Sodium requirement for capacitation and membrane fusion during the guinea-pig sperm acrosome reaction

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Summary. Guinea-pig spermatozoa, incubated in a minimal culture medium (MCM-PL) containing 150 mM- Na^+ and no added K^+ , capacitate within 2 h and respond to added Ca^{2+} with maximal percentage of acrosome reactions. When spermatozoa, initially capacitated in Medium MCM-PL, were washed and resuspended in saline-based media, the motile spermatozoa showed acrosome reactions only in response to added Ca^{2+} at an extracellular pH > 7.4. In contrast, resuspension of capacitated spermatozoa into isotonic sucrose or choline-based media containing no added Na^+ and K^+ (pH 7.8) did not support acrosome reactions in response to the addition of Ca^{2+} . Examination under the electron microscope showed that these spermatozoa responded to added Ca^{2+} with swelling or cavitation of the acrosomal matrix but fusion of the plasma membrane and acrosomal membrane did not occur. Treatment of spermatozoa with monensin, a monovalent cationic ionophore, induced rapid acrosome reactions in the presence of extracellular Ca^{2+} and Na^+ . In contrast, spermatozoa incubated for 3 h in a sucrose-based Na^+ -deficient medium or Medium MCM-PL containing 10 mM-KCl failed to give a significant percentage of acrosome reactions in response to the addition of Ca^{2+} , but the addition of ouabain (0.1 mM) prevented the K^+ inhibition. Amiloride, a sodium channel blocker in various other tissues, retarded the development of acrosome reactions of spermatozoa incubated in Medium MCM-PL. Based on these data, it appears that an increase in intracellular Na^+ has a functional role in acquisition of guinea-pig sperm fertilizing ability.

Introduction

To acquire fertilizing capacity, spermatozoa must undergo the 'acrosome reaction', a fusion and vesiculation of the outer acrosomal membrane and overlying plasma membrane (Barros, Bedford, Franklin & Austin, 1967). The involvement of a regulatory mechanism consisting of, in part, changes in the movement of ions across the sperm membranes has been suggested (Schackmann & Shapiro, 1981). Like other membrane fusion events (Poste & Allison, 1973), the acrosome reaction of mammalian (Yanagimachi & Usui, 1974; Miyamoto & Ishibashi, 1975) and invertebrate (Dan, 1954, 1967) spermatozoa is completely dependent on the presence of extracellular Ca^{2+} . Altered Ca^{2+} permeability appears to represent one of the primary signals for the induction of the sperm acrosome reaction (Yanagimachi, 1975; Singh, Babcock & Lardy, 1978), although the mechanism for the change in Ca^{2+} transport is unknown. Biochemically and ultrastructurally the acrosome reaction has many similarities to the release of secretory products from intracellular granules by exocytosis (Poste & Allison, 1973). The requirement for Ca^{2+} in the stimulus–secretion of zymogen

granules is well established. Recent studies indicate that a rise in the cytoplasmic Na^+ concentration also stimulates the secretory activity of cells (Suchard, Lattanzio, Rubin & Pressman, 1982).

The acrosome reaction of sea-urchin spermatozoa requires extracellular Na^+ (Schackmann, Eddy & Shapiro, 1978). It is inhibited by an increase in extracellular K^+ (Schackmann *et al.*, 1978), is pH dependent (Collins & Epel, 1977; Hyne & Garbers, 1982) and is also accompanied by uptake of $^{45}\text{Ca}^{2+}$ and $^{22}\text{Na}^+$, as well as release of K^+ and H^+ (Schackmann & Shapiro, 1981). Induction of the sea-urchin sperm acrosome reaction leads to depolarization of the membrane potential (Schackmann, Christen & Shapiro, 1981).

The acrosome reaction in mammals occurs only after the spermatozoa have undergone a series of currently undefined biochemical changes described as 'capacitation'. An increase in the ratio of K^+ to Na^+ in the incubation medium has been shown to stimulate fertilization of rat oocytes *in vitro* (Toyoda & Chang, 1974) and a K^+ influx appears to accompany the hamster sperm acrosome reaction (Mrsny & Meizel, 1981), but whether these effects were on sperm capacitation and/or the acrosome reaction is not clear. In contrast, capacitation of guinea-pig spermatozoa is inhibited by extracellular K^+ (Rogers, Ueno & Yanagimachi, 1981). Other studies also suggest that inorganic ions have a role in the initiation and maintenance of sperm motility. The requirement of Na^+ for the maintenance of the motility of rat spermatozoa, after removal from the epididymis, has been attributed to an increase in the intracellular pH via a Na^+/H^+ exchange (Wong, Lee & Tsang, 1981).

