

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^{2+} by the sperm occurs in two distinguishable phases. An initial loose association of Ca^{2+} , possibly to surface sites, is unaffected by agents (Mg^{2+} , inhibitors of mitochondrial function) that prevent or delay the exocytotic spermatozoal acrosome reaction. The time course of a secondary Ca^{2+} 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^{2+} , low external Ca^{2+}) the acrosome reaction. We conclude that the secondary Ca^{2+} 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^{2+} -deficient medium, but the exocytotic acrosome reaction and motility activation rapidly and synchronously follow the re-addition of Ca^{2+} , it was inferred that cellular alterations independent of external Ca^{2+} precede the more-visible Ca^{2+} -dependent events in the capacitation sequence (Yanagamachi & Usui, 1974). That these initial alterations resulted in increased membrane permeability to Ca^{2+} 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^{2+} during sperm capacitation in the presence and absence of membrane-directed agents. A primary and a secondary Ca^{2+} flux are identified and partially characterized. Several agents that either promote or inhibit the acrosome reaction have a corresponding effect on the secondary Ca^{2+} influx. We conclude that this Ca^{2+} influx is the component of capacitation that apparently links membrane alterations to activation of spermatozoal motility and the acrosome reaction.

Experimental

Materials

$^{45}\text{CaCl}_2$ 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 5 ml 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 2×20 ml of saline by centrifugation at 600g (for 7 min each). Either medium NKM (110 mM-NaCl, 5 mM-KCl, 10 mM-sodium pyruvate, 10 mM-morpholinopropanesulphonic acid, pH 7.4) or the minimal capacitation medium (MCM) of Rogers & Yanagamachi (1975) (110 mM-NaCl, 25 mM- NaHCO_3 , 1 mM-sodium pyruvate, 1 mM- CaCl_2) was used for final suspension at 1×10^7 – 2×10^7 spermatozoa/ml. All incubations were carried out in a scintillation vial at 37°C; those in minimal capacitation medium were maintained under CO_2 /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 [$100 \times (\text{motile spermatozoa without acrosome} / \text{total number of motile spermatozoa})$] was that used by others (Yanagamachi & Usui, 1974; Rogers *et al.*, 1976). The percentage of CO_2 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 0.5–4 min before addition of Ca^{2+} , unless otherwise indicated.

$^{45}\text{Ca}^{2+}$ -uptake measurements. Incubations were performed in the presence of $^{45}\text{CaCl}_2$ (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- Ca^{2+} in 2.0 ml of saline. After immediate centrifugation (1800g for 4 min), 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 the above procedure (less than 5% of that observed when spermatozoa were included). The kinetics of exchange of accumulated $^{45}\text{Ca}^{2+}$ with non-radioactive extracellular Ca^{2+} were studied by a modification of the technique described by Borle (1972) for isolated kidney cells.

Ca^{2+} -electrode studies. For these studies we used a multichannel analyser of the type described by Pressman (1967), utilizing ion-selective electrodes

(Radiometer A.S., Copenhagen, Denmark) to monitor net movements of Ca^{2+} in spermatozoal suspensions. The system was calibrated by addition of known amounts of Ca^{2+} to the cell suspension at the end of each experiment. The sensitivity of the electrode varies with the Ca^{2+} concentration of the medium, but the method can detect changes of approx. 1 μM when the extracellular Ca^{2+} 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 fully 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 Ca^{2+} or Mg^{2+} , but either of these bivalent cations supported more vigorous flagellar motility. Spermatozoa incubated in medium devoid of Ca^{2+} or medium that contained both Ca^{2+} and 5 mM- Mg^{2+} 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 1. Assessment of mitochondrial and non-mitochondrial $^{45}\text{Ca}^{2+}$ uptake by bovine and guinea-pig spermatozoa. Spermatozoa were incubated in the presence of 1 mM- $^{45}\text{CaCl}_2$ in either NKM medium or minimal capacitation medium with or without 0.5 mM-sodium phosphate. Identical incubations were performed in the presence of 5 μM -carbonyl cyanide *p*-fluorophenylhydrazine (FCCP). Triplicate samples (50 μl) were removed after incubation for 20 min and processed as described in the text. The results are the means \pm S.E.M. from three experiments.

