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Induction of acrosomal reaction and calcium uptake in ram spermatozoa by ionophores

Pazit Ben-Av, Sara Rubinstein and Haim Breitbart

Health Sciences Research Center, Department of Life Sciences, Bar-Ilan University, Ramat-Gan (Israel)

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Ram spermatozoa incubated in the presence of Ca^{2+} and the Ca^{2+}-ionophore A23187 undergo a process which is known as the acrosome reaction. This reaction is characterized by fusion of the outer acrosomal membrane and the overlying plasma membrane to form mixed vesicles which can be seen in the electron microscope. As a result, the trypsin-like acrosin is released from the cells to the medium. The occurrence of the acrosome reaction was determined by following acrosin activity in the medium. After 2 h of incubation of the cells in the presence of ionophore and Ca^{2+}, the released acrosin activity is related to the ionophores according to the sequence: A23187 > monensin > valinomycin > FCCP = without ionophore. The study of Ca^{2+} uptake by the cells revealed that Ca^{2+} enters the cell prior to the release of acrosin. Monensin can induce Ca^{2+} uptake and acrosin release only when Na^+ is present in the incubation medium. There is no increase in Ca^{2+} uptake with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). We suggest 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. Since monensin can induce an increase in Ca^{2+} uptake only in the presence of Na^+, FCCP enhances Ca^{2+} uptake in the presence of valinomycin, and A23187 is a $Ca^{2+}/2H^+$ exchanger, we suggest that alkalization of the intracellular space is involved in the acrosome reaction. Calcium uptake in the presence of monensin is not affected by the uncoupler FCCP, a result which indicates that Ca^{2+} is not accumulated in the mitochondria. Incubation of cells for 3 h in the absence of Ca^{2+} or ionophore caused a 3-fold increase in the rate of acrosin release when monensin and Ca^{2+} were added together. There was no change in this rate when A23187 was used. We suggest that during the preincubation time (known as capacitation) the permeability of the plasma membrane to Ca^{2+} is enhanced. This study shows that acrosin release and Ca^{2+} uptake can be used as a quantitative asay for the determination of the acrosome reaction.

Introduction

Capacitation and the acrosome reaction are primary physiological changes in the spermatozoa necessary for mammalian fertilization [1,2]. The Ca^{2+}-dependent breakdown and fusion of the plasma and the outer acrosomal membranes are major events in the acrosome reaction and result in the release into the medium of some hydrolytic enzymes like acrosin [4,5]. The majority of potential acrosin present in mammalian spermatozoa is in a zymogen form, proacrosin [33–35]. In vitro, proacrosin spontaneously and rapidly converts into acrosin at neutral pH [33]. It was found that

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; BAEE, benzoylarginine ethyl ester.

Correspondence: H. Breitbart, Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52100, Israel.

uterine glycosaminoglycan [36] and a sperm-associated non-proteolytic protein [37] stimulate the conversion of proacrosin to acrosin.

Spermatozoa require a period of incubation in the female reproductive tract or in defined culture media prior to the acrosome reaction. Without this capacitation process, the cells do not respond to Ca^{2+} and the acrosome reaction does not occur [5,6]. Although the molecular mechanism of capacitation is as yet unclear, it is generally accepted that this process involves a restructuring of the sperm surface [5,7]. Initial alterations in capacitation include modification and redistribution or loss of proteins from the sperm plasma membrane [7,15], and enhanced influx of Ca^{2+} [16].

There are difficulties in assaying quantitatively the occurrence of the acrosome reaction in ram spermatozoa, since the morphological changes cannot be seen clearly under the light microscope. Therefore, we used quantitative biochemical assays of Ca^{2+} uptake and acrosin release, events which take place in capacitation and the acrosome reaction.

The acrosome reaction was induced by various ionophores for Ca^{2+}, Na^+, H^+ or K^+. A possible mechanism which includes the involvement of these ions in capacitation and the acrosome reaction is described herein.

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Materials and Methods

Semen collection and sperm preparation

Semen was collected from rams by electric induction. The fresh semen was immediately diluted (1 : 6) with minimal capacitation medium (buffer I) containing 101 mM NaCl, 2.68 mM KCl, 0.36 mM NaH_2PO_4, 35.7 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 4.5 mM glucose, 0.09 mM pyruvate, 9.0 mM L-lactate, 0.5 mM taurine, 0.05 mM L-epinephrine, 3.0 mg/ml bovine serum albumin, 10.0 IU/ml penicillin (pH 7.6) [17]. The sperm cells were washed twice by centrifugation at $780 \times g$ for 10 min at room temperature. The final cell pellet was resuspended in buffer I to reach a final concentration of $1 \cdot 10^8$ cells/ml.