Synchronous acrosome reactions in a large number of motile guinea-pig spermatozoa can be obtained by preincubation of spermatozoa to capacitate them in Ca^{2+} -free medium and then stimulation of the acrosome reaction by the addition of Ca^{2+} as described by Yanagimachi & Usui (1974). In the present study, experiments were undertaken to establish the minimal ionic requirements that would support the occurrence of acrosome reactions of spermatozoa that had previously been capacitated and to gain information on how Ca^{2+} transport is controlled and altered during the capacitating procedure.

Materials and Methods

Media. The basic sperm capacitating medium (MCM-PL) was a modified Ca^{2+} -free minimal culture medium of Barros (1974) containing 106 mM-NaCl, 1.0 mM- MgCl_2 , 25.1 mM- NaHCO_3 , 0.25 mM-sodium pyruvate, 20 mM-sodium lactate, penicillin (100 units/ml) and streptomycin sulphate (50 $\mu\text{g}/\text{ml}$). It was essential to include 1 mM- Mg^{2+} in all Ca^{2+} -free media to maintain sperm motility. In some experiments attempts were made to capacitate the spermatozoa by incubating the cells in a Na^+ -deficient medium (~ 45 mM- Na^+) containing 212 mM-sucrose, 1.0 mM- MgCl_2 , 25.1 mM- NaHCO_3 , 0.25 mM-sodium pyruvate, 20 mM-sodium lactate, penicillin (100 units/ml) and streptomycin sulphate (50 $\mu\text{g}/\text{ml}$) or a modified MCM-PL medium containing 10 mM-KCl.

In some experiments, after the initial preincubation in Medium MCM-PL, the spermatozoa were centrifuged twice and resuspended in medium containing 145 mM-NaCl, 1 mM- MgCl_2 and 10 mM-Tris buffered between 6.6 (301 mosmol/kg) and 8.2 (312 mosmol/kg) with 1 M-HCl at 24°C . In the experiments using Na^+ -deficient media, the medium had a similar composition except that various concentrations of choline chloride were substituted for NaCl and the pH adjusted to 7.8. The Na^+ - and K^+ -free media contained 178 mM-choline chloride or 300 mM-sucrose together with 1 mM- MgCl_2 and 10 mM-Tris buffered at pH 7.8 and no added Na^+ or K^+ . The osmolality of all media varied between 300 and 315 mosmol/kg.

Sperm preparation and incubation procedure. Adult male guinea-pigs (Dunkin-Hartley or Monash University outbred strain) of > 700 g weight were anaesthetized with ether and killed by cervical dislocation. A testis was removed, the distal portion of one cauda epididymidis attached to

the vas deferens was obtained and the spermatozoa were isolated by flushing the lumen with 0.9% (w/v) NaCl as described previously (Hyne & Garbers, 1979, 1981). The spermatozoa were washed once by centrifugation (400 g, 10 min) and resuspended in 0.9% NaCl. When Na⁺-free experiments were undertaken, the cells were collected and washed in an isotonic sucrose-based medium containing 1 mM-MgCl₂ and 10 mM-Tris, pH 7.8. Aliquants (0.1 ml) of the sperm suspension were added to 0.9 ml of the capacitating medium in sterile polystyrene tubes with caps (Falcon Plastics, No. 2003, Oxnard, California). All incubations were initiated by the addition of spermatozoa (0.5×10^7 spermatozoa/ml) to the assay mixtures. The tubes were placed horizontally on a shaker (Yankee, Clay Adams, Parsippany, New Jersey), rotating at 60 strokes per min in an incubator at 37°C with air as the atmosphere.

In some experiments, after an initial preincubation in Medium MCM-PL for 135 min (unless otherwise stated), the spermatozoa were washed twice by centrifugation (700 g, 10 min) and resuspended in a different medium. Then Ca²⁺ (5 mM, final concentration) was added and after a 10-min incubation the percentages of motile spermatozoa and of acrosome reactions were assessed.

Determination of sperm motility, capacitation and acrosome reactions. At various times during an incubation after the addition of Ca²⁺, small aliquants of the sperm suspension were transferred to a ceramic ring slide (Clay Adams) and mounted under a coverslip. At least 100 spermatozoa were examined to estimate the percentage of motile cells within the entire population of spermatozoa and the percentage of cells without a visible acrosomal cap within the motile sperm population by phase-contrast microscopy at $\times 320$. The time required for capacitation is defined here as the incubation time required before the acrosome reaction can be observed in response to the acute addition of Ca²⁺.

Addition of ionophores, ouabain and amiloride. Ionophores were dissolved in ethanol in stock solutions of 2–20 mM. The ionophores or ethanol (5 μ l) were added to culture tubes containing 900 μ l Medium MCM-PL or a medium consisting of 300 mM-sucrose, 1 mM-MgCl₂ and 10 mM-Tris (pH 7.8). In some cases, 5 mM-CaCl₂ (final concentration) were also included in the treatments. Incubations were initiated by the addition of 100 μ l of the stock sperm suspension to the culture tubes.

