Increased Calcium-Ion Influx is a Component of Capacitation of Spermatozoa

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Capacitation (modifications required for gamete fusion) is produced by incubating guinea-pig spermatozoa in vitro in a chemically defined medium. It is shown that during such incubation a net uptake of Ca⁺⁺ by the sperm occurs in two distinguishable phases. An initial loose association of Ca⁺⁺, possibly to surface sites, is unaffected by agents (Mg⁺⁺, inhibitors of mitochondrial function) that prevent or delay the exocytotic spermatozoal acrosome reaction. The time course of a secondary Ca⁺⁺ uptake parallels or slightly precedes the time course of the acrosome reaction. This parallelism is maintained during a variety of treatments that either expedite (local anaesthetics, ionophore A23187, Triton X-100) or delay (Mg⁺⁺, low external Ca⁺⁺) the acrosome reaction. We conclude that the secondary Ca⁺⁺ influx described herein apparently serves to link alterations of the spermatozoal membrane to subsequent contractile and secretory components of the capacitation sequence.

In the last few years it has become possible to produce, by extended incubation in vitro, a terminal maturational sequence for spermatozoa of several mammalian species (Yanagamachi & Chang, 1964; Bavister, 1969; Barros et al., 1973; Oliphant & Brackett, 1973). The modifications of the spermatozoa occurring in vitro are apparently similar to or identical with those that take place in vivo in the period between mating and fertilization and are known as capacitation (for reviews see Bedford, 1970; Barros, 1974; Chang & Hunter, 1975).

Because capacitation of guinea-pig spermatozoa in vitro does not occur in Ca⁺⁺-deficient medium, but the exocytotic acrosome reaction and motility activation rapidly and synchronously follow the re-addition of Ca⁺⁺, it was inferred that cellular alterations independent of external Ca⁺⁺ precede the more-visible Ca⁺⁺-dependent events in the capacitation sequence (Yanagamachi & Usui, 1974). That these initial alterations resulted in increased membrane permeability to Ca⁺⁺ was further suggested by the ability of a variety of membrane-directed agents to expedite motility activation and the acrosome reaction (Yanagamachi, 1975).

In the present paper we directly examine membrane permeability to Ca⁺⁺ during sperm capacitation in the presence and absence of membrane-directed agents. A primary and a secondary Ca⁺⁺ flux are identified and partially characterized. Several agents that either promote or inhibit the acrosome reaction have a corresponding effect on the secondary Ca⁺⁺ influx. We conclude that this Ca⁺⁺ influx is the component of capacitation that apparently links membrane alterations to activation of spermatozoal motility and the acrosome reaction.

Experimental

Materials

⁴⁰CaCl₂ was purchased from New England Nuclear Corp., Boston, MA, U.S.A., and fatty acid-free bovine serum albumin from Pentex Research Products, Kankakee, IL, U.S.A. CIBA Pharmaceuticals Co., Summit, NJ, U.S.A., provided nupercaine. Other chemicals were obtained from sources previously described (Babcock et al., 1975, 1976).

Methods

Spermatozoal preparation and acrosome reaction. Bovine epididymides were obtained from a local slaughterhouse. The details of isolation of spermatozoa and washing procedures are given elsewhere (Babcock et al., 1975, 1976). Freshly excised guinea-pig caudal epididymides were held under the pressure of a haemostat in a Petri dish containing 5ml of saline (0.9% NaCl) and the spermatozoa were extruded by puncturing the distal tubules with a stainless-steel needle. The spermatozoa isolated as rouleaux were dispersed by passage through a wide-bore pipette and washed with 2×20ml of saline by centrifugation at 600g (for 7min each). Either medium NKM (110mm-NaCl, 5mm-KCl, 10mm-sodium pyruvate, 10mm-morpholinopropanesulfonic acid, pH7.4) or the minimal capacitation medium (MCM) of Rogers & Yanagamachi (1975) (110mm-NaCl, 25mm-NaHCO₃, 1mm-sodium pyruvate, 1mm-CaCl₂) was used for final suspension at 1×10⁷–2×10⁷ spermatozoa/ml. All incubations were carried out in a scintillation vial at 37°C; those in minimal capacitation medium were maintained under CO₂/ air (1:39), those in NKM medium under air alone.
Periodically 200–300 cells in a small sample of the spermatozoal suspension were examined under a phase-contrast microscope to assess the proportion of motile cells and the proportion of motile cells without a visible acrosomal cap.