Acrosin release measurements

Sperm cells (10^8 cells/ml) suspended in buffer I were incubated at 37 °C, with continuous agitation. The tubes were covered with two layers of parafilm. At appropriate time intervals, 1 ml samples was removed and immediately centrifuged at $7500 \times g$ for 20 min at 4 °C. The pH of the supernatant was adjusted to pH 3.0 with 1 M HCl and was kept overnight at −20 °C. The acrosin activity in this solution was determined according to Brown et al. [18] using the substrate BAEE and recording increase in absorbance at 259 nm with time. The molar absorption coefficient was taken as 1150.

Calcium uptake measurements

^{45}Ca uptake by the cells was determined by the filtration technique [19]. The incubation conditions are as described for acrosin release measurements, but here 5 μCi/ml of $^{45}CaCl_2$ was added. At appropriate time intervals, 0.1 ml samples were removed and immediately vacuum-filtered on GF/C filters that had been prewashed with buffer composed of 150 mM NaCl, 2 mM $CaCl_2$ and 10 mM Tris (pH 7.4) (buffer A). The cells trapped on the filter were washed three times with 5 ml of ice-cold buffer A. The dry filters were placed in scintillation vials with 4 ml of Lumax (Lumac) solution for measurement of β-radioactivity. All data are expressed as the experimental value corrected for the zero time control.

Electron microscopy

Cells were incubated as described for acrosin release measurement. At the appropriate time, 0.15 ml sample was transferred into 2.0 ml of 1% (w/v) osmium tetroxide (see Fig. 1). This cell suspension was incubated for 20 min at room temperature, and then centrifuged at $780 \times g$ for 10 min. The pellet was dehydrated through ethanol to propylene oxide in 10 min stages and embedded in Epon 812. Thin sections were cut with an LKB Ultratome III, stained with uranyl acetate/lead citrate and examined with a JEOL 1200 EX transmission electron microscope.

Materials

A23187 was obtained from Calbiochem, monensin from Eli-Lilly, valinomycin and FCCP

from Sigma and $^{45}CaCl_2$ from New England Nuclear.

Results

The acrosome reaction in washed ram spermatozoa was induced by Ca^{2+} and the Ca^{2+}-ionophore A23187. The electron micrograph of intact and acrosomal reacted cells can be seen in Fig. 1. This revealed that the plasma membrane and the outer acrosomal membrane were fused to form mixed vesicles which later disappeared from the cell. For each electron-microscope preparation, at least 100 cells were followed for the determination of the number of cells that undergo acrosome-reaction. It was found that after 1 h incubation 5.0% and 95.0% of the cells were acrosome-reacted in the presence of Ca^{2+} or Ca^{2+} plus A23187, respectively. After 30 min incubation 45% of the cells were acrosome-reacted in the presence of Ca^{2+} plus A23187.

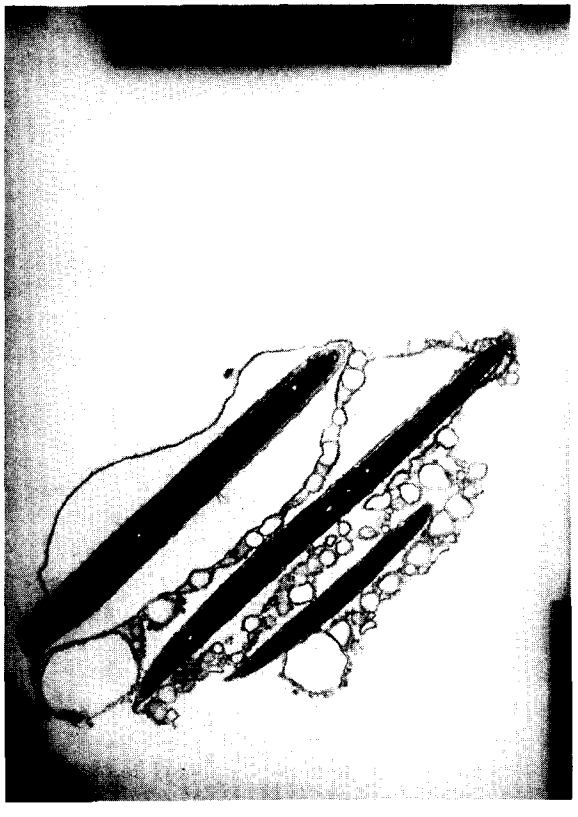

Fig. 1. Transmission electron micrograph of the head of an intact cell (upper cell) and acrosome-reacted cells (lower cells). The intact acrosome and the plasma membrane can be seen in the intact cell, whereas in the acrosome-reacted cells, the plasma membrane and the outer acrosomal membrane were fused to form mixed vesicles. Magnification × 12000.