Amiloride (0.5 mM, final concentration) was added to 1.0 ml of a sperm suspension preincubated for 135–195 min in Medium MCM-PL and the mixture was incubated for an additional 30 min. In other experiments, amiloride was added to the medium before the addition of the spermatozoa, and the final mixture was incubated for 3 h. Ca²⁺ (5 mM, final concentration) was then added to the sperm suspension and after 10 min the sperm motility and the sperm acrosome reaction rate were assessed.

Ouabain (0.1 mM, final concentration) and/or KCl (10 mM, final concentration) was added to culture tubes containing 900 μ l Medium MCM-PL and incubations were initiated by the addition of 100 μ l of the stock sperm suspension to the culture tubes.

Acid efflux measurements during sperm acrosome reactions. Sperm suspensions (1.5 ml), initially preincubated in Medium MCM-PL for 140 min, were washed twice by centrifugation (700 g, 10 min) and resuspended in medium containing 150 mM-NaCl or 178 mM-choline chloride with 1 mM-MgCl₂ and 1 mM-NaHCO₃ as a weak buffer and then transferred to a 2.0-ml capacity temperature-control chamber fitted with a semi-micro-combination pH electrode (N. J. Jones Scientific Instruments, Melbourne, Australia). The sperm concentration was 0.5×10^7 ml and the suspension was gently stirred with a magnetic stirrer. Changes in pH in response to the addition of Ca²⁺ (5 mM, final concentration) was measured with a pH meter (N. J. Jones Scientific Instruments).

Chemicals. Ouabain octahydrate, and gramicidin S were purchased from Sigma. Amiloride hydrochloride was a gift from Merck, Sharp & Dohme (Australia) Pty Ltd and monensin (sodium salt) was a gift from Eli Lilly (Australia) & Co. Choline chloride was obtained from Hopkin and Williams (Chadwell Heath, Essex, U.K.). All common chemicals were purchased from Ajax (Melbourne, Australia) or Sigma (St Louis, Missouri, U.S.A.).

Sperm preparation for electron microscopy. Spermatozoa were fixed in 3.5% glutaraldehyde in 0.1 M-cacodylate buffer, 1.5 mM- CaCl_2 (pH 7.3) for 30 min at room temperature. Fixed spermatozoa were centrifuged (1000 g, 10 min), resuspended in 0.1 M-cacodylate (0.5 ml), transferred to a glass test-tube (18 × 5 mm) and stored at 4°C until embedded. The spermatozoa were centrifuged and the pellet post-fixed with 1% osmium tetroxide in 0.1 M-cacodylate buffer for 1 h. After a brief buffer wash, the pellets were dehydrated through a series of graded ethanol to absolute ethanol, followed by absolute acetone, infiltrated with Spurr resin and embedded in Beem capsules.

For light microscopy, survey sections of 0.5 μm were cut (Reichert OMU4 Ultracut) and stained with toluidine blue and viewed under a Leitz Dialux microscope under oil (× 1250).

For electron microscopy, gold-coloured sections were stained with uranyl acetate, followed by lead citrate and examined with a Philips 401 transmission electron microscope.

Results

Effect of pH on the response of capacitated spermatozoa to Ca^{2+}

The occurrence of acrosome reactions within 10 min of the addition of Ca^{2+} was the standard indicator of prior capacitation (Hyne & Garbers, 1979, 1981). After a preincubation period of 0.5 h in Medium MCM-PL motile spermatozoa showed acrosome reactions within 10 min of the addition of Ca^{2+} , indicating that capacitation had occurred in the absence of added Ca^{2+} . The acrosome reaction was maximal after a 2 h preincubation, when 60–80% of the motile spermatozoa responded.

Experiments were undertaken to establish the minimal ionic requirements of culture media that could not inhibit acrosome reactions of spermatozoa that had previously been capacitated by incubation in Medium MCM-PL. After incubation in Medium MCM-PL for 135 min the spermatozoa were centrifuged twice and resuspended in a saline-based medium containing 1 mM- MgCl_2 and 10 mM-Tris buffered at various pH values between pH 6.6 and 8.2. When Ca^{2+} was added, the motile spermatozoa showed acrosome reactions within 10 min only with a pH > 7.4, with a maximal response at pH > 7.8 (Text-fig. 1a). If the capacitated spermatozoa were incubated longer in medium buffered to a pH of 7.0 or less, no acrosome reactions occurred even after 1 h.

