Is a Ca^{2+} -ATPase involved in Ca^{2+} regulation during capacitation and the acrosome reaction of guinea-pig spermatozoa?

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Summary. The Ca^{2+} -ATPase antagonists quercetin and ethacrynic acid accelerated the onset of the acrosome reaction in guinea-pig spermatozoa incubated in the continuous presence of Ca^{2+} , whereas furosemide had no effect, and sodium orthovanadate only affected sperm motility. When spermatozoa were preincubated in a ' Ca^{2+} -free' medium, quercetin and ethacrynic acid shortened capacitation time: spermatozoa incubated for 1 h in 100–200 μM -ethacrynic acid showed 60–80% acrosome reactions when Ca^{2+} was added. Such spermatozoa were able to fertilize zona-free hamster eggs. Our results therefore point to the possible involvement of a Ca^{2+} -ATPase in the regulation of intracellular Ca^{2+} in spermatozoa. Cysteine and dithiothreitol, both disulphide reducing agents, prevented the effects of quercetin and ethacrynic acid, suggesting that sulphhydryl groups may be important for the expression of Ca^{2+} -ATPase activity. Lysophosphatidylserine (LS) also prevented the stimulatory effect of ethacrynic acid, an effect similar to that shown by LS on lysophosphatidylcholine (LC). It is argued that both LS and LC could exert their action through an effect on the Ca^{2+} -ATPase.

Keywords: calcium; Ca^{2+} -ATPase; spermatozoa; capacitation; acrosome reaction; guinea-pig

Introduction

Mammalian spermatozoa need to undergo both capacitation and the acrosome reaction to be able to fertilize eggs. Although it is well established that extracellular Ca^{2+} is essential for the acrosome reaction (Yanagimachi & Usui, 1974), only recently has it been suggested that Ca^{2+} might also be needed for capacitation of spermatozoa (hamster: Yanagimachi, 1982; mouse: Fraser, 1987). It is nevertheless likely that species will differ in the minimum concentrations of extracellular Ca^{2+} needed to support capacitation.

Our understanding of Ca^{2+} regulation during capacitation and the acrosome reaction is still largely fragmentary. Ionic exchanges in mammalian spermatozoa are probably not regulated by changes in membrane potential (Rink, 1977) and voltage-gated channels do not appear to participate in Ca^{2+} influx (Roldan *et al.*, 1986). Instead, a $\text{Na}^+/\text{Ca}^{2+}$ antiport (Hyne *et al.*, 1984; Bhattacharyya *et al.*, 1986) and ion ATPases (Mrsny & Meizel, 1981; Ashraf *et al.*, 1982; Breitbart *et al.*, 1983, 1984; Usui & Yanagimachi, 1986), working alone or in conjunction, may be responsible for the regulation of the intracellular concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$).

In several cell systems, a Ca^{2+} -ATPase located in the plasma membrane helps to maintain low $[\text{Ca}^{2+}]_i$ by pumping this ion out of the cell (reviewed by Penniston, 1983; Schatzmann, 1985). In

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addition, some cell systems also have Ca^{2+} -ATPases on subcellular organelles that contribute to keeping a low $[\text{Ca}^{2+}]_i$ by pumping this ion into the organelle (e.g. sarcoplasmic reticulum: Shamoo, 1985). Using cytochemical methods, Ca^{2+} -ATPase activity has been detected in sperm membranes of different mammalian species (Gordon & Barrnett, 1967; Gordon, 1973). Further studies have identified and measured enzyme activity in sperm membranes of ram (Voglmayr *et al.*, 1969; Breitbart *et al.*, 1983), bull (Vijayasarathy *et al.*, 1980; Breitbart *et al.*, 1984), boar (Ashraf *et al.*, 1982, 1984) and man (Abla *et al.*, 1974). It has been proposed that, since a Ca^{2+} -ATPase may regulate $[\text{Ca}^{2+}]_i$ in spermatozoa, a decrease in Ca^{2+} -ATPase activity may lead to the rise in Ca^{2+} that is needed for the acrosome reaction (Santos-Sacchi & Gordon, 1982). On the other hand, Bradley & Forrester (1985) postulated that Ca^{2+} -ATPases might only be responsible for the fine tuning of Ca^{2+} levels, other mechanisms (e.g. antiporters) being the major regulators of Ca^{2+} fluxes. So far, no studies have been undertaken on intact sperm cells to elucidate whether a Ca^{2+} -ATPase is actually involved in ion regulation during capacitation and the acrosome reaction.