Parallel to the electron microscopic study, the release of acrosin into the medium was determined. The results in Fig. 2 show the time-dependency of acrosin release. Only when Ca^{2+} plus the Ca^{2+}-ionophore A23187 were present was a high release of acrosin found. There was almost no increase in the rate of acrosin release in the absence of Ca^{2+} or with Ca^{2+} but without A23187. The activity of the acrosin preparation after freezing was sensitive to the pH at which the acrosin preparation was kept. The results in Fig. 3 show that only the acrosin preparation from cells that had been incubated in the presence of Ca^{2+} plus A23187 were highly sensitive to the pH of preservation. The acrosin preparations from cells incubated without Ca^{2+} or with Ca^{2+} but without A23187 show a completely different behaviour towards the pH of preservation. The same results were obtained with the unfrozen material (data not shown). These results suggest that the basal acrosin activity that had been found at zero time in the absence of Ca^{2+} or A23187 is not related to the acrosomal reaction.

The acrosome reaction is defined in the litera-

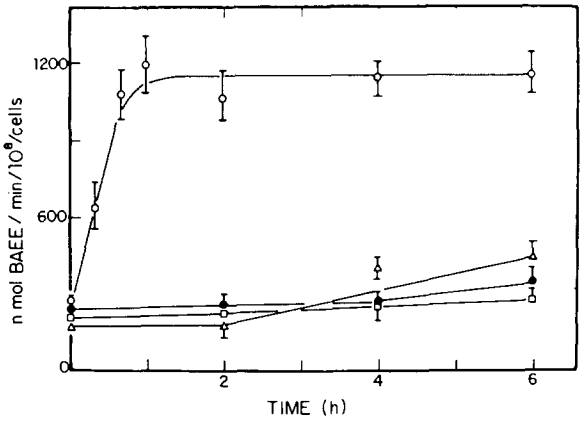

Fig. 2. Time course of acrosin release. Sperm cells (10^8/ml) were incubated in buffer I and the activity of the released acrosin was determined in the supernatant after precipitating the cells. Values represent: no additions (□), 2.0 mM Ca^{2+} (△), 1.0 μM A23187 (●), 2.0 mM Ca^{2+}, 1 μM A23187 (○). Each point represents the mean ± S.E. of duplicates from three experiments.

ture as a Ca^{2+}-dependent process. It was shown in Fig. 2 that in the absence of Ca^{2+} there is no increase in acrosin release with time. In Fig. 4 we show the dependency of acrosin release on extracellular Ca^{2+} concentrations. It can be seen that the optimal Ca^{2+} concentration for acrosin release is 1–2 mM.

It was shown before that monensin and valinomycin plus FCCP can stimulate the occurrence of the acrosome reaction in guinea pig and hamster sperm [20,21]. The effect of various ionophores on acrosin release from ram spermatozoa is shown in Fig. 5. In the presence of Ca^{2+} and ionophore, the rate of acrosin release is related to the ionophores according to the sequence: A23187 > monensin > valinomycin > FCCP = without ionophore. There is no increase in acrosin release in the absence of Ca^{2+}. The highest rate is seen with the Ca^{2+}-ionophore, which suggests that the permeability of the plasma membrane to Ca^{2+} is increased during the incubation period. In order to test this possibility, cells were incubated for 3 h without ionophore, and then ionophore and Ca^{2+} were added. It can be seen in Fig. 6A that after 3 h of preincubation in the absence of Ca^{2+}, the rate of acrosin release induced by adding monensin and Ca^{2+} is 3.0-times faster in comparison to the rate observed without preincubation (monensin added at zero time). When an identical experiment was performed with A23187, there was no effect of the preincubation time on the rate of acrosin release (Fig. 6B). The data indicate that during the 3 h of preincubation the cells undergo the capacitation process by which the permeability of the plasma membrane to Ca^{2+} is increased. The