Text-fig. 1. The response of capacitated guinea-pig spermatozoa to Ca^{2+} at various extracellular pH values or Na^+ concentrations. Spermatozoa were capacitated by incubation in Medium MCM-PL for 135 min before being washed twice and re-suspended in (a) an isotonic saline-based medium containing 1 mM- MgCl_2 and 10 mM-Tris buffered at the various pH values indicated or (b) isotonic choline-based media containing various concentrations of Na^+ as indicated. Ca^{2+} (5 mM) was then added and the percentage of motile spermatozoa without acrosomal caps was assessed after 10 min. The percentage of motile spermatozoa was not different for any of the treatments and varied between 50 and 65%. Values shown are the mean ± s.e.m. of (a) 3 animals or (b) 4 animals.

Therefore, all further experiments with capacitated spermatozoa were conducted with media buffered at a pH of 7.8. It was essential to include 1 mM-Mg²⁺ in all media to maintain sperm motility in Ca²⁺-free media. The capacitated spermatozoa maintained good motility in media containing no exogenous substrates for up to 2 h.

Na⁺ requirement for the acrosome reaction

Spermatozoa initially capacitated by preincubation for 135 min in Medium MCM-PL were washed twice by centrifugation and resuspended in isotonic Na⁺ and K⁺-free choline- or sucrose-based media (containing no added Na⁺ and K⁺) buffered at pH 7.8. After the addition of Ca²⁺ (5 mM), no acrosome reactions were observed after incubation for 10 min (Table 1). Resuspending the capacitated spermatozoa in an isotonic K⁺-containing medium with Ca²⁺ but no added Na⁺ gave acrosome reactions, although the percentage was considerably more variable (Table 1). If capacitated spermatozoa were resuspended in choline-based media containing various concentrations of Na⁺ and no added K⁺ buffered at pH 7.8, the spermatozoa gave acrosome reactions in response to the addition of Ca²⁺ only when the Na⁺ concentration was > 125 mM (Text-fig. 1b). If the incubation was continued for up to 1 h after the addition of Ca²⁺ in the low Na⁺ media, no increase in the percentage of acrosome reaction was observed in any of the treatments.

Table 1. The response of capacitated spermatozoa to Ca²⁺ when suspended in various isotonic media buffered at pH 7.8

Resuspension medium	% Acrosome reactions
150 mM-NaCl	62.3 ± 4.9
150 mM-KCl	43.5 ± 17.6
178 mM-Choline chloride	<1
300 mM-Sucrose	<1

Spermatozoa were capacitated by incubation in Medium MCM-PL for 135 min before being washed twice by centrifugation and resuspended in the saline-based or Na⁺-free media indicated. The media contained in addition 1 mM-MgCl₂ and 10 mM-Tris buffered to pH 7.8. Ca²⁺ (5 mM) was then added and the percentage of motile spermatozoa without acrosomal caps was assessed after 10 min. The percentage of motile spermatozoa was not different for any of the treatments and varied between 50 and 60%. Values shown are the mean ± s.e.m. for 4 animals.

The dependence of the guinea-pig sperm acrosome reaction on extracellular Na⁺ suggested that Na⁺ transport could be involved in the activation process. To determine whether the membrane fusion was associated with a Na⁺/H⁺ exchange, capacitated spermatozoa, suspended in an isotonic medium containing no added Na⁺ and K⁺, were incubated with 25 mM-NH₄Cl and 5 mM-Ca²⁺. Ammonium is known to cause an increase in intracellular pH and can activate cells even in Na⁺-free media (Johnson, Epel & Paul, 1976; Winkler & Grainger, 1978). However, this treatment did not overcome the block to the induction of the acrosome reaction. Direct measurement of acid efflux from capacitated spermatozoa suspended in saline-based medium or medium containing no added Na⁺ showed that the addition of Ca²⁺ caused slight acidification (Δ pH = 0.1) of the extracellular medium in both treatments.

Treatment of capacitated spermatozoa suspended in Medium MCM-PL with 0.5 mM-amiloride, a sodium channel blocker in various other tissues (Salako & Smith, 1970; Smith, Macara, Levenson, Housman & Cantley, 1982), for 30 min before the addition of Ca²⁺ retarded

induction of the acrosome reactions compared with untreated spermatozoa (Text-fig. 2a). Higher concentrations of amiloride inhibited the sperm motility. If amiloride was added at the start of the incubation, the sperm acrosome reaction was reduced in a dose-dependent manner, with 0.5 mM-amiloride reducing the percentage of acrosome reactions by approximately 65% after 3 h (Text-fig. 2b).

