The present study was designed in order to examine the effect of Ca^{2+} and Ca^{2+}-ATPase on acrosome reaction during capacitation by means of CTC fluoresence technique. As the experimental results, the higher concentration

of the extracellular concentration of Ca^{2+}, the more increment of the intracellular concenration of Ca^{2+} which facilitate the capacitation of spermatozoa: this finding was supported by an evidence that the type AR spermatozoa increased in the medium of 3.6 mM that those in the medium of 1.8 mM.

It has been known by Yanagimachi[14] that Ca^{2+} influx from the extracellular fluid into the cytoplasm increase to bring about acrosome reaction. Based on some experimental reports, when the Ca^{2+} influx through Ca^{2+}-ATPase into the cytoplasm is larger than those efflux from the higher Ca^{2+} concentration in the extracellular fluid, transition into the type B and the type AR spermatozoa would occur rapidly, with special regard to the type AR.

However, it has been generally understood that how much the type AR spermatozoa affect the capacitation, which have been observed by means of CTC fluorescence technique would not be clarified by the present time. There are some evidence to support these uncertainties. Fraser[5] reported that when acrosome reactioned spermatozoa are observed with the electron microscope, most of them were found to be deformed and dead, in spite of that most of them had first been recognized to be adequeate to fertilize. On the other hand, White *et al.*[12] reported that on the contrary to the general understanding, during spermatozoa incubation the acrosome reaction rate neither increased nor necessary to get fertilization with the ovum.

Based on the experimental results obtained in the present study, it is deduced that the Ca^{2+} influx occurred within a definite period of time to induce transition toward the capacitation, finally to bring about the acrosome reaction, and this deduction supports the former experimental results[1,6].

It has been reported by Roldan & Fleming[10] that Ca^{2+}-ATPase and Ca^{2+}-Na^{+} exchangers play an important role during Ca^{2+} influx into the spermatozoan cytoplasm. It is well known that Ca^{2+}-ATPase exists within the plasma membrane, from which the Ca^{2+} is exerted to keep the intracellular Ca^{2+} concentration in low level. A Ca^{2+}-ATPase located in the plasma membrane, in somatic cell, helps to maintain low $[Ca^{2+}]i$ by pumping Ca^{2+} out of the cell. Such an Ca^{2+}-ATPase has been identified in mammalian spermatozoa. Recent experiments have been suggested that mouse spermatozoa in the presence of compounds known to inhibit Ca^{2+}-ATPase activity in the somatic cells shortents acrosome reaction, indicating that the enzyme activity may play a role in regulating the events of acrosome reaction[5]. In the present study, a certain degree of possibility of influence of Ca^{2+}-ATPase on capacitation has been verified by means of FITC-PNA and H33258 method[8] using quercetin known as a Ca^{2+}-ATPase inhibitor. There was found significant increase of the type B and the type AR spermatozoa in the 50 to 200 μl concentration (Table 4 and 5). This increment suggests a possibility that Ca^{2+}-ATPase may play an important role in the capacitation and the acrosome reaction during which efflux and influx of Ca^{2+} may occur vigorously and that procedure of lowering the Ca^{2+}-ATPase activity may be a prerequsite physiological step in the acrosome reaction.

Being based upon Fraser[4], it is likely to occur that inhibiting factors for capacitation might facilitate Ca^{2+}-ATPase activities. On the contrary for Fraser's methodology, the present authors have demonstrated to keep the intracellular Ca^{2+} concentration in high level by means of getting rid of the inhibiting factor (quercetin) and to transit quickly toward the capacitation: Ca^{2+}-ATPase working as a Ca^{2+}-pump could play an important role to control and accelerate the spermatozoan acrosome reaction. The quercetin has been known not only to make Ca^{2+}-ATPase inactive but also to attach to the postacrosomal region where is though to be a site of discriminating standard for capacitation.

Conclusively it is deduced from the experimental results obtained in the present study

that steady maintenance of high level for the extracellular Ca^{2+} concentration may be an important factor to get acrosome reaction quickly, and that Ca^{2+}-ATPase may control the slopes of the intra- and extracellular Ca^{2+} concentration to induce the spermatozoa into capacitation and ultimately and to get acrosome reaction for fertilization.

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=국문초록=

세포외 Ca^{2+}과 Ca^{2+}-ATPase가 정자의 첨체반응에 미치는 영향

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세포내, 외 Ca^{2+} 농도구배 유지에는 Ca^{2+}-ATPase와 Ca^{2+}-Na^{+} exachangers가 주요한 기능을 한다고 알려져 있는데 특히 Ca^{2+}-ATPase의 기능에 대해 많은 연구가 행해지고 있다. Ca^{2+}-ATPase는 체세포에서 세포막에 위치하고 있으며 Ca^{2+}을 세포외부로 배출하는 기능을 함으로써 세포내부의 Ca^{2+}농도를 낮게 유지할 수 있도록 하는 기능을 담당하고 있다. 이러한 Ca^{2+}-ATPase는 포유동물의 정자에도 존재하고 있지만 그 기능에 대해서는 아직 많은 설명이 되어있지 않다.

본 연구에서 정자가 수정을 하기위한 기능적인 능력이 Ca^{2+} 농도와 관련된 변화와 얼마나 연관되어 있는가를 규명하고, 이러한 Ca^{2+}농도 조절이 원형질막의 중요인자인 Ca^{2+}-ATPase와는 어떠한 연관성이 있는가를 알기위해 시도한 결과, Ca^{2+}-ATPase는 세포내, 외 Ca^{2+}의 농도구배를 조절함으로써 세포내 Ca^{2+}의 농도를 증가시켜 정자가 수정능 획득과정으로 빨리 전환하도록 유도하고 첨체반응에 중요한 역할을 하는 것으로 판단되며, 세포외 Ca^{2+}농도가 높게 유지될 경우에도 정자의 첨체반응이 유도됨으로써 난자와 용이하게 수정을 할 수 있는 생리적 환경이 제공될수 있다고 사료된다.

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