Spermatozoa and incubation media	$^{45}\text{Ca}^{2+}$ uptake (nmol/10 ⁸ cells)			
	With phosphate		Without phosphate	
	+FCCP	-FCCP	+FCCP	-FCCP
Bovine (in NKM medium)	0.5 \pm 0.1	25 \pm 10	0.6 \pm 0.1	4 \pm 2
Guinea pig (in NKM medium)	6.0 \pm 2	15 \pm 3	6.0 \pm 2	8 \pm 2
Bovine (in minimal capacitation medium)	1.0 \pm 0.2	6 \pm 2	0.6 \pm 0.1	2 \pm 1
Guinea pig (in minimal capacitation medium)	16.0 \pm 2	16 \pm 3	20.0 \pm 2	20 \pm 2

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. 2*b*). 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. 2*a*). It is probable that the two aspects of ⁴⁵Ca²⁺ uptake observed in the presence of ionophore (one associated with and one

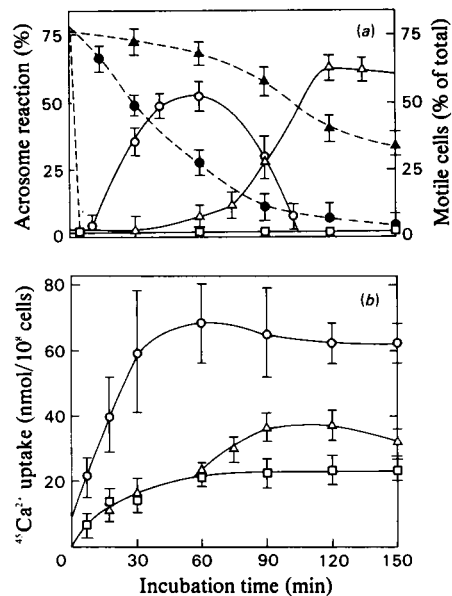


Fig. 1. Acrosome reaction, motility and ⁴⁵Ca²⁺ uptake during capacitation of guinea-pig spermatozoa *in vitro*. Spermatozoa were incubated as described in the text with the noted additions. (a) The proportion of motile cells (----) and motile cells without acrosomal caps (—) were assessed at intervals and (b) samples were removed for radioactive isotopic determinations. Symbols: △ and ▲, controls; ○ and ●, +70 nM-ionophore A23187; □, +5 μM-carbonyl cyanide *p*-fluorophenylhydrazine. Values shown are the mean ± S.E.M. (*n* = 3).

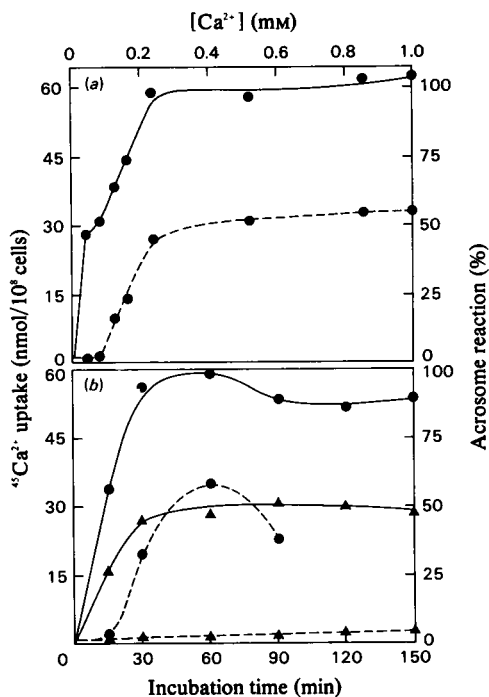


Fig. 2. Dependence of the ionophore-induced acrosome reaction and $^{45}\text{Ca}^{2+}$ uptake on extracellular Ca^{2+} concentration