Text-fig. 2. Effect of amiloride, a sodium channel blocker, on the guinea-pig sperm acrosome reaction. (a) Spermatozoa were capacitated by incubation in Medium MCM-PL for 135–195 min before the addition of 0.5 mM-amiloride ($\circ--\circ$) and the incubation was then continued for an additional 30 min. Control treatment ($\Delta-\Delta$) consisted of spermatozoa incubated in Medium MCM-PL with no other addition for the same time periods. Ca^{2+} (5 mM, final conc.) was then added and the percentage of motile spermatozoa without acrosomal caps was assessed at the times indicated. (b) Spermatozoa were incubated for 3 h in Medium MCM-PL in the presence of the various concentrations of amiloride indicated. Ca^{2+} (5 mM, final conc.) was added for the last 10 min of the incubation and the percentage of acrosome reactions ($\Delta-\Delta$) was then assessed. The percentage of motile spermatozoa was not different for any of the treatments and varied between 50 and 60%. Values are mean $\pm$ s.e.m. for 4 (a) and 3 (b) animals.

These results indicate that the sperm capacitation process may involve an increase in Na^+ influx. Cells suspended in an isotonic medium containing no extracellular Na^+ and K^+ may lose intracellular Na^+ due to an uncoupled efflux of Na^+ through $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Robinson & Flashner, 1979). Whether this effect contributes to the inhibition of the sperm acrosome reaction was examined by treating capacitated spermatozoa suspended in a Na^+ - and K^+ -free medium (containing no added Na^+ and K^+) with 1 mM-ouabain, a specific inhibitor of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Robinson & Flashner, 1979). However, neither this treatment nor the addition of 5 mM- K^+ to the Na^+ -free medium removed the inhibition of the acrosome reaction.

Effect of ionophores and ouabain on sperm capacitation

Guinea-pig spermatozoa incubated in Medium MCM-PL underwent rapid acrosome reactions when incubated with Ca^{2+} and monensin (30–50 μM), a monovalent cationic ionophore which catalyses Na^+/H^+ exchange across cell membranes. The acrosome reactions induced by treatment with monensin were completely dependent upon the presence of both extracellular Ca^{2+} and Na^+ and occurred within 10 min after addition of the ionophore (Text-fig. 3). The spermatozoa with acrosome reactions induced by treatment with monensin initially showed good motility, but after 20–45 min the spermatozoa were immotile. If the spermatozoa were treated with monensin in the absence of extracellular Ca^{2+} , the motile spermatozoa did not undergo rapid acrosome reactions, but maintained good motility after 45 min.

Text-fig. 3. Induction of guinea-pig sperm acrosome reactions by various concentrations of monensin. Freshly collected spermatozoa were incubated in Medium MCM-PL (+ Na⁺) or an isotonic sucrose-based medium containing no added Na⁺ and K⁺ (– Na⁺). All treatments had in addition 5 mM-Ca²⁺ (unless otherwise indicated) and monensin at the final concentrations indicated. As a control, absolute ethanol (0.5%) was added to the treatment containing no ionophore. The percentage of motile spermatozoa without acrosomal caps in each treatment was assessed after incubation for 10 min. The percentage of motile spermatozoa was not different for any of the treatments and varied between 60 and 70%. Values shown are the mean $\pm$ s.e.m. of 5 animals.

Gramicidin S (10 μ M), a monovalent cationic ionophore which shows little selectivity between Na⁺ and K⁺ (Goodall, 1970), also induced rapid acrosome reactions in a Ca²⁺-dependent manner, but the sperm motility declined rapidly.

Treatment of spermatozoa suspended in Medium MCM-PL with ouabain (0.1 mM) did not induce a shorter capacitation time compared with control spermatozoa suspended in Medium MCM-PL with a similar concentration of sodium chloride. Spermatozoa in both treatments were incubated for 0.5, 1.0, 1.5 and 2.0 h, with Ca²⁺ (5 mM, final concentration) being added for the final 10 min. The percentages of sperm acrosome reactions were similar for both treatments at all incubation times.

Spermatozoa incubated for 3 h in a sucrose-based Na⁺-deficient Medium MCM-PL (~45 mM-Na⁺) with no added K⁺ did not undergo acrosome reactions in response to the addition of Ca²⁺. If the spermatozoa were centrifuged and transferred to complete Medium MCM-PL before the addition of Ca²⁺, the motile spermatozoa still gave no acrosome reactions (data not shown). Similarly, if spermatozoa were incubated for 3 h in Medium MCM-PL containing an extra 10 mM-K⁺, the percentage of motile spermatozoa that underwent acrosome reactions in response to the addition of Ca²⁺ was significantly reduced (Table 2). However, the addition of ouabain (0.1 mM)

Table 2. The effect of K⁺ and ouabain on the guinea-pig sperm acrosome reaction

Medium	% Acrosome reactions
Control	56.7 $\pm$ 5.8
10 mM-KCl	11.0 $\pm$ 2.7
10 mM-KCl + 0.1 mM-ouabain	55.3 $\pm$ 2.7
0.1 mM-ouabain	68.3 $\pm$ 8.8

Spermatozoa were incubated for 3 h in Medium MCM-PL with or without K⁺ and/or ouabain as indicated. Ca²⁺ (final concentration 5 mM) was added for the last 10 min of the incubation and the percentage acrosome reaction was then assessed. The percentage of motile spermatozoa was not significantly different for any of the treatments and varied between 50 and 70%, although the medium containing 10 mM-KCl supported better sperm motility in comparison with the other treatments. Values shown are the mean $\pm$ s.e.m. of 3 animals.

and K^+ (10 mM) to the spermatozoa suspended in Medium MCM-PL overcame the K^+ inhibition of the sperm acrosome reaction after incubation for 3 h (Table 2).