Several Ca^{2+} -ATPase antagonists have been described and used in different cell systems. Quercetin, a flavonoid compound, inhibits Ca^{2+} -dependent ATPase activity in sarcoplasmic reticulum (Fewtrell & Gomperts, 1977) and red blood cells (Wuethrich & Schatzmann, 1980); it also inhibits Ca^{2+} -ATPase activity in ram and boar sperm plasma membrane preparations (Breitbart *et al.*, 1983; Ashraf *et al.*, 1984). Sodium orthovanadate is an effective inhibitor of the Ca^{2+} -ATPase activity present in human and dog red blood cell plasma membranes (Ortiz & Sjodin, 1984), and adipocyte membranes (Delfert & McDonald, 1985). Finally, ethacrynic acid and furosemide inhibit a Ca^{2+} -ATPase present in renal microsomes and plasma membranes (Moore & Landon, 1979) and in membrane preparations from guinea-pig placenta (McKercher *et al.*, 1984). In addition, it has been shown that sulphhydryl groups are important for the expression of Ca^{2+} -ATPase activity (McKercher *et al.*, 1984).

We examined the ability of these putative Ca^{2+} -ATPase antagonists to influence capacitation and the acrosome reaction in guinea-pig spermatozoa.

Materials and Methods

Reagents. Inorganic salts were purchased from Mallinckrodt (St Louis, MO, USA) or Baker (Philadelphia, PA, USA). D-Glucose was purchased from Baker; sodium lactate and sodium pyruvate from Sigma Chemical Co. (St Louis, MO, USA); lysophosphatidylcholine from Avanti Biochemicals (Birmingham, AL, USA); lysophosphatidylserine, cysteine-HCl, dithiothreitol (DTT) and gentamicin sulphate from Sigma; bovine serum albumin (BSA, fraction V, fatty-acid poor) from Calbiochem (La Jolla, CA, USA); and mineral oil from Squibb and Sons (Princeton, NJ, USA).

The Ca^{2+} -ATPase antagonists quercetin, sodium orthovanadate, ethacrynic acid and furosemide were all purchased from Sigma. Stock solutions (10 mM) were prepared by first dissolving each reagent in acetone, followed by further dilution with double-distilled water, except for sodium orthovanadate which was dissolved directly in distilled water. Stock solutions were kept frozen at -20°C .

Media. The modified Tyrode's media described by Fleming & Yanagimachi (1981) and modified by Roldan *et al.* (1986) were used. The basic medium composition was: 108.76 mM-NaCl, 2.70 mM-KCl, 0.49 mM-MgCl₂, 25.07 mM-NaHCO₃, 5.56 mM-glucose, 10.00 mM-sodium lactate, 1.00 mM-sodium pyruvate, and 3 mg BSA/ml. Media with three different concentrations of Ca^{2+} were prepared: (a) a medium with no added Ca^{2+} (Ca^{2+} -free mT; strictly, this medium is Ca^{2+} -deficient, contaminated by about $25\text{ }\mu\text{M-Ca}^{2+}$, according to our measurements with a Ca^{2+} selective electrode); (b) a regular medium containing 2 mM-CaCl₂ (Regular mT); and (c) a medium containing 4 mM-CaCl₂ ($2 \times \text{Ca}^{2+}$ mT). The concentrations of NaCl were adjusted in the first and last medium to keep osmolality constant. When spermatozoa were incubated for 10 h or longer, 50 μg gentamicin sulphate/ml were included in the medium. The osmolality of all media was about 308 mosmol/kg and the pH 7.8–8.0 when incubated at 37°C under mineral oil (gas phase, pure air). In some experiments, Minimal Capacitation Medium (MCM) was used. The composition of this medium was: 102.9 mM-NaCl, 1.7 mM-CaCl₂, 25 mM-NaHCO₃, 20 mM-sodium lactate, and 0.25 mM-sodium pyruvate (Barros, 1974). Osmolality of this medium was about 302 mosmol/kg and the pH 7.9–8.2. All media were prepared immediately before use.

Preparation of spermatozoa. Spermatozoa were collected from the vas deferens and the distal segments of the cauda epididymidis of mature guinea-pig males ($>700\text{ g}$ body weight), and were suspended in 0.9% (w/v) NaCl. The

concentration of spermatozoa at this stage was approximately 3.5×10^7 /ml. Spermatozoa were used for experiments only when more than 90% were actively motile.