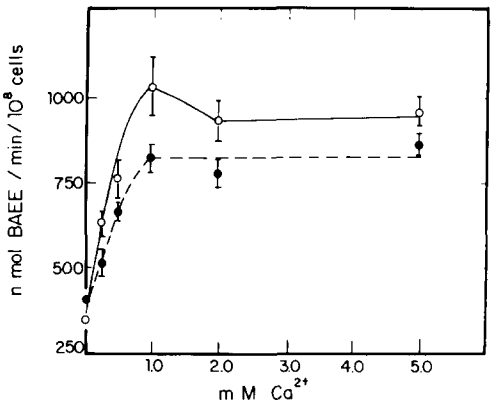

Fig. 4. The effect of extracellular Ca^{2+} concentration on acrosin release. Sperm cells (10^8/ml) were incubated in buffer I which contained increased concentrations of Ca^{2+}. Acrosin release was induced by incubation for 1 h with 1 μM A23187 (○) or 4 h with 50 μM monensin (●). Each point represents the mean ± S.E. of duplicates from two experiments.

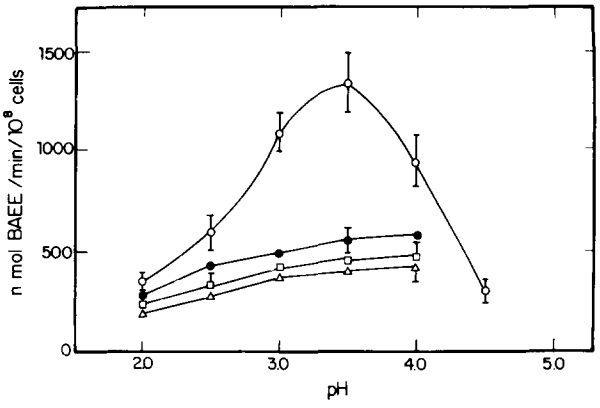

Fig. 3. Effect of pH of preservation on acrosin activity. Acrosin preparations were achieved as described in the legend to Fig. 2. The pH values of these preparations were changed to the desired pH by adding diluted HCl solution. The samples were kept at −20 °C overnight and then the acrosin activity was measured. The values represent the condition of incubating the cells for 2 h at 37 °C: no addition (□), 2.0 mM Ca^{2+} (△), 1.0 μM A23187 (●), 2.0 mM Ca^{2+}, 1 μM A23187 (○). Each point represents the mean ± S.E. of duplicates from two experiments.

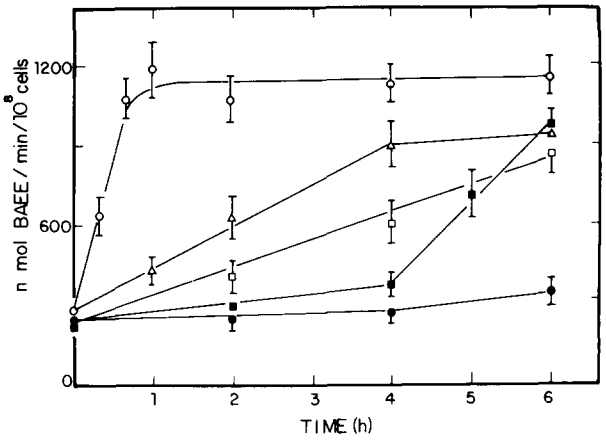

Fig. 5. Effect of various ionophores on acrosin release. Sperm cells (10^8/ml) were incubated in buffer I with 2.0 mM Ca^{2+}, and acrosin activity was determined in the cell medium after centrifugation to precipitate the cells. Values represent: no ionophore (●), 20 μM FCCP (■), 5 μM valinomycin (□), 50 μM monensin (△) and 1 μM A23187 (○). Each point represents the mean ± S.E. of duplicates from four experiments.