Spermatozoa were incubated in the standard manner but with 70 nM-ionophore A23187 and various concentrations of Ca^{2+} in the medium. (a) $^{45}\text{Ca}^{2+}$ uptake (—) and the extent of acrosome reaction (---) were assessed after 50 min of incubation. (b) The time course of the $^{45}\text{Ca}^{2+}$ uptake (—) and the acrosome reaction (---) were followed for incubations containing 0.06 mM- Ca^{2+} (\blacktriangle) and 1 mM- Ca^{2+} (\bullet).

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 $^{45}\text{Ca}^{2+}$ 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

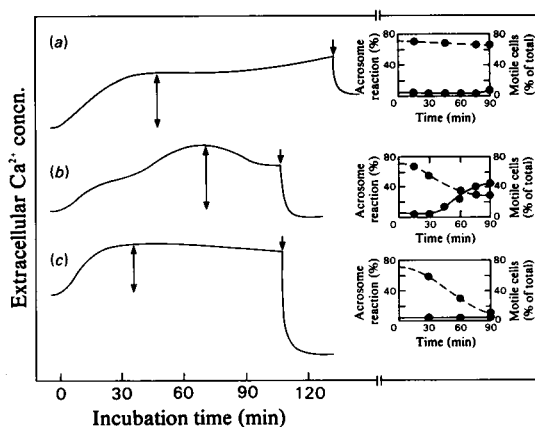


Fig. 3. Net Ca^{2+} movements during capacitation *in vitro*

The conditions of incubation were as described in the text except that extracellular Ca^{2+} was decreased to improve the sensitivity of the Ca^{2+} -selective electrode and the spermatozoal concentration was increased to produce a larger response. An upward deflection of the trace indicates removal of Ca^{2+} from the medium, and the magnitude of the removal, indicated by the double-headed vertical arrows, was: (a) 15 nmol of $\text{Ca}^{2+}/10^8$ cells; (b) 65 nmol of $\text{Ca}^{2+}/10^8$ cells; (c) 21.4 nmol of $\text{Ca}^{2+}/10^8$ cells. At intervals small samples were removed for microscopic examination; motility (---) and the acrosome reaction (—) are shown in the insets. (a) The initial external Ca^{2+} concentration was 0.2 mM. The 2 ml of medium contained 10^8 spermatozoa. (Under these conditions the acrosome reaction was substantially delayed.) (b) The medium contained 2×10^7 spermatozoa, 70 nM-ionophore A23187 and 0.1 mM- Ca^{2+} . (c) The medium contained 2×10^7 spermatozoa, 70 nM-ionophore A23187 and 0.06 mM- Ca^{2+} . Addition of 10 nmol of Ca^{2+} is indicated by a vertical arrow.

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 $^{45}\text{Ca}^{2+}$ 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 $^{45}\text{Ca}^{2+}$ 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