Examination of the effects of Ca²⁺-ATPase antagonists on capacitation/acrosome reaction of spermatozoa. Two sperm incubation methods were used to examine the effects of Ca²⁺-ATPase antagonists (Roldan *et al.*, 1986). In Method 1, spermatozoa were incubated in a Ca²⁺-containing medium, in the presence or absence of different test reagents, and both the percentages of motile spermatozoa and the incidence of the acrosome reaction were determined at various times after the start of incubation. Two different Ca²⁺-containing media, MCM or Regular mT, were used to examine whether medium composition had any effect on the response of spermatozoa to the Ca²⁺-ATPase antagonists. An aliquant of sperm suspension in 0.9% NaCl (3.5×10^7 /ml) was placed in 0.3 or 3.0 ml MCM or Regular mT which had been previously placed under mineral oil in a plastic Petri dish (Falcon Plastics, Oxnard, CA, USA). The final sperm concentration was $5-7 \times 10^6$ /ml. The preparations were incubated at 37°C. Motility of spermatozoa was evaluated using a microscope with pseudo-dark-field or phase-contrast optics. The incidence of the acrosome reaction was determined by examining at least 100 motile spermatozoa under phase-contrast optics ($\times 400$). The percentage of acrosome-reacted spermatozoa in the entire population was calculated as described by Roldan *et al.* (1986).

In Method 2 spermatozoa were incubated in the Ca²⁺-free medium (Ca²⁺-free mT) for various times, in the presence or absence of different test reagents, before they were exposed to 2 mM-Ca²⁺ (Fleming & Yanagimachi, 1981; Roldan *et al.*, 1986). Briefly, an aliquant of the sperm suspension in 0.9% NaCl (3.5×10^7 /ml) was added to 1.5 ml Ca²⁺-free mT with or without test reagents. The final concentration of spermatozoa was $5-7 \times 10^6$ /ml. Positive controls consisted of spermatozoa incubated in Ca²⁺-free mT with 85 μ g lysophosphatidylcholine/ml. After various periods of incubation at 37°C an equal volume of 2 $\times$ Ca²⁺ mT was added and motility and the acrosome reactions of spermatozoa were scored 15–20 min later.

Examination of the fertilizing capacity of spermatozoa. Spermatozoa were treated by Method 2 (see above) and at 20 min after exposure to 2 mM Ca²⁺, sperm suspensions were diluted with Regular mT to give a final sperm concentration of $6-8 \times 10^4$ cells/ml. An aliquant (0.3 ml) of this suspension was placed in a plastic dish and covered with mineral oil. The final concentration of the reagents had been reduced by 1/200 at this stage. Then 10–20 zona-free hamster eggs, prepared as described by Yanagimachi (1972), were introduced into the sperm suspension. After 1 h of incubation at 37°C, the eggs were examined for evidence of fertilization (see Yanagimachi, 1972).

Statistical analysis. Mean $\pm$ s.d. values are given throughout, and statistical analyses of the data were carried out using Student's *t* test.

Results

Effect of Ca²⁺-ATPase antagonists on capacitation/acrosome reaction of spermatozoa incubated in Ca²⁺-containing medium

When incubated in Regular mT containing 2 mM-Ca²⁺, guinea-pig spermatozoa started to undergo the acrosome reaction in about 10 h and about 25% spermatozoa were acrosome-reacted by 20 h (Fig. 1). The motility of spermatozoa decreased slightly during this incubation period. When quercetin at 1 and 10 μ M was included in the medium from the beginning of the incubation, the percentage of acrosome-reacted spermatozoa at 10 h and 20 h remained similar to control values. A slight increase was seen when 100 μ M-quercetin was added but the difference was not statistically significant (Fig. 1a). A higher concentration of quercetin (200 μ M) significantly increased the rate of acrosome-reacted spermatozoa at 10 and 20 h of incubation. Quercetin did not have any apparent effect on sperm motility. Similarly, low concentrations of ethacrynic acid did not seem to have any effect (Fig. 1b). However, a concentration of 100 μ M resulted in about 50% spermatozoa having reacted acrosomes at 10 h of incubation. A higher concentration (200 μ M) or a prolonged incubation of spermatozoa in the presence of ethacrynic acid resulted in a decline in sperm motility (Fig. 1b). Furosemide, a compound structurally related to ethacrynic acid, had no significant effect on either motility or the acrosome reaction at any concentration, even after 20 h of incubation (Fig. 1c). Finally, sodium orthovanadate was detrimental to sperm motility, without any apparent effect on the acrosome reaction (Fig. 1d).