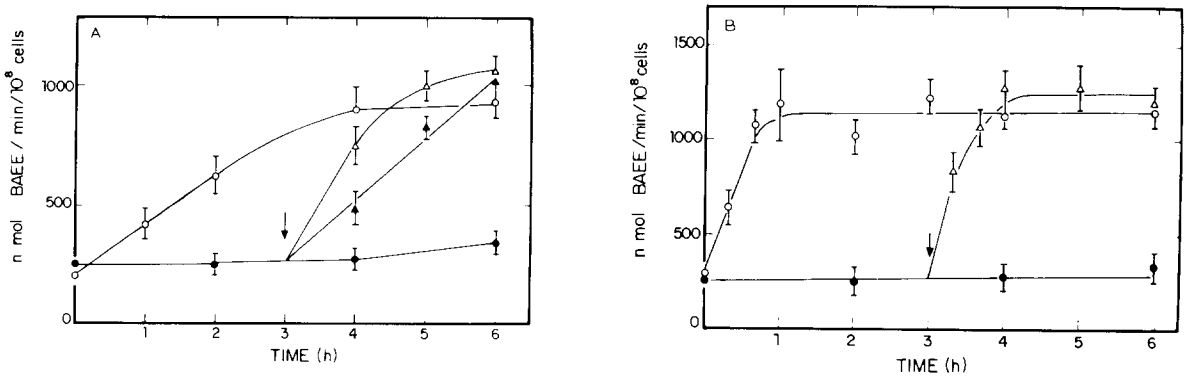

Fig. 6. Effect of preincubation (capacitation) on the rate of acrosin release. Sperm cells (10^8/ml) were preincubated in buffer I for 3 h and then ionophore and Ca^{2+} were added, and the activity of the released acrosin was determined. The arrows indicate time of addition of 50 μM monensin (A) or 1 μM A23187 (B). The values represent: 2.0 mM Ca^{2+} at zero time (●), 2.0 mM Ca^{2+} and ionophore at zero time (○), 2.0 mM Ca^{2+} and ionophore after 3 h of preincubation (△), 2.0 mM Ca^{2+} at zero time and monensin after 3 h of preincubation (▲). Each point represents the mean ± S.E. of duplicates from three experiments.

results also show that Ca^{2+} is not required in order to capacitate the cells. We can see that there was a 50% inhibition in the capacitation rate when the cells were capacitated in the presence of Ca^{2+} (see Fig. 6A).

In order to show that Ca^{2+} enters the cell during capacitation, the uptake of Ca^{2+} into the cells was measured. The data in Fig. 7 show an increase in Ca^{2+} uptake with time in the presence of A23187 or monensin. There was no increase in Ca^{2+} uptake with FCCP or Ca^{2+} alone. These results are in good correlation with acrosin release data (see Fig. 5). When valinomycin was used, there was a high increase in Ca^{2+} uptake only after 4 h of incubation, and by adding FCCP on top of it there was a 1.4-fold increase in Ca^{2+} uptake which reached the level that was found with A23187 (Table I). These results suggest that efflux of H^+ from the cells, probably from the acrosome, is involved in the mechanism of capaci-

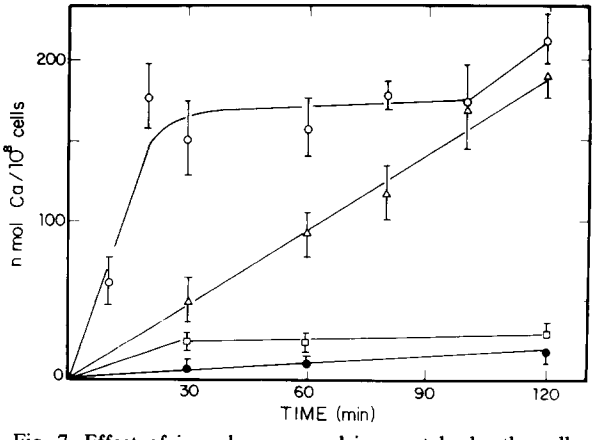

Fig. 7. Effect of ionophores on calcium uptake by the cells. Sperm cells (10^8/ml) were incubated in buffer I which contained 2.0 mM Ca^{2+} and the uptake of Ca^{2+} into the cell was determined as described under Materials and Methods. The values indicate the following: control (□), 20 μM FCCP (●), 50 μM monensin (△), and 1 μM A23187 (○). Each point represents the mean ± S.E. of duplicates from three experiments.

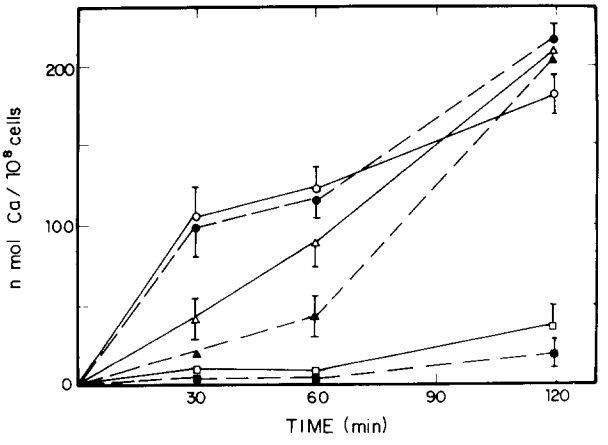

Fig. 8. The effect of FCCP on calcium uptake by the cells, induced by A23187 or monensin. Conditions as in legend to Fig. 7. The values represent: open signals are without FCCP, closed signals with 20 μM FCCP. The boxes are the controls, the triangles contain 50 μM monensin and the circles 1 μM A23187. Each point represents the mean ± S.E. of duplicates from two experiments.