Ultrastructural observations

Guinea-pig spermatozoa isolated from the epididymis agglutinate spontaneously to form well ordered stacks by head-to-head association (Green, 1978; Bearer & Friend, 1982). After capacitation of the spermatozoa by incubation in Medium MCM-PL for > 2 h, spermatozoa lying in mid- or para-sagittal section were scored for evidence of the acrosome reaction. Sections were initially examined under the light microscope ($\times 1250$) and were classified as (a) acrosome intact, (b) swelling of the acrosome or (c) acrosome reaction complete and acrosomal matrix no longer apparent. Spermatozoa capacitated in Medium MCM-PL showed 65–80% cells with acrosome intact, 15–25% cells with acrosomal swelling and <10% spermatozoa had reacted acrosomes. Within 30 sec of addition of Ca^{2+} to spermatozoa capacitated in Medium MCM-PL, 40–45% had intact acrosomes, 50–55% showed acrosomal swelling and <10% had reacted acrosomes. By 5 min after the addition of Ca^{2+} , <10% of spermatozoa had intact acrosomes, 30–35% showed acrosomal swelling and 50–60% had reacted acrosomes.

Upon the addition of Ca^{2+} to spermatozoa capacitated in medium MCM-PL, the initial changes in the morphology of spermatozoa undergoing acrosome reactions were characterized by the formation of membrane-bound vesicles in the acrosomal cap region as well as swelling (i.e. cavitation) of the acrosome matrix (Pl. 1, Fig. 1). As the acrosome reaction proceeded, the contents of the acrosomal matrix became less electron dense as the acrosomal material dispersed and numerous vesicles were formed, particularly posterior to the acrosomal cap where the acrosome overlies the sperm nucleus. The vesicles appeared to form by multiple fusion of the plasma membrane and outer acrosomal membrane and proceeded posteriorly to the equatorial segment and eventually detached from the sperm head (Pl. 1, Fig. 2). Thus, when the acrosome reaction was completed, all the acrosomal components were absent from the sperm head except for the inner acrosomal membrane and the equatorial segment of the acrosome.

When capacitated spermatozoa were washed and resuspended in media containing no added Na^+ and then incubated in the presence of Ca^{2+} for 5–10 min before fixation, many spermatozoa showed swelling of the acrosomal matrix (Pl. 2, Fig. 3). When sections were examined under the light microscope, these spermatozoa showed 15–25% acrosomal swelling before the addition of Ca^{2+} , 30–40% acrosomal swelling 5 min after, and 45–65% acrosomal swelling 10 min after the addition of Ca^{2+} . However, little membrane vesiculation had occurred except for a series of vesicles which formed adjacent to the inner acrosomal membrane overlying the nucleus and within the acrosomal matrix (Pl. 2, Fig. 4). These vesicles were membrane-bound and were possibly formed by vesiculation of the outer acrosomal membrane as the swollen acrosomal matrix collapsed to lie adjacent to the sperm nucleus.

There was no observation of vesicles formed by fusion of the outer acrosomal membrane with the plasma membrane, indicating that Na^+ is required for this process. If the spermatozoa were fixed 5 min after the addition of Ca^{2+} , the spermatozoa showing swelling of the acrosomal matrix appeared to have an intact plasma membrane overlying the acrosome (Pl. 2, Fig. 3); however, 10 min after the addition of Ca^{2+} many of these spermatozoa had lost the plasma membrane overlying the acrosome and the swollen acrosomal matrix was bound by the outer acrosomal membrane (Pl. 2, Figs 4, 5 & 6).

Spermatozoa capacitated in Medium-PL before being twice washed and re-suspended in a saline-based medium buffered at pH 6.6 and subsequently incubated for an additional 10 min in the presence of Ca^{2+} showed 35–40% spermatozoa with intact acrosomes, 50–60% spermatozoa with acrosomal swelling, and <10% of spermatozoa that had reacted acrosomes, when sections were examined by light microscopy. In contrast, sections of spermatozoa that were incubated for 3 h in Na^+ -deficient Medium MCM-PL (~ 45 mM- Na^+) showed 65–80% cells with intact acrosomes, 15–

PLATE 1

Response of a stack of guinea-pig spermatozoa to Ca^{2+} after incubation in Medium MCM-PL. Spermatozoa were incubated in Medium MCM-PL for 135 min, then 5 mM- Ca^{2+} (final conc.) was added and the incubation continued for an additional 10 min before fixation.