The composition of the incubation medium appeared to influence the effect of the reagents on spermatozoa. Thus, 100 μ M-quercetin could not accelerate the onset of the acrosome reaction significantly when spermatozoa were incubated in Regular mT (Fig. 1a), but the same concentration greatly accelerated the onset of the acrosome reaction in a simpler saline medium (MCM)

Fig. 1. Effect of Ca^{2+} -ATPase antagonists on motility and the onset of the acrosome reaction of guinea-pig spermatozoa incubated in Ca^{2+} -containing (2 mM) Regular mT medium. Results are mean $\pm$ s.d. of at least 3 experiments using 3 different males. Compared with control: * $P < 0.05$; ** $P < 0.01$.

without BSA (Table 1). Ethacrynic acid also accelerated the onset of the acrosome reaction in MCM, but spermatozoa became motionless very quickly (not shown).

The importance of sulphhydryl groups in the ability of Ca^{2+} -ATPase antagonists to modify the onset of the acrosome reaction was investigated by incubating spermatozoa in the presence of antagonists and cysteine or dithiothreitol (DTT) for 10 h (Table 2). Cysteine did not affect the quercetin-stimulated onset of the acrosome reaction, but it was able to prevent the stimulatory effect of 100 μM -ethacrynic acid. On other hand, DTT was able to prevent the stimulatory ability of both quercetin (200 μM) and ethacrynic acid (100 μM). In addition, DTT was also able to prevent the deleterious effect of high concentrations of ethacrynic acid (200 μM) on sperm motility. However, the deleterious effect of sodium orthovanadate on motility was not prevented by DTT.

Effect of Ca^{2+} -ATPase antagonists on capacitation of spermatozoa incubated in Ca^{2+} -free medium

When guinea-pig spermatozoa were incubated in Ca^{2+} -free mT for 10 h and then exposed to 2 mM- Ca^{2+} , 10–30% of spermatozoa underwent the acrosome reaction (see Figs 2a, b). When lysophosphatidylcholine (85 $\mu\text{g}/\text{ml}$) was added to Ca^{2+} -free mT, most spermatozoa exhibited an acrosome reaction when Ca^{2+} was added, even after only 1 h of preincubation. Exposure of spermatozoa to lysophosphatidylcholine for 10 h had detrimental effects on sperm motility (see Fig. 2).

Since quercetin and ethacrynic acid were the only Ca^{2+} -ATPase antagonists that accelerated the onset of the acrosome reaction in the previous series of experiments, further experiments were undertaken using these two reagents. When spermatozoa were incubated in Ca^{2+} -free mT with different concentrations of quercetin for different periods of time, only a concentration of 200 μM and a preincubation time of 6 h showed a significant increase in the number of spermatozoa that underwent the acrosome reaction after Ca^{2+} addition (Fig. 2a); quercetin did not have any apparent effect on sperm motility. Ethacrynic acid showed a dose- and time-dependent effect. For example, when spermatozoa were preincubated for 1 h, there was a dose-dependent increase in the

Table 1. Effects of quercetin on the onset of the acrosome reaction of guinea-pig spermatozoa incubated in a Minimal Capacitation Medium (MCM; Ca²⁺ concentration, 1.7 mM) at 37°C under pure air

Antagonist (conc.)	Spermatozoa after 4 h incubation in MCM	
	% Motile	% with reacted acrosomes
None (control)	82 ± 4.4	18 ± 4.7
Quercetin		
1 µM	82 ± 2.6	16 ± 3.5
10 µM	82 ± 2.6	29 ± 9.6*
100 µM	77 ± 6.1	52 ± 17.8**

Results are mean ± s.d. of at least 3 experiments using 3 different males.

Compared with control: **P* < 0.05; ***P* < 0.01.

Table 2. Effects of cysteine and dithiothreitol (DTT) on the quercetin- and ethacrynic acid-stimulated onset of the acrosome reaction and motility of guinea-pig spermatozoa incubated for 10 h in the continuous presence of Ca²⁺ (regular mT, 2 mM-Ca²⁺) at 37°C under air

Antagonist (conc.)	Conc. of cysteine or DTT (mM)	Cysteine		DTT	
		% motile spermatozoa	% with reacted acrosomes	% motile spermatozoa	% with reacted acrosomes
—	0	83 ± 5.8	8 ± 8.2	87 ± 5.8	8 ± 7.1
—	2	83 ± 5.8	8 ± 9.3	87 ± 5.8	1 ± 0.6*
Quercetin (200 µM)	0	83 ± 5.8	21 ± 9.1	87 ± 5.8	31 ± 9.2
	2	77 ± 11.5	20 ± 21.2	87 ± 5.8	0*
Ethacrynic acid (100 µM)	0	73 ± 5.8	53 ± 9.3	80 ± 10.0	55 ± 10.5
	2	70 ± 10.0	7 ± 1.0**	90 ± 0	0**
Ethacrynic acid (200 µM)	0	23 ± 31.7	18 ± 31.2	2 ± 2.9	—
	2	53 ± 37.8	14 ± 12.5	87 ± 5.8**	0
Sodium ortho-vanadate (200 µM)	0	0	—	0	—
	2	0	—	0	—

Results are mean ± s.d. of 3 experiments using 3 different males.