TABLE I

EFFECT OF FCCP ON CALCIUM UPTAKE INDUCED BY VALINOMYCIN

Ca^{2+} uptake into the cells was determined in buffer I with 20 mM Ca^{2+}. Each point represents the mean ± S.E. of duplicates from three experiments.

Time (h) of incubation	nmol $Ca^{2+}/10^8$ cells			
	no addition	1 μM A23187	5 μM valinomycin	5 μM valinomycin + 20 μM FCCP
1	7.89 ± 1.4	111.10 ± 10.5	7.31 ± 1.6	8.10 ± 2.1
2	8.75 ± 1.6	151.30 ± 22.4	5.77 ± 0.9	18.80 ± 4.2
4	28.67 ± 3.5	271.60 ± 23.2	193.20 ± 12.5	276.03 ± 19.8

tation. The protonophore FCCP does not affect Ca^{2+} uptake in the presence of A23187 ($Ca^{2+}/2H^+$ exchanger) or monensin (Na^+/H^+ exchanger), since these ionophores already transport H^+ (see Fig. 8). It is interesting to mention that under non-capacitating conditions, FCCP causes above 90% inhibition of Ca^{2+} uptake into the cells [19], which indicates that above 90% of the Ca^{2+} taken up is accumulated in the mitochondria. The fact that FCCP does not inhibit Ca^{2+} uptake in capacitation medium suggests that most of the Ca^{2+} that is taken up is not accumulated in the mitochondria. The 50% inhibition in Ca^{2+} uptake by FCCP found after 30 and 60 min, in the presence of monensin (see Fig. 8), suggests that under these conditions about 50% of the Ca^{2+} is accumulated in the mitochondria, and this Ca^{2+} might later be transported to other regions of the cells. These results might explain the very low acrosin release found with monensin after 1 h incubation (see Fig. 5). We also found that although FCCP causes 50% inhibition in Ca^{2+} uptake induced by monensin, there is no effect of FCCP on acrosin release induced by monensin (see Table II). The data with FCCP suggest that there is no need for the mitochondria to be coupled in order to capacitate the cells.

In order to find out whether Na^+ is involved in the mechanism by which monensin induces capacitation, the cells were incubated in medium without Na^+. The data in Table III show that when NaCl was replaced by choline chloride, monensin could not induce acrosin release or Ca^{2+} uptake. Thus, we suggest that influx of Na^+ and

TABLE II

EFFECT OF FCCP ON ACROSIN RELEASE INDUCED BY A23187 AND MONENSIN

Sperm cells (10^8/ml) were incubated in buffer I with 2.0 mM Ca^{2+}, and the released acrosin was determined after pelleting the cells. Each point represents the mean ± S.E. of duplicates from two experiments.

Conditions	nmol BAEE/min per 10^8 cells	
	1 h incubation	2 h incubation
Control	173.3 ± 15.1	159.6 ± 13.8
20 μM FCCP	174.3 ± 16.3	146.3 ± 12.9
1 μM A23187	851.2 ± 64.1	957.6 ± 78.2
1 μM A23187 + 20 μM FCCP	837.9 ± 72.2	838.3 ± 75.1
50 μM monensin	425.6 ± 36.1	571.9 ± 41.2
50 μM monensin + 20 μM FCCP	545.3 ± 38.4	678.3 ± 50.1

TABLE III

EFFECT OF A23187 AND MONENSIN ON ACROSIN RELEASE AND CALCIUM UPTAKE IN THE ABSENCE OF Na^+ IN THE MEDIUM

The release of acrosin into the medium and Ca^{2+} uptake into the cells was determined in buffer I in which NaCl was replaced by choline chloride. The cells were incubated for 2 h with 2.0 mM Ca^{2+}. Each point represents the mean ± S.E. of duplicates from three experiments.