Fig. 1. In the centre is a sperm head which has not yet undergone an acrosome reaction. Immediately above and below the intact spermatozoon are two spermatozoa which are in an early stage of the acrosome reaction. The acrosomal matrix of these spermatozoa has expanded to form a large cavity, and vesiculation of the plasma membrane and outer acrosomal membrane is evident.

Fig. 2. Membrane fusion of another spermatozoon that is in the terminal stage of an acrosome reaction, showing a series of membrane-bound vesicles apparently formed by fusion of the plasma membrane overlying the outer acrosomal membrane.

PLATE 2

25% cells with acrosomal swelling and <10% spermatozoa with reacted acrosomes. Similar results were obtained in the presence or absence of added Ca^{2+} .

Discussion

This report presents evidence suggesting that one component of the capacitation process in guinea-pig spermatozoa *in vitro* requires an increase in intracellular Na^+ . This is indicated by experiments in which freshly collected spermatozoa treated with the monovalent ionophore monensin showed rapid acrosome reactions in the presence of both extracellular Ca^{2+} and extracellular Na^+ . In addition, spermatozoa incubated in an isotonic sucrose-based Na^+ -deficient medium ($\sim 45 \text{ mM-Na}^+$) for 3 h did not undergo acrosome reactions in response to the addition of Ca^{2+} , compared with spermatozoa incubated in capacitating medium containing Na^+ ($\sim 150 \text{ mM-Na}^+$). It has been demonstrated previously that sucrose does not inhibit the development of the guinea-pig acrosome reaction (Rogers & Yanagimachi, 1975).

The intracellular Na^+ concentration of freshly collected guinea-pig epididymal spermatozoa is very low ($2\text{--}3 \text{ mM-Na}^+$), but increases approximately 4-fold (12 mM-Na^+) after incubation for 2 h in physiological medium containing Na^+ and K^+ (Rink, 1977). This increase in intracellular Na^+ is even more substantial after incubation in K^+ -free medium containing ouabain, an inhibitor of $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ (Rink, 1977). At pH 7.0, a $(\text{Na}^+, \text{K}^+)\text{-ATPase}$, without divalent ion requirement, has been demonstrated cytochemically to be present on the plasmalemma that overlies the guinea-pig sperm acrosome (Gordon, Dandekar & Eager, 1978). However, treatment of the spermatozoa with ouabain when suspended in K^+ -free medium did not induce a more rapid onset of the acrosome reaction rate, possibly because the efflux of Na^+ is inhibited to about the same extent, either by the removal of K^+ or the addition of ouabain (Robinson & Flashner, 1979). Such a result is consistent with the proposal that the Na^+, K^+ pump participates in the sperm capacitation process (Mrsny & Meizel, 1981). In contrast, the addition of K^+ to capacitating medium inhibits the development of a high percentage of acrosome reactions (Rogers *et al.*, 1981) possibly because the sperm $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ can now effect Na^+/K^+ exchange and thus maintain low intracellular Na^+ concentrations. The ability of ouabain to prevent the K^+ inhibition is consistent with this proposal.

Monensin is known to catalyse an initial electrical neutral Na^+ in/ H^+ out exchange across cell membranes (Henderson, McGivan & Chappell, 1969; Reed, 1982) so it is possible that the requirement of guinea-pig spermatozoa for extracellular Na^+ during capacitation is primarily linked to an increase in intracellular pH resulting from the Na^+/H^+ exchange. However, treatment of capacitated spermatozoa suspended in Na^+ -free medium with Ca^{2+} in the presence of NH_4Cl ,

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PLATE 2

Response of capacitated spermatozoa, suspended in Na^+ -free media, to Ca^{2+} after preincubation for 135 min in Medium MCM-PL.

Fig. 3. A stack of spermatozoa suspended in medium composed of 300 mM-sucrose, 1 mM- MgCl_2 , 10 mM-Tris (pH 7.8) and 5 mM- CaCl_2 for 5 min after preincubation in Medium MCM-PL. The acrosomal matrices of these spermatozoa have expanded, but the plasma membranes appear intact.

Fig. 4. A spermatozoon suspended in medium composed of 178 mM-choline chloride, 1 mM- MgCl_2 , 10 mM-Tris (pH 7.8) and 5 mM- CaCl_2 , for 10 min after preincubation in Medium MCM-PL. The acrosomal matrix has expanded and is bound by the outer acrosomal membrane.

Fig. 5. A spermatozoon incubated in the sucrose-based medium for 10 min. The acrosomal matrix has expanded.