Compared with 0 mM cysteine or DTT: **P* < 0.05; ***P* < 0.01.

number of spermatozoa that underwent the acrosome reaction upon the addition of Ca²⁺ (Fig. 2b). On the other hand, ethacrynic acid at 50 µM produced a time-dependent increase in acrosome reactions. During long preincubation times in ethacrynic acid, the motility of spermatozoa was affected (Fig. 2b). Furosemide, at similar concentrations, had no effect on either motility or the acrosome reaction (data not shown). Thus, ethacrynic acid was able to shorten specifically the time that spermatozoa had to be preincubated in Ca²⁺-free medium (capacitation) to be able to respond to millimolar levels of Ca²⁺.

Fig. 2. Effect of quercetin and ethacrynic acid on the motility and capacitation of guinea-pig spermatozoa incubated in a medium without added Ca^{2+} . Lysophosphatidylcholine (LC), 85 $\mu\text{g}/\text{ml}$, was used as positive control. After incubation in a Ca^{2+} -free mT medium for different periods of time, Ca^{2+} (final concentration: 2 mM) was added and spermatozoa examined 15–20 min later. Values are mean $\pm$ s.d. of at least 3 different experiments using 3 different males. Compared with control: * $P < 0.05$; ** $P < 0.01$.

The effects of cysteine and DTT on the lysophosphatidylcholine- and ethacrynic acid-induced capacitation are presented in Table 3. Spermatozoa preincubated with lysophosphatidylcholine for 1 h showed about 50–60% acrosome reactions upon Ca^{2+} addition. However, if cysteine or DTT were added from the beginning of the preincubation, few spermatozoa showed acrosome reactions. Similarly, spermatozoa preincubated in ethacrynic acid for 1 h showed about 70–75% acrosome reactions when exposed to Ca^{2+} , but no reactions occurred if cysteine or DTT had been present during preincubation.

Effect of lysophosphatidylserine (LS) on the Ca^{2+} -ATPase antagonist-mediated capacitation of spermatozoa

Since lysophosphatidylserine can inhibit the lysophosphatidylcholine-mediated capacitation of guinea-pig spermatozoa (Table 4), we examined whether lysophosphatidylserine also interfered

Table 3. Effects of cysteine and dithiothreitol (DTT) on the lysophosphatidylcholine (LC)- and ethacrynic acid-stimulated capacitation of guinea-pig spermatozoa incubated in a medium without added Ca²⁺†

Antagonist (conc.)	Conc. of cysteine or DTT (mM)	Cysteine		DTT	
		% motile spermatozoa	% with reacted acrosomes	% motile spermatozoa	% with reacted acrosomes
—	0	92 ± 2.9	1 ± 0.6	93 ± 2.9	1 ± 1.0
—	2	93 ± 2.9	1 ± 0.6	93 ± 2.9	0
LC (85 µg/ml)	0	73 ± 5.8	52 ± 4.0	95 ± 1.0	59 ± 4.6
	2	62 ± 16.1	9 ± 10.3*	87 ± 5.8	10 ± 2.0*
Ethacrynic acid (200 µM)	0	92 ± 2.9	70 ± 14.4	90 ± 0.0	75 ± 10.4
	2	83 ± 5.8	1 ± 1.1*	93 ± 2.9	1 ± 0.3*

Results are mean ± s.d. of 4 experiments using 4 different males.

*Compared with 0 mM cysteine or DTT: $P < 0.01$.

†Spermatozoa were incubated in Ca²⁺-free mT medium for 1 h at 37°C under air; Ca²⁺ (final concentration, 2 mM) was then added and spermatozoa examined 15–20 min later.

with the ethacrynic acid-mediated effect. When spermatozoa were incubated for 1 h in Ca²⁺-free medium with both ethacrynic acid and lysophosphatidylserine, no acrosome reactions occurred upon addition of Ca²⁺. These results clearly indicate that lysophosphatidylserine is able to inhibit the ethacrynic acid-mediated capacitation of spermatozoa (Table 4). Quercetin showed no effect

Table 4. Effects of lysophosphatidylcholine (LC), Ca²⁺-ATPase antagonists, and lysophosphatidylserine (LS) on motility and the acrosome reaction of guinea-pig spermatozoa incubated in a medium without added Ca²⁺*