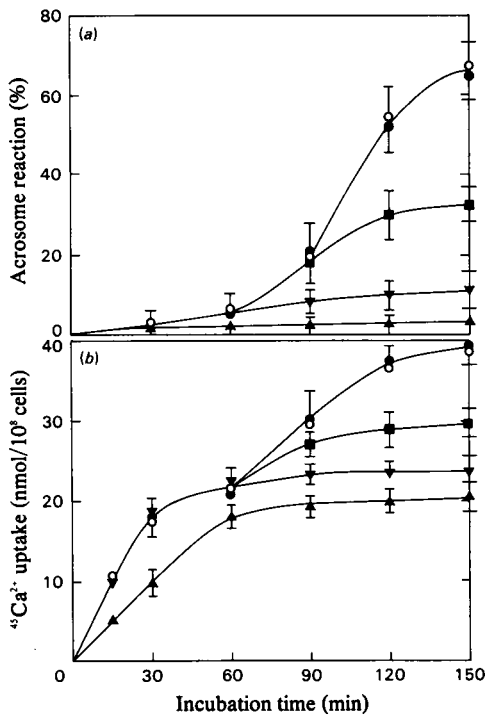


Fig. 4. Effect of Mg²⁺ on ⁴⁵Ca²⁺ uptake and the acrosome reaction

Incubations were performed as in Fig. 1, except that 5 mM-Mg²⁺ was added at 0 min (▲), 35 min (▼), 70 min (■) or 100 min (●). A control preparation (○) contained no Mg²⁺. Values shown are means ± S.E.M. (*n* = 3).

phase of ⁴⁵Ca²⁺ uptake and arrested the further progress of the acrosome reaction. If Mg²⁺ 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 Ca²⁺ 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 ⁴⁵Ca²⁺ 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 Ca²⁺.

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 ⁴⁵Ca²⁺ uptake. The acrosome reaction did not take place in the presence of nupercaine when Ca²⁺ 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.

Table 2. Effect of various treatments on the acrosome reaction, motility and ⁴⁵Ca²⁺ uptake of guinea-pig spermatozoa. Incubations were performed under standard conditions described in the text with the noted additions or deletions. Small samples were removed at 15–20 min intervals for microscopic examination or radioactive isotopic determinations throughout the 150 min duration of these experiments. Values reported are means ± S.E.M. (*n* = 3) for ⁴⁵Ca²⁺ uptake and the range for the other parameters. Abbreviation: FCCP, carbonyl cyanide *p*-fluorophenylhydrazone.

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	
				Begins	Maximum
Control	40–50	60–70	35 ± 4	80–90	130–140
-Ca ²⁺	60–65	1–2	—	—	—
+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	50–60
Ruthenium Red (20–40 μM)	50–55	60–65	36 ± 2	60–70	100–120
Triton X-100 (0.003%)	25–30	70–75	36 ± 2	60–70	100–120
Nupercaine (50 μM)	45–50	50–60	37 ± 2	50–60	90–100
(400 μM)	—	—	8 ± 1	—	—

However, 1–2mM-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^{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).

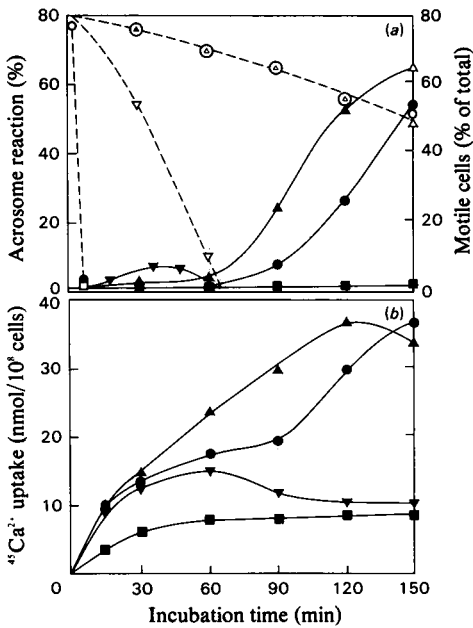


Fig. 5. Effect of nupercaine on the acrosome reaction, motility and $^{45}Ca^{2+}$ uptake

Incubations were performed as in Fig. 1. The suspensions contained nupercaine at 50 μM (Δ, ▲) 100 μM (▽, ▼) or 400 μM (□, ■). The control contained no nupercaine (○, ●). (a) Motility (----) and the acrosome reaction (—) were assessed as before. (b) $^{45}Ca^{2+}$ uptake was determined as described in the text.

Dilution loss and equilibrium exchange kinetics

A single wash in Ca^{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 $^{45}Ca^{2+}$ was retained by cells that had undergone secondary uptake before washing in Ca^{2+} -free medium. After correction for initial Ca^{2+} uptake, it was apparent that there was a loss of only 33% of the $^{45}Ca^{2+}$ incrementally accumulated during the secondary phase of uptake.