The calculation of percentage acrosome reaction [100×(motile spermatozoa without acrosome/total number of motile spermatozoa)] was that used by others (Yanagamachi & Usui, 1974; Rogers et al., 1976). The percentage of CO2 in the gaseous mixture, pH and temperature are important factors affecting the time course of the acrosome reaction and thus were carefully controlled. Pharmacological agents were added to the spermatozoal suspension 0.5–4min before addition of Ca2+, unless otherwise indicated.

45Ca2+ uptake measurements. Incubations were performed in the presence of 45CaCl2 (sp. radioactivity 25000 c.p.m./nmol). At intervals, triplicate samples (50 μl) of spermatozoal suspension were transferred to 9 cm Pasteur pipettes (sealed at the narrow end) containing 7% bovine serum albumin and 1 mM-Ca2+ in 2.0 ml of saline. After immediate centrifugation (1800g for 4min), the cells at the bottom were transferred by breaking the narrow end of the pipette directly into a scintillation vial. After addition of 5 ml of a Triton X-100/toluene-based scintillation fluid (Patterson & Green, 1965), the vials were shaken, cooled, and counted for radioactivity in an Isocap/300 (Nuclear–Chicago Corp., Des Plaines, IL, U.S.A.) liquid-scintillation counter. The results were corrected for the background obtained when cell-free medium was subjected to that procedure (less than 5% of that observed when spermatozoa were included). The kinetics of exchange of 45Ca2+ with non-radioactive extracellular Ca2+ were studied by a modification of the technique described by Borle (1972) for isolated kidney cells.

Ca2+-electrode studies. For these studies we used a multichannel analyzer of the type described by Pressman (1967), utilizing ion-selective electrodes (Radiometer A.S., Copenhagen, Denmark) to monitor net movements of Ca2+ in spermatozoal suspensions. The system was calibrated by addition of known amounts of Ca2+ to the cell suspension at the end of each experiment. The sensitivity of the electrode varies with the Ca2+ concentration of the medium, but the method can detect changes of approx. 1 μM when the extracellular Ca2+ is 100 μM.

Results

Acrosome reaction

Guinea-pig spermatozoa offer several advantages as a model system to study the acrosome reaction; the spermatozoa that are capacitated in simple media are capable of egg penetration (Yanagamachi, 1972; Barros et al., 1973), the morphological (see Barros, 1974) and ultrastructural (see Friend et al., 1977) aspects of the acrosome reaction have been well described, and the large dimensions of the acrosome allow an easy visual estimation of the progress of the acrosome reaction.

In the light microscope we found that the acrosome of freshly collected epididymal spermatozoa appears compact and tightly surrounds the anterior of the head. In agreement with Barros’s (1974) observations, we also found that most cells exhibited head-to-head agglutination within 5–10 min of incubation. Agglutination did not require the presence of Ca2+ or Mg2+, but either of these bivalent cations supported more vigorous flagellar motility. Spermatozoa incubated in medium devoid of Ca2+ or medium that contained both Ca2+ and 5 mM-Mg2+ remained agglutinated until the experiments were terminated at 5 h of incubation.

The acrosomes of spermatozoa incubated in minimal capacitation medium swelled and then underwent vesiculation beginning after 60–70 min of incubation. Subsequently these vesicles and residual membrane fragments were dissociated from the spermatozoa. In some cases the membranes over the swollen

<table>
<thead>
<tr>
<th>Spermatozoa and incubation media</th>
<th>With phosphate</th>
<th>Without phosphate</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+FCCP</td>
<td>-FCCP</td>
</tr>
<tr>
<td>Bovine (in NKM medium)</td>
<td>0.5 ± 0.1</td>
<td>25 ± 10</td>
</tr>
<tr>
<td>Guinea pig (in NKM medium)</td>
<td>6.0 ± 2</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Bovine (in minimal capacitation medium)</td>
<td>1.0 ± 0.2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Guinea pig (in minimal capacitation medium)</td>
<td>16.0 ± 2</td>
<td>16 ± 3</td>
</tr>
</tbody>
</table>

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acrosome formed separate vesicles or membrane whorls, which were finally ruptured and released. An increasing number of spermatozoa with activated motility were found freely swimming after 60–70 min. However, many spermatozoa remained agglutinated for as long as 100–150 min, at which time the number of motile cells without an acrosome reached a maximum.