At 20 min after exposure to Ca ²⁺		
Additions to Ca ²⁺ -free mT†	% motile	% with reacted acrosomes
— (control)	90 ± 0.0	3 ± 2.1
LC	83 ± 5.8	64 ± 8.0
Quercetin	90 ± 0.0	5 ± 3.0
EA	89 ± 6.3	67 ± 16.9
LC + quercetin	77 ± 5.8	59 ± 4.6
EA + quercetin	87 ± 5.8	75 ± 5.2
LC + EA	60 ± 20.0	31 ± 21.3
LC + LS	90 ± 0.0	1 ± 1.5
Quercetin + LS	92 ± 2.9	8 ± 9.6
EA + LS	92 ± 2.9	2 ± 2.4

Results are mean ± s.d. of 3 experiments using 3 different males.

*Spermatozoa were incubated in Ca²⁺-free mT for 1 h at 37°C under pure air, Ca²⁺ was then added, and spermatozoa examined 20 min later.

†LC: lysophosphatidylcholine (85 µg/ml); quercetin (200 µM); EA: ethacrynic acid (200 µM); LS: lysophosphatidylserine (40 µg/ml).

when added together with lysophosphatidylcholine or ethacrynic acid. However, the combination of lysophosphatidylcholine and ethacrynic acid produced a decline in sperm motility; this, in turn, resulted in a drop in the percentage of acrosome-reacted spermatozoa found in the whole population (Table 4).

Fertilizing ability of acrosome-reacted spermatozoa

When spermatozoa were preincubated in Ca^{2+} -free medium containing lysophosphatidylcholine or ethacrynic acid, they were capable of fertilizing zona-free hamster eggs *in vitro*. The majority of spermatozoa preincubated in quercetin for a short period of time did not exhibit an acrosome reaction when Ca^{2+} was added. The fact that these spermatozoa only fertilized a very small proportion of eggs was consistent with this finding (Table 5).

Table 5. Fertilizing capacity of guinea-pig spermatozoa preincubated with lysophosphatidylcholine (LC) or Ca^{2+} -ATPase antagonists*

Addition to Ca^{2+} -free mT	% acrosome-reacted spermatozoa after Ca^{2+} addition	No. of eggs inseminated (3 expts)	No. (%) of eggs fertilized	Mean $\pm$ s.d. sperm heads per fertilized egg
Control	< 1	50	1 (2)	1.0 $\pm$ 0.0
LC (85 $\mu\text{g/ml}$)	60 $\pm$ 17.3	56	54 (96)	4.2 $\pm$ 0.9
Quercetin (200 μM)	3 $\pm$ 1.7	47	5 (11)	1.0 $\pm$ 0.0
Ethacrynic acid (200 μM)	76 $\pm$ 5.8	52	52 (100)	3.3 $\pm$ 1.6

*Spermatozoa were incubated for 1.5 h in Ca^{2+} -free mT with different reagents; Ca^{2+} (final concentration, 2 mM) was then added and incubation continued for 20 min before insemination. Spermatozoa were allowed to interact with zona-free hamster eggs for 1 h.

Discussion

We have investigated whether a Ca^{2+} -ATPase is involved in Ca^{2+} regulation during capacitation and the acrosome reaction of spermatozoa by using Ca^{2+} -ATPase antagonists. As an active Ca^{2+} -ATPase has already been identified in sperm plasma membranes (Gordon & Barnett, 1967; Ashraf *et al.*, 1982, 1984; Breitbart *et al.*, 1983, 1984), we anticipated that Ca^{2+} -ATPase antagonists, by blocking its activity (thus preventing Ca^{2+} efflux and prompting a rise in $[\text{Ca}^{2+}]_i$), could shorten capacitation time and/or accelerate the onset of the acrosome reaction. Our results show that quercetin and ethacrynic acid, which are known to inhibit Ca^{2+} -ATPase activity in membrane preparations from different cell systems (Wuethrich & Schatzmann, 1980; McKercher *et al.*, 1984; Breitbart *et al.*, 1984), can accelerate the onset of the acrosome reaction when spermatozoa are incubated in the continuous presence of Ca^{2+} . Furthermore, these reagents can shorten capacitation time if spermatozoa are preincubated in a medium without added Ca^{2+} .