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

Table 3. Kinetics of equilibrium exchange of the Ca^{2+} accumulated during capacitation *in vitro* and its loss in Ca^{2+} -free medium. Spermatozoa (0.5×10^7 – 1.0×10^7 /ml) were incubated in minimal capacitation medium containing $^{45}Ca^{2+}$ as described in the text. After 45 and 120 min, portions were removed and the cells collected by centrifugation (at 2000 g for 1 min), then resuspended in minimal capacitation (MC) medium containing non-radioactive Ca^{2+} . At 45 s intervals thereafter, duplicate samples (50 μl) were processed as in previous experiments. Alternatively the cells were resuspended in Ca^{2+} -free medium and immediately processed as before. Mean values ± S.E.M. are shown, with the numbers of determinations in parentheses k and $t_{1/2}$ are respectively the first-order rate constants and associated half-times for exchange.

Incubation time (min)	$^{45}Ca^{2+}$ accumulation during incubation in MC medium (nmol of Ca^{2+} /10 ⁸ cells)	$^{45}Ca^{2+}$ loss after transfer to:		
		Ca ²⁺ -free MC medium (%)	Unlabelled MC medium	
			$t_{1/2}$ (min)	k (min ⁻¹)
45 (initial phase)	19 ± 2 (5)	71 ± 2 (3)	13 (2)	0.054 (2)
120 (secondary phase)	31 ± 2 (5)	33 ± 6 (3)	38 (2)	0.017 (2)

respiratory (Iritani *et al.*, 1969) rates. Various exogenously supplied energy sources increase or decrease the time required for capacitation *in vitro* (Bavister & Yanagimachi, 1977; Hoppe, 1976; Rogers & Yanagimachi, 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 (Yanagimachi, 1975; Talbot *et al.*, 1976) suggests that increased permeability of the spermatozoal membranes to Ca²⁺ 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 (Yanagimachi, 1975) and hamster spermatozoa (Talbot *et al.*, 1976) rapidly follow treatment with Ca²⁺ and agents whose pharmacological actions are thought to increase membrane permeability. It was also found that removal of Ca²⁺ prevents the acrosome reaction that follows incubation in the presence (Yanagimachi, 1975) or absence (Yanagimachi & Usui, 1974) of such agents that increase membrane permeability.

We have here directly measured an initial uptake of Ca²⁺ by guinea-pig spermatozoa that is apparently unrelated to capacitation and detected a secondary net inward movement of Ca²⁺ 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 Ca²⁺ into mitochondrial sites when extracellular phosphate is available (Table 1, and Babcock *et al.*, 1975, 1976, 1978), an initial uptake of Ca²⁺ 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 Ca²⁺ may instead initially associate with the spermatozoal surface during incubation in the minimal capacitation medium.

The Ca²⁺ 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 Ca²⁺ that is only one-third of that for Ca²⁺ accumulated during the initial phase.

The secondary Ca²⁺ uptake is also distinguished from the initial uptake by the fact that it does not occur in the presence of 5mM-Mg²⁺ or in the presence of ionophore A23187 and low external Ca²⁺ 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 Ca²⁺.

The importance of the secondary Ca²⁺ 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 Ca²⁺ concentrations) both accelerate the acrosome reaction and expedite the secondary uptake of Ca²⁺.

Ruthenium Red forms complexes with mucopolysaccharides at membrane surfaces and blocks the uptake of Ca²⁺ 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 Ca²⁺ uptake and the acrosome reaction of guinea-pig spermatozoa. It should be noted that Ca²⁺/Mg²⁺-dependent adenosine triphosphatases are located in the membranes surrounding the spermatozoal acrosome (Gordon & Barnett, 1967; Gordon *et al.*, 1975; Yanagimachi, 1975) and that movements of Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ 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 (Meizel & 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 (Meizel & Lui, 1976).

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