Fig. 6. The equatorial segment of the same spermatozoon as in Fig. 5 at higher magnification showing the loss of the plasma membrane overlying the acrosome.

which has been used to increase intracellular pH in various cell types (Winkler & Grainger, 1978), did not allow induction of the acrosome reaction. Therefore, an increase in intracellular pH is not likely to be the prime stimulator of the acrosome reaction. In addition, measurements of acid efflux from capacitated spermatozoa, suspended in saline-based medium or Na^+ -free medium, were similar in both treatments in response to the addition of Ca^{2+} .

Changes in Na^+ permeability produced by monensin could activate the sperm acrosome reaction because of an elevation of intracellular Na^+ concentrations. Moreover, amiloride, a compound that inhibits passive Na^+ transport in a number of mammalian cell types (Salako & Smith, 1970; Smith *et al.*, 1982), retarded the onset of acrosome reactions of spermatozoa incubated in capacitating medium. A reversible $\text{Na}^+/\text{Ca}^{2+}$ carrier or antiporter has been demonstrated on plasma membrane vesicles isolated from ram spermatozoa (Bradley & Forrester, 1980) and boar spermatozoa (Ashraf, Peterson & Russell, 1982), and is similar to the $\text{Na}^+/\text{Ca}^{2+}$ exchange system operating across the plasma membrane of cells from a variety of excitable tissues and erythrocytes (Reeves & Sutko, 1979; Smith *et al.*, 1982; Philipson & Nishimoto, 1982). Studies on the $\text{Na}^+/\text{Ca}^{2+}$ antiporter of cardiac sarcolemmal vesicles indicate that the intracellular Na^+ -dependent Ca^{2+} influx in an intact myocardial cell has an especially steep dependence on a Na^+ concentration between 8 and 12 mM (Philipson & Nishimoto, 1982). This is close to in-vivo intracellular Na^+ activity and indicates that small changes in intracellular Na^+ levels can have a substantial effect on Ca^{2+} influx (Smith *et al.*, 1982) and hence cell response.

Spermatozoa capacitated in Medium MCM-PL before being washed twice and resuspended in Na^+ -deficient media do not give acrosome reactions in response to addition of Ca^{2+} , as seen by phase-contrast microscopy. Examination under the electron microscope shows that some of the spermatozoa respond to the addition of Ca^{2+} and undergo swelling or cavitation of the acrosomal matrix, but no membrane fusion occurs between the plasma membrane and the outer acrosomal membrane. Cavitation of the acrosome of spermatozoa suspended in these Na^+ -deficient media appeared to occur before disruption of the plasma membrane overlying the acrosome. The results suggest that cavitation of the acrosome is a Ca^{2+} -dependent event that precedes membrane fusion during the acrosome reaction of guinea-pig spermatozoa, as suggested previously (Yanagimachi & Usui, 1974). Ca^{2+} entry possibly occurs in these capacitated spermatozoa suspended in Na^+ -deficient media as evidenced by disruption of the plasma membrane overlying the acrosome. However, extracellular Na^+ ions are required at relatively high concentration (> 125 mM) for fusion of the plasma membrane with the outer acrosomal membrane.

A working model to explain the effects of inducers such as monensin or serum (Hyne & Garbers, 1981) on the guinea-pig acrosome reaction can be postulated, based on the mechanisms discussed above. In the absence of inducer, the $(\text{Na}^+, \text{K}^+)\text{-ATPase}$ maintains a low intracellular Na^+ and high intracellular K^+ concentration by pumping Na^+ out of the cell and K^+ into the cell. The consequent Na^+ gradient can then be used to pump Ca^{2+} out of the spermatozoon by coupling the two ion movements via a $\text{Na}^+/\text{Ca}^{2+}$ antiporter. During sperm capacitation in medium containing no added K^+ or in medium supplemented with serum, reduced $(\text{Na}^+, \text{K}^+)\text{ pump}$ activity causes a sufficient increase in cytoplasmic Na^+ to reverse the direction of the $\text{Na}^+/\text{Ca}^{2+}$ antiporter. This allows Ca^{2+} to enter the spermatozoon and the increased cellular Ca^{2+} concentration activates a series of events which lead to the acrosome reaction. The demonstration of a $\text{Na}^+/\text{Ca}^{2+}$ antiporter in the guinea-pig sperm plasma membrane overlying the acrosome and a description of a functional role in the acrosome reaction would support this model.

A characteristic feature of the fluid from the cauda epididymidis of mammals, including the guinea-pig, is a low Na^+ concentration (~ 20 mM) and an acidic pH, in contrast to the composition of blood or other reproductive tract fluids (Jones, 1978). The results from these studies indicate that the low concentration of Na^+ and acidic pH of the luminal fluid would prevent the premature acquisition of fertilizing ability by spermatozoa stored in the cauda epididymidis.

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