Although the reagents used in this study can inhibit Ca^{2+} -ATPases of different cell systems, only some of them had an effect on spermatozoa. Moreover, whereas some of the antagonists had a definite effect on capacitation/acrosome reaction (e.g. ethacrynic acid), others only affected sperm motility (e.g. sodium orthovanadate). It can be argued that Ca^{2+} -ATPases may vary from one cell system to another, or even at different subcellular levels (Penniston, 1983; Fischer *et al.*, 1985), in such a way that they show different sensitivities to the antagonists. On the other hand, some reagents could be acting on ATPases other than the Ca^{2+} pump. For instance, vanadate inhibits a

Na⁺,K⁺-ATPase, a proton pump, and dynein ATPase (Gibbons *et al.*, 1978; Macara, 1980) and this may account for the decrease in motility seen after exposure of spermatozoa to this reagent.

Even though ethacrynic acid was able to shorten capacitation time and to accelerate the onset of the acrosome reaction, furosemide, a structurally related compound, had no effect. Bize & Santander (1985) found that 5 mM-furosemide inhibited the spontaneous and adrenaline-induced capacitation/acrosome reaction of golden hamster spermatozoa. However, our results cannot be directly compared with those of Bize & Santander (1985) because of the differences in ionic regulatory mechanisms underlying the golden hamster and guinea-pig acrosome reactions. Furthermore, the high concentration of furosemide used by Bize & Santander (1985) could have inhibited other regulatory mechanisms, such as Na⁺-K⁺ cotransport, Na⁺,K⁺ exchange, and anion transport.

The ability of some Ca²⁺-ATPase antagonists to modify the onset of the acrosome reaction was influenced by the composition of the medium in which spermatozoa were incubated; more pronounced effects were observed in media with a simpler composition and no BSA. The reasons for this remain unclear. The efficiency of the Ca²⁺-ATPase antagonists also varied according to the concentration of Ca²⁺ in the medium. Thus, when incubated with 100 µM-ethacrynic acid, spermatozoa needed about 10 h in a medium with 2 mM external Ca²⁺ to show 50% acrosome reactions, whereas a similar percentage of spermatozoa was able to acrosome react after a preincubation of only 1 h with this reagent in a medium with low levels of Ca²⁺ (about 25 µM-Ca²⁺). A similar phenomenon occurs when spermatozoa are incubated with some lysophospholipids such as lysophosphatidylcholine (Fleming & Yanagimachi, 1981). Even in the absence of reagents, guinea-pig spermatozoa respond faster if they are first preincubated in a low Ca²⁺ medium (see Figs 1 and 3 in Fleming & Yanagimachi, 1981). The mechanisms underlying these effects await elucidation. One possible explanation is that cells incubated in low Ca²⁺ may be rendered more permeable to ions. But the opposite may also be true; capacitation could be seen as 'delayed' in a medium with high extracellular Ca²⁺ (see Fleming & Yanagimachi, 1981, for discussion). Hunter (1987) has proposed that, under natural conditions, although capacitation could be completed in a short period of time, to adjust to the time-lag existing between mating and ovulation (and hence fertilization), the female tract has mechanisms that prevent premature capacitation. It may be that spermatozoa remain unresponsive to high Ca²⁺ until they go through a 'microenvironment' of low Ca²⁺, which is present in some portions of the female tract (see Borland *et al.*, 1977). As several enzymes and membrane fusion processes are Ca²⁺-dependent, it is possible that different levels of available Ca²⁺ might be the thresholds that regulate different stages of the exocytotic process in spermatozoa.

The fact that reagents known to regulate Ca²⁺ fluxes can shorten the preincubation time of guinea-pig spermatozoa strongly suggests that Ca²⁺ does play a role in capacitation in this species. Previous work has regarded guinea-pig sperm capacitation as a phenomenon needing no external Ca²⁺, because it can occur in so-called 'Ca²⁺-free' media (Yanagimachi & Usui, 1974; Fleming & Yanagimachi, 1981; Hyne *et al.*, 1984). However, the results of the present study, together with the finding that capacitation does not occur if spermatozoa are incubated in Ca²⁺-free medium containing lysophosphatidylcholine in which residual Ca²⁺ has been chelated with EGTA (E. R. S. Roldan, unpublished results), show that guinea-pig spermatozoa do need Ca²⁺ during capacitation, even though the minimum concentration of Ca²⁺ needed might be lower than for other species. Although capacitation can occur in low Ca²⁺, spermatozoa will not undergo the acrosome reaction unless the extracellular concentration of Ca²⁺ is increased to millimolar levels. It may well be that, as mentioned above, there are different Ca²⁺ thresholds for the different steps of the exocytotic process. Small rises in [Ca²⁺]_i may be enough to activate some enzymes, and thus generate other messengers (Abdel-Latif, 1986), but higher levels of Ca²⁺ may be needed for the actual fusion of membranes (Papahadjopoulos, 1978).