45Ca²⁺ uptake by guinea-pig and bovine spermatozoa

Both bovine and guinea-pig epididymal spermatozoa accumulated 45Ca²⁺ during incubation in vitro in NKM medium (Table 1). Uptake of 45Ca²⁺ was stimulated by the presence of phosphate and inhibited by uncouplers of oxidative phosphorylation, and probably is the result of a mitochondrial sequestration (Babcock et al., 1975, 1978). In minimal capacitation medium, which contains the permeant anions pyruvate and HCO₃⁻, but not phosphate, guinea-pig spermatozoa accumulated 15–20 nmol of 45Ca²⁺/10⁸ cells within 20–30 min of incubation (Table 1 and Fig. 1). However, treatment with uncouplers of oxidative phosphorylation (Table 1) or inhibitors of the mitochondrial electron-transport chain (results not shown) had no effect on this initial uptake. Fig. 1 also depicts a secondary phase of 45Ca²⁺ uptake, the time course of which parallels that of the acrosome reaction.

The secondary phase of 45Ca²⁺ uptake was prevented by treatment with uncouplers of oxidative phosphorylation or by passing the cells several times through a fine-bore pipette, either of which immobilizes the spermatozoa. These control experiments suggest that the secondary 45Ca²⁺ uptake is not a result of cell damage during incubation. In all experiments it was found that as the acrosome reaction progressed, the amount of 45Ca²⁺ associated with the spermatozoa eventually decreased. It is possible that part of the Ca²⁺ is bound to the outer acrosomal or plasma membranes and then later released with the vesicles formed during the acrosome reaction.

Ionophore-induced 45Ca²⁺ uptake and acrosome reaction

A relationship between 45Ca²⁺ uptake and the acrosome reaction is further suggested from experiments (Fig. 1) in which the spermatozoa were treated with the bivalent-cation ionophore A23187 (Reed & Lardy, 1972). The ionophore elicited maximum acrosome reaction within 30–40 min, compared with 100–150 min for the control preparations. The uptake of 45Ca²⁺ was similarly expedited. Induction of the acrosome reaction did not occur if ionophore A23187 was added in the absence of Ca²⁺. Uncouplers did not block the 45Ca²⁺ uptake induced by ionophore; rather, a larger uptake was observed when uncoupler and the antibiotic were both present in the medium (results not shown).

When the Ca²⁺ concentration of the medium was varied from 0.02 to 1 mM in the presence of a fixed concentration of ionophore, two components of 45Ca²⁺ uptake were observed. When the medium contained 0.06 mM-Ca²⁺, 45Ca²⁺ uptake attained an equilibrium value within 20 min, but the acrosome reaction did not occur (Fig. 2b). When the external Ca²⁺ concentration exceeded 0.1 mM the acrosome reaction took place and was accompanied by additional 45Ca²⁺ influx. Thus the acrosome reaction is dependent on a critical concentration of exogenous Ca²⁺ even in the presence of ionophore. The additional uptake that accompanied the acrosome reaction in the presence of ionophore remained relatively constant in amount over a broad range of Ca²⁺ concentration (Fig. 2a). It is probable that the two aspects of 45Ca²⁺ uptake observed in the presence of ionophore (one associated with and one

![Fig. 1. Acrosome reaction, motility and 45Ca²⁺ uptake during capacitation of guinea-pig spermatozoa in vitro](image)
unrelated to the acrosome reaction) correspond to those observed in its absence.

Net uptake: Ca\(^{2+}\)-electrode studies

It was necessary to clarify whether the observed \(^{45}\)Ca\(^{2+}\)-influx represents a net uptake or an exchange of extracellular Ca\(^{2+}\) with intracellular Ca\(^{2+}\) pools. Attempts to measure alterations of total cellular calcium by atomic-absorption spectroscopy were unsuccessful. However, the Ca\(^{2+}\)-sensitive electrode provided sufficient sensitivity to observe net Ca\(^{2+}\) fluxes during incubations in vitro, when lower external Ca\(^{2+}\) and higher spermatozoal concentration were used. The acrosome reaction was substantially delayed under these conditions, but the continuous measurements of Ca\(^{2+}\) movements provided by the electrode were consistent with the fluxes previously observed by radioisotopic techniques. Biphasic net influxes of Ca\(^{2+}\) were observed in the absence (Fig. 3a) or presence (Fig. 3b) of ionophore A23187. When the external Ca\(^{2+}\) concentration was maintained at a value too low to support the acrosome reaction, only the early phase of uptake was observed (Fig. 3c).