Conditions	Acrosin activity (nmol BAEE/ min per 10^8 cells)	Ca^{2+} uptake nmol Ca^{2+}/ h per 10^8 cells
Control	201.4 ± 19.8	15.2 ± 1.6
1 μM A23187	1055.2 ± 74.1	241.2 ± 23.2
50 μM monensin	208.8 ± 20.2	26.1 ± 3.5

efflux of H^+ are involved in the mechanism of capacitation. The absence of Na^+ in the medium does not affect acrosin release or Ca^{2+} uptake induced by A23187 (Table III). These data indicate that there is not a direct inhibitory effect of choline chloride or absence of Na^+ on the acrosome reaction.

Discussion

The acrosome of ram spermatozoa is relatively small, therefore it is difficult to determine the occurrence of acrosome reaction under the light microscope. Our first objective in this study was to develop a reliable quantitative biochemical assay for the determination of the acrosome reaction in ram sperm. The divalent cation-ionophore A23187 [22] has been used to induce a calcium-dependent acrosome reaction [23,24]. The results from the electron microscope revealed that 95% of the cells undergo the Ca^{2+}-dependent acrosome reaction. In the absence of added Ca^{2+}, only 5.1% of the cells are acrosome-reacted. In order to determine acrosome reaction in a more rapid and quantitative method the release of acrosin from acrosome-reacted cells was measured. The data show (Figs. 2 and 4) that the release of acrosin from the cells is dependent upon the presence of Ca^{2+} in the incubation medium. We also show (Fig. 3) that acrosin released from cells incubated with Ca^{2+} plus A23187 is highly sensitive to the pH of preservation, while acrosin released in the absence of Ca^{2+} is not. These results indicate that acrosin release from the cells can be used as a tool to determine acrosome reaction. We shall see later that when acrosome reaction was determined by acrosin release, the results obtained were comparable to the data described by others who had used morphological criteria. Acrosin release can be induced with monovalent ionophores, in a Ca^{2+}-dependent process (Fig. 5). It has been shown before that monovalent ionophores such as monensin, nigericin and valinomycin plus FCCP can induce acrosome reaction [20,21].

A number of reports have clearly demonstrated that the enhanced influx of Ca^{2+} is an early event required for the acrosome reaction in guinea-pig spermatozoa [16]. Our data with ram spermatozoa (Fig. 7) demonstrate for the first time the correlation between acrosin release and Ca^{2+} uptake when A23187 or monensin were used. With A23187, which is a Ca^{2+} ionophore, acrosin release or Ca^{2+} uptake are completed after 60 or 20 min, respectively. When monensin, a Na^+/H^+ exchanger, was used, acrosin release and Ca^{2+} uptake were completed after 4 h or 2 h, respectively. These data indicate that Ca^{2+} is taken up by the cells prior to acrosome reaction. Since the rates with A23187 are much faster in comparison to the rates with monensin, it is suggested that the permeability of the plasma membrane to Ca^{2+} is the rate-limiting step. The rate of acrosin release induced by monensin is enhanced 3-times after 3 h of preincubation (capacitation) in the absence of Ca^{2+} or ionophore (Fig. 6). There is no change in this rate when A23187 is used. These data indicate that the permeability of the plasma membrane to Ca^{2+} has been increased in the capacitation time. We also suggest that the presence of extracellular Ca^{2+} is not required for capacitation, and capacitation in the presence of Ca^{2+} causes a 50% inhibition in the rate of acrosin release (Fig. 6A). Yanagimachi and Usui [3] have also reported that Ca^{2+} is not required for the capacitation of guinea-pig sperm, while Frazer [25] has shown that Ca^{2+} is required for the capacitation of mouse spermatozoa. Thus, it seems that there are differences among various species regarding the requirement for extracellular Ca^{2+} for capacitation.

The K^+-ionophore, valinomycin, can induce acrosin release which is linearly increased up to 6 h of incubation (Fig. 5). When the effect of valinomycin on Ca^{2+} uptake was measured (Table I), it was found that only after 4 h was there a high increase in Ca^{2+} uptake. This Ca^{2+} uptake was low in comparison to that found with A23187, and it was enhanced by the inclusion of both valinomycin and FCCP in the medium. Valinomycin, as an electrogenic K^+-ionophore, creates a cross-membrane potential, and FCCP as electrogenic protonophore can relax this limitation. It is not clear what the mechanism is by which valinomycin can induce acrosin release or Ca^{2+} uptake. It was reported that noncapacitated hamster spermatozoa do not undergo an acrosomal pH change in the presence of K^+ and valinomycin, but do so if FCCP is also present [21]. Thus, it appears that acrosin release and Ca^{2+} uptake by the cells might be dependent upon alkalization of