The ability of ethacrynic acid to shorten capacitation time was completely prevented by lysophosphatidylserine. Serine phospholipids are known to greatly stimulate the energy-dependent Ca²⁺

transport (Noack & Greeff, 1971; Niggli *et al.*, 1981), so it could be that lysophosphatidylserine competes with ethacrynic acid by stimulating the Ca^{2+} -ATPase present in the sperm plasma membrane. Comparable systems could be those of lysophospholipids such as lysophosphatidylcholine whose stimulatory action is also prevented by lysophosphatidylserine (Fleming & Yanagimachi, 1981). How lysophosphatidylcholine works is not clear; it has been suggested that it may directly perturb the lipid components of the sperm membranes, thus leading to membrane fusion (see Yanagimachi, 1981, 1988, for review). However, since lysophosphatidylcholine can inhibit Ca^{2+} -ATPases in other cell systems (Mostafa *et al.*, 1984) it is tempting to propose that, in spermatozoa, both lysophosphatidylserine and lysophosphatidylcholine may act on the same regulatory mechanism, namely the Ca^{2+} -ATPase. Thus, lysophosphatidylcholine may inhibit a Ca^{2+} -ATPase present in the sperm plasma membrane and this may be followed by a rise in $[\text{Ca}^{2+}]_i$ and other cellular processes. Further similarities between ethacrynic acid and lysophosphatidylcholine include the fact that the effects of both reagents can be prevented by cysteine and DTT (see also Fleming *et al.*, 1986). Ethacrynic acid contains an α,β -unsaturated ketone moiety, which confers high degree of reactivity toward sulphhydryl groups (Koechel, 1981). Since free SH groups are important for the expression of Ca^{2+} -ATPase activity (McKercher *et al.*, 1984), ethacrynic acid might inhibit the sperm Ca^{2+} -ATPase by binding to -SH groups. The fact that disulphide reducing agents such as cysteine and DTT are able to prevent the effects of ethacrynic acid is consistent with this hypothesis. However, whether the mechanism of action of lysophosphatidylcholine (and its prevention by cysteine and DTT) is also related to Ca^{2+} -ATPase-SH groups remains to be elucidated.

In conclusion, how can one envisage a role for a Ca^{2+} -ATPase during capacitation and the acrosome reaction? It has been already recognized that, by continuously pumping Ca^{2+} out of the cell, a Ca^{2+} -ATPase may, alone or in conjunction with a $\text{Na}^+/\text{Ca}^{2+}$ antiporter, regulate $[\text{Ca}^{2+}]_i$ (Bradley & Forrester, 1985), but the question remains as to whether the Ca^{2+} -ATPase has a primary or secondary role during the acquisition of fertilizing ability in spermatozoa. One possible way in which a Ca^{2+} -ATPase may be involved in capacitation is if a ligand, either directly or through a signal transduction system, is able to inhibit its activity. It has been found, for example, that the activity of Ca^{2+} -ATPases can be modulated by both cAMP (Holmes, 1985) and components of the polyphosphoinositide cycle (Abdel-Latif, 1986; Putney, 1986; Kuo & Tsang, 1988). After Ca^{2+} -ATPase inactivation, $[\text{Ca}^{2+}]_i$ could then rise by passive influx and other cellular processes may follow. Capacitation is accompanied by a slow and steady rise in $[\text{Ca}^{2+}]_i$ (Singh *et al.*, 1978; Coronel & Lardy, 1987) but the acrosome reaction is a very rapid event and perhaps passive diffusion would be too slow to account for the velocity of this phenomenon. A second possibility is that the Ca^{2+} -ATPase may be important only to keep $[\text{Ca}^{2+}]_i$ low (as proposed by Bradley & Forrester, 1985). During capacitation and the acrosome reaction, no active role for a Ca^{2+} -ATPase would exist if the effectiveness of the pump was simply overridden by other mechanisms such as a reversed $\text{Na}^+/\text{Ca}^{2+}$ antiporter (Hyne *et al.*, 1984) that would allow a rapid influx of Ca^{2+} . Finally, a third possibility is that even though other mechanisms may account for Ca^{2+} influx, a simultaneous inhibition of the Ca^{2+} -ATPase may prolong or sustain the elevation of cytosolic free Ca^{2+} (Lotersztajn *et al.*, 1985). Further studies are needed to determine which of these mechanisms is at work. In any case, the finding that, by using Ca^{2+} -ATPase antagonists, the time needed for capacitation can be dramatically shortened may be useful in the preparation of spermatozoa for experimental or clinical in-vitro fertilization.

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