Influence of some pharmacological agents on acrosome reaction and \(^{45}\)Ca\(^{2+}\)uptake

We have confirmed that extracellular Mg\(^{2+}\) inhibits the guinea-pig spermatozoal acrosome reaction (Rogers & Yanagamachi, 1976). Although 5 mM-Mg\(^{2+}\) was beneficial to survival and motility of spermatozoa, only 2–5 % of the spermatozoa underwent the acrosome reaction during incubation for 150 min under these conditions. The initial phase of \(^{45}\)Ca\(^{2+}\) uptake was unaffected, but the secondary phase was almost abolished in the presence of Mg\(^{2+}\) (Fig. 4). If Mg\(^{2+}\) was added before 60–70 min of incubation, it effectively inhibited the secondary
Fig. 4. Effect of Mg$^{2+}$ on $^{45}$Ca$^{2+}$ uptake and the acrosome reaction

Incubations were performed as in Fig. 1, except that 5 mM-Mg$^{2+}$ was added at 0 min (▲), 35 min (▼), 70 min (●) or 100 min (●). A control preparation (○) contained no Mg$^{2+}$. Values shown are means ± S.E.M. (n = 3).

Table 2. Effect of various treatments on the acrosome reaction, motility and $^{45}$Ca$^{2+}$ uptake of guinea-pig spermatozoa

| Incubation conditions | Motile cells at maximum acrosome reaction (%) | Maximum acrosome reaction (%) | Maximum $^{45}$Ca$^{2+}$ uptake (nmol/10$^8$ cells) | Time (min) when acrosome reaction
|-----------------------|-----------------------------------------------|-------------------------------|-----------------------------------------------|-------------------------------
| Control               | 40–50                                         | 60–70                         | 35 ± 4                                        | Begins                         |
| −Ca$^{2+}$            | 60–65                                         | 1–2                           | —                                            | 80–90                          |
| +Mg$^{2+}$ (5 mM)     | 60–65                                         | 2–5                           | 24 ± 3                                       | 130–150                        |
| +FCCP (5 μM)          | —                                             | —                             | 21 ± 3                                       | —                             |
| Ionophore A23187 (0.5 nmol/mg of protein) | 30–40                                       | 50–60                         | 65 ± 10                                      | 20–30                          |
| Ruthenium Red (20–40 μM) | 50–55                                   | 60–65                         | 36 ± 2                                       | 60–70                          |
| Triton X-100 (0.003%) | 25–30                                       | 70–75                         | 36 ± 2                                       | 60–70                          |
| Nupercaine (50 μM)    | 45–50                                         | 50–60                         | 37 ± 2                                       | 50–60                          |
| (400 μM)              | —                                             | —                             | 8 ± 1                                        | —                             |

Phases of $^{45}$Ca$^{2+}$ uptake and arrested the further progress of the acrosome reaction. If Mg$^{2+}$ was added after about 80–90 min of incubation, it produced little or no effect on these two parameters. Various other pharmacological agents that are known to alter Ca$^{2+}$ fluxes across biological and artificial lipid membranes were also assessed for their effects on the guinea-pig spermatozoal acrosome reaction. These results are summarized in Table 2. Inclusion of 20–40 μM-Ruthenium Red in the incubation medium slightly diminished sperm motility, but no inhibitory effect on the $^{45}$Ca$^{2+}$ uptake was observed. When compared with controls, an early incidence of the acrosome reaction was observed in the presence of Ruthenium Red. The non-ionic detergent Triton X-100 (0.003%) produced a more pronounced acceleration of the acrosome reaction in the presence, but not in the absence, of external Ca$^{2+}$.

The local anaesthetic nupercaine exhibited concentration-dependent effects on spermatozoa incubated under capacitation conditions. A low concentration of nupercaine (50 μM) expedited both the acrosome reaction and the associated $^{45}$Ca$^{2+}$ uptake. The acrosome reaction did not take place in the presence of nupercaine when Ca$^{2+}$ was absent. Higher concentrations of nupercaine considerably suppressed the flagellar movement, and soon most cells became immotile (Fig. 5a). Concentrations in excess of 400 μM caused immediate immobilization of guinea-pig spermatozoa. Nupercaine similarly suppressed motility and inhibited respiration of bovine spermatozoa suspended in minimal capacitation medium, or NKM medium adjusted to pH 8.0.
However, 1–2 mm-nupercaine stimulated the motility and respiration of bovine spermatozoa suspended in NKM medium adjusted to pH 7.4. These observations are not completely understood, but may be analogous to pH-dependent effects of local anaesthetics on contractile responses of muscle tissue (Bianchi & Bolton, 1967) that apparently result from different actions of the charged and uncharged forms of the drug on intracellular Ca\textsuperscript{2+} distributions (Chen, 1974). Alternatively these observations may simply result from an enhanced proportion of the active uncharged form present under alkaline conditions (Papahadjopoulos, 1972).