the acrosome content. It was reported recently that epididymal ram sperm plasma membrane contains a voltage-dependent calcium channel which can be operated by depolarization of the membrane with high $[K^+]_0$ [32]. Under our conditions this Ca^{2+} channel is not operated since the $[K^+]_0$ is too low, but in the presence of valinomycin depolarization of the membrane might occur and the Ca^{2+} channel could then be operated. FCCP itself can induce acrosin release only after 5–6 h of incubation (Fig. 5), and no increase in Ca^{2+} uptake was found after 2 h (Fig. 7) or 6 h of incubation (data not shown). It has been published [21] that FCCP cannot induce a change in acrosomal pH in noncapacitated hamster sperm, but it does in capacitated sperm. Assuming that the cells in the present study are capacitated after 4 h of incubation and that we can only then see acrosin release, why can we not see Ca^{2+} uptake in parallel? Thus, it appears that acrosin release shown after 6 h incubation in the presence of FCCP is not related to acrosome reaction. It is important to mention that in noncapacitated ram spermatozoa, 90% of the uptaken Ca^{2+} is accumulated in the sperm mitochondria [19]. We also found that FCCP causes above 90% inhibition of Ca^{2+} uptake into noncapacitated bull sperm [26]. In this study, under capacitated conditions, there is no inhibition of Ca^{2+} uptake (Fig. 8) or acrosin release (Table II) in the presence of FCCP after 2 h of incubation. These data indicate that under these conditions Ca^{2+} is accumulated in extramitochondrial regions, maybe in the head of the sperm. When Ca^{2+} uptake is induced by monensin, there is 50% inhibition by FCCP after 60 min of incubation. This suggests that monensin can enhance Ca^{2+} transport via the flagellar plasma membrane as well as via the head plasma membrane. Till 60 min of incubation, about 50% of the Ca^{2+} taken up is accumulated in the mitochondria, and later on this Ca^{2+} is probably released from the mitochondria and transferred to the other regions of the sperm. Since we could not reveal any morphological alterations in the flagellar plasma membrane, and we did see a tremendous change in the sperm head, we suggest that the high Ca^{2+} uptake is due to the binding of Ca^{2+} to sites which have been exposed after the acrosome reaction. We are now trying to give a more direct answer to this question.

In order to obtain a better understanding of the mechanism by which monensin induces acrosome reaction, cells were incubated in medium without Na^+. Under these conditions, monensin cannot induce acrosin release or Ca^{2+} uptake, but A23187 does (see Table III). The data suggest that an Na^+/H^+ exchange across the plasma membrane is involved in the mechanism of acrosome reaction. An influx of Na^+ and efflux of H^+ will alkalize the cytosol, which is an important step in acrosome reaction mechanisms [27]. Since alkalization is not enough, we suggest that the relatively high intracellular Na^+ concentration, which has been created by this exchange [20], might operate a Ca^{2+}/Na^+ antiporter which will exchange inner Na^+ for outer Ca^{2+} [28,29]. As a result, Ca^{2+} will start to accumulate in the cells, and acrosome reaction will occur. Another possible mechanism by which monensin may enhance Ca^{2+} transport into the cell takes into consideration the existence of $(Na^+ + K^+)$-ATPase and an ATP-dependent Ca^{2+}-pump in the head of the bull and ram spermatozoa [30]. An increase in cytosolic Na^+ induced by monensin will enhance $(Na^+ + K^+)$-ATPase activity and the ATP level in the cell will drop. As a result of the ATP depletion, the ATP-dependent Ca^{2+}-pump activity is decreased and intracellular Ca^{2+} is enhanced, thus stimulating acrosome reaction as suggested by Santos-Sacchi and Gordon [31].

Since A23187 can induce acrosome reaction in the absence of Na^+ (Table III), it is suggested that Na^+ is only an intermediate to enhance H^+ efflux and Ca^{2+} influx into the cell. This $Ca^{2+}/2H^+$ ionophore can enhance efflux of H^+ and influx of Ca^{2+}, even in the absence of extracellular Na^+.

In conclusion, we suggest that any treatment which leads to intracellular alkalization and increase in intracellular Ca^{2+} concentration will result in acrosome reaction. Our data also suggest that there is no need for the sperm mitochondria to be coupled in order for the acrosome reaction to occur. From our respiration and Ca^{2+}-uptake study we know that FCCP is a very good uncoupler in ram spermatozoa (data not shown). This study shows that acrosin release and Ca^{2+} uptake can be used as a quantitative assay for the determination of acrosome reaction.

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