### Dilution loss and equilibrium exchange kinetics

A single wash in Ca\textsuperscript{2+}-free medium resulted in the loss of 70–80% of the radioactivity associated with spermatozoa that had completed the initial phase of uptake (Table 3). In contrast, half of the 45Ca\textsuperscript{2+} was retained by cells that had undergone secondary uptake before washing in Ca\textsuperscript{2+}-free medium. After correction for initial Ca\textsuperscript{2+} uptake, it was apparent that there was a loss of only 33% of the 45Ca\textsuperscript{2+} incrementally accumulated during the secondary phase of uptake.

The two components of Ca\textsuperscript{2+} uptake by guinea-pig spermatozoa were also distinguished by different rates of exchange of accumulated 45Ca\textsuperscript{2+} with extracellular Ca\textsuperscript{2+}. On transfer to non-radioactive medium, spermatozoa that had undergone both the initial and the secondary uptake of Ca\textsuperscript{2+} lost the label in an exponential manner, apparently from a single compartment. The half-times for exchange were approx. 13 and 38 min respectively. Thus dilution loss and equilibrium-exchange studies each indicate that the initial uptake of Ca\textsuperscript{2+} is a rather loose association, possibly representing the binding of Ca\textsuperscript{2+} to surface components of the cell, but that the properties of the Ca\textsuperscript{2+} accumulated during the secondary uptake are characteristic of a more internal location.

### Discussion

During capacitation in vivo, changes in energy metabolism in spermatozoa are manifested by increased glycolytic (Hamner & Williams, 1963) and

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**Table 3. Kinetics of equilibrium exchange of the Ca\textsuperscript{2+} accumulated during capacitation in vitro and its loss in Ca\textsuperscript{2+}-free medium**

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>45Ca\textsuperscript{2+} accumulation during incubation in MC medium (nmol of Ca\textsuperscript{2+}/10\textsuperscript{9} cells)</th>
<th>45Ca\textsuperscript{2+} loss after transfer to:</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Ca\textsuperscript{2+}-free MC medium (%): MC medium Unlabelled MC medium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>t\textsubscript{4} (min)</td>
</tr>
<tr>
<td>45 (initial phase)</td>
<td>19 ± 2 (5)</td>
<td>71 ± 2 (3)</td>
</tr>
<tr>
<td>120 (secondary phase)</td>
<td>31 ± 2 (5)</td>
<td>33 ± 6 (3)</td>
</tr>
</tbody>
</table>
respiratory (Iritani et al., 1969) rates. Various exogenously supplied energy sources increase or decrease the time required for capacitation in vitro (Bavister & Yanagamachi, 1977; Hoppe, 1976; Rogers & Yanagamachi, 1975), and inhibitors of mitochondrial function prevent the acrosome reaction of hamster spermatozoa, even though motility and other cellular functions are maintained by glycolysis (Rogers et al., 1976). Thus it was hypothesized that a particular metabolic programme may be required to achieve capacitation.

Indirect evidence (Yanagamachi, 1975; Talbot et al., 1976) suggests that increased permeability of the spermatozoal membranes to Ca\(^{2+}\) is a crucial component of capacitation that results from this required metabolic programme and that allows the subsequent occurrence of the acrosome reaction and motility activation. It was found that motility activation of bovine spermatozoa (Babcock et al., 1976) and the acrosome reaction and motility activation of guinea pig (Yanagamachi, 1975) and hamster spermatozoa (Talbot et al., 1976) rapidly follow treatment with Ca\(^{2+}\) and agents whose pharmacological actions are thought to increase membrane permeability. It was also found that removal of Ca\(^{2+}\) prevents the acrosome reaction that follows incubation in the presence (Yanagamachi, 1975) or absence (Yanagamachi & Usui, 1974) of such agents that increase membrane permeability.

We have here directly measured an initial uptake of Ca\(^{2+}\) by guinea-pig spermatozoa that is apparently unrelated to capacitation and detected a secondary net inward movement of Ca\(^{2+}\) that takes place during incubation under conditions that produce capacitation in vitro. Similar fluxes are closely associated with the acrosome reaction that takes place in the presence of several pharmacological agents that hasten the occurrence of capacitation in vitro. These fluxes were partially characterized by examining their susceptibility to various inhibitors and their equilibrium-exchange kinetics.

Although both bovine and guinea-pig spermatozoa apparently sequester Ca\(^{2+}\) into mitochondrial sites when extracellular phosphate is available (Table 1, and Babcock et al., 1975, 1976, 1978), an initial uptake of Ca\(^{2+}\) occurs during capacitation in vitro in the absence of phosphate and even in the presence of inhibitors of mitochondrial function. The exchange properties of the radioactive isotope accumulated during this period suggest that Ca\(^{2+}\) may instead initially associate with the spermatozoal surface during incubation in the minimal capacitation medium.

The Ca\(^{2+}\) that is accumulated during the secondary phase of uptake is apparently more stably associated with the spermatozoa, as evidenced by a rate of exchange with extracellular Ca\(^{2+}\) that is only one-third that for Ca\(^{2+}\) accumulated during the initial phase. The secondary Ca\(^{2+}\) uptake is also distinguished from the initial uptake by the fact that it does not occur in the presence of 5 mM-Mg\(^{2+}\) or in the presence of ionophore A23187 and low external Ca\(^{2+}\) concentrations. Although uncouplers of oxidative phosphorylation also selectively prevent the secondary influx, there is no strong evidence to suggest that the spermatozoal mitochondria represent the site of either the primary or the secondary uptake of Ca\(^{2+}\).

The importance of the secondary Ca\(^{2+}\) uptake is indicated by the observation that its time course parallels or slightly precedes that of the acrosome reaction and the observation that treatments that prevent its occurrence also prevent the acrosome reaction. It is equally important that Triton X-100, nupercaine or ionophore A23187 (plus high external Ca\(^{2+}\) concentrations) both accelerate the acrosome reaction and expedite the secondary uptake of Ca\(^{2+}\).

Ruthenium Red forms complexes with mucopolysaccharides at membrane surfaces and blocks the uptake of Ca\(^{2+}\) by isolated mitochondria (Moore, 1971), intact ascites-tumour cells (Cittadini et al., 1973; Landry & Lehninger, 1976), isolated hepatocytes (Kleineke & Stratman, 1974) and bovine spermatozoa (Babcock et al., 1976). However, Ruthenium Red promotes both secondary Ca\(^{2+}\) uptake and the acrosome reaction of guinea-pig spermatozoa. It should be noted that Ca\(^{2+}/\)Mg\(^{2+}\)-dependent adenosine triphosphatases are located in the membranes surrounding the spermatozoal acrosome (Gordon & Barnett, 1967; Gordon et al., 1975; Yanagamachi, 1975) and that movements of Ca\(^{2+}\) mediated by adenosine triphosphatases present in erythrocyte (Dormand et al., 1974) and sarcoplasmic reticulum (Vale & Carvalho, 1973) membranes are resistant to the action of Ruthenium Red.

It is generally accepted that local anaesthetics bind to and displace Ca\(^{2+}\) from acidic phospholipids (for review see Papahadjopoulos, 1972). However, the action of these drugs in biological systems is complex and may also reflect fluidizing actions on membrane lipids (Papahadjopoulos et al., 1975) and increases in the passive permeability of membranes (Browning & Nelson, 1976; DeBoland et al., 1975).

Thus it is possible that the potentiating action of low concentrations of nupercaine on the guinea-pig spermatozoal acrosome reaction may result from displacement of Ca\(^{2+}\) from an internal site such as that visualized by Friend et al. (1977), from direct increases in membrane permeability or fragility or from mediation by still unknown mechanisms. It is curious that local anaesthetics inhibit secretory responses in some other tissues (Feinstein et al., 1976; Berridge & Prince, 1975).

We can also only speculate about the site of localization and the consequences of the secondary Ca\(^{2+}\) entry that is observed during capacitation. If entry
was restricted to the acrosomal volume, high regional concentrations of Ca\(^{2+}\) might directly promote membrane fusion (Miller et al., 1976; Papahadjopoulos et al., 1977). Alternatively, such acrosomal Ca\(^{2+}\) might activate proteolytic zymogens (Meisel & Mukerji, 1975; Schluening et al., 1976) or phospholipases (Allison & Hartree, 1968; Scott & Dawson, 1968; Conway & Metz, 1976) that may themselves participate in the acrosome reaction (Meisel & Lui, 1976).

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