CALCIUM IONS AND CONTROL OF THE MOTILITY OF SEA URCHIN SPERMATOOZOA

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Summary. Both excess and insufficient calcium ions in the medium adversely affected the swimming performance of sea urchin spermatozoa. Sperm cells suspended in calcium-free artificial sea water swam at less than half the speed of those suspended in artificial sea water containing the optimum of 9 mmol CaCl_2/l . Excess Ca^{2+} added to natural sea water decreased motility by 20% at 36 mM (four times the normal amount) while the same amount of CaCl_2 in artificial sea water depressed motility by 45%. The chelating agent, EGTA, caused greater depression than equivalent amounts of EDTA, though at millimolar concentration or higher both caused complete cessation of motility with the flagella in a rigidly extended position. If LaCl_3 was added to artificial sea water containing 9 mM- CaCl_2 , it caused an even greater depression of motility. The effects were detectable at 50 nmol LaCl_3/l and complete inhibition occurred at 20 $\mu\text{mol}/\text{l}$. The flagella ceased beating in a coiled seemingly 'relaxed' state. Sperm motility appears to require maintenance of Ca^{2+} gradients, reversible sequestration and release of Ca^{2+} , and Ca^{2+} -dependent metabolic regulation of the flagellar contractile system.

INTRODUCTION

Changing patterns of sperm cell metabolism and motility usually reflect changes in the environmental condition. Heavy metal ions and sulphydryl reagents influence the duration and speed of swimming as well as respiration and glycolysis. There have been occasional reports that calcium ions also adversely affect mammalian sperm cell activity. Lardy & Phillips (1943) found that an increase in the calcium content of the suspension medium inhibited the respiration and glycolysis of bull spermatozoa. Quinn, White & Voglmayr (1970) recently reported that an excess of extracellular calcium was inhibitory to the motile activity of ram spermatozoa. Bredderman & Foote (1971), using cell volume as an index, found that excess calcium caused

both structural and permeability changes in bovine spermatozoa. McGrady & Nelson (1972) observed that excess calcium in the diluent reversed the polarity of the bull sperm cell membrane potentials from negative inside to positive inside.

A considerable amount is known about the effects of heavy metal ions on the respiration, glycolysis and motility of the spermatozoa of invertebrates (Barron, Nelson & Ardao, 1948; Rothschild & Tuft, 1950; Rothschild & Tyler, 1954; Young & Nelson, 1974), but there are relatively few data available on the effect of calcium ions on the activity of these spermatozoa. While Tyler (1953) reported that changes in the concentration of external calcium had no effect on the metabolism of *Strongylocentrotus purpuratus* spermatozoa, he did not investigate the effect of calcium ions on their motility.

In this study, the effects of alterations in the concentration of environmental calcium ions on the motile activity of *Arbacia punctulata* spermatozoa have been examined.

MATERIALS AND METHODS

Male *Arbacia punctulata* were induced to spawn by injection of isotonic KCl (0.53 M) into the perivisceral cavity. The shed spermatozoa were collected under filtered sea water, centrifuged in a clinical centrifuge for 3 min at 1000 g and the supernatant fluid was discarded. The packed spermatozoa were kept at 4°C and were used within 4 hr of collection. Immediately before the motility rating tests, samples of the spermatozoa were adjusted to a final concentration of $10^7 \pm 10\%$ spermatozoa/ml (optical density of 0.500 at 540 nm).

A 5-ml vol. of a suspension of spermatozoa in artificial sea water (ASW) was added to each of several round colorimeter tubes containing either: (1) 0.5 ml distilled water or 0.5 ml of 90 mM-CaCl₂, (2) 0.5 ml of 1% formaldehyde to kill the cells, or (3) 0.5 ml of the reagent being tested. The tube contents were mixed thoroughly by inverting twice.

The swimming speed of spermatozoa, linearly orientated in a mild centrifugal field (120 g), was determined by measuring the change in optical density and correcting for the sedimentation of the formalin-killed cells (Nelson, 1972). All experimental procedures were carried out in an air-conditioned room (22 to 23°C) and initial swimming speeds were obtained within 5 min of dilution of the spermatozoa.

Unbuffered calcium-free ASW (Cavanaugh, 1956) obtained from the chemical room of the Marine Biological Laboratory (MBL) was buffered with 0.18 mg NaHCO₃/l immediately before use, except when lanthanum chloride was included in the experimental medium.

The MBL calcium-free artificial sea water contained (per litre) 25.55 g NaCl, 9.0 mmol KCl, 22.94 mmol MgCl₂ and 25.50 mmol MgSO₄, and 0.18 mg NaHCO₃/l was added just before use. The reagents tested included calcium chloride (CaCl₂), 0 to 36 mM; lanthanum chloride (LaCl₃, obtained from K & K Laboratories, Inc., Plainview, New York), 50 nM to 100 µM; ethylenediamine tetraacetic acid (EDTA) disodium salt (Sigma Chemical Co.), 0.1 mM to 4.5 mM; ethyleneglycol-bis (β-amino-ethyl ether) N,N'-tetraacetic acid

(EGTA) (Sigma Chemical Co.), 0.1 to 4.5 mM. The solutions of EDTA and EGTA were adjusted with NaOH so that a 1:10 dilution in filtered sea water gave a final pH of 7.8. Suspensions of the spermatozoa were observed with an American Optical Phase-Star microscope at a magnification of $\times 400$.

TEXT-FIG. 1. The effect of CaCl_2 on the swimming speed of *Arbacia punctulata* spermatozoa. The CaCl_2 was added to (●) Ca^{2+} -free artificial sea water (Marine Biological Laboratory, Woods Hole) or to (▲) filtered 'natural' sea water from Woods Hole containing $10^7 \pm 10\%$ spermatozoa/ml. The points and ranges represent the mean and standard deviations of individual determinations made on spermatozoa collected from a minimum of four individual sea urchins. The temperature was 22 to 23°C.

RESULTS

Calcium chloride

The importance of calcium ions for the motility of *Arbacia punctulata* spermatozoa became evident as the concentrated sperm cells were diluted into buffered Ca^{2+} -free ASW to which various amounts of CaCl_2 were added. Since Woods Hole sea water contains approximately 9 mM calcium ions (Cavanaugh, 1956), the swimming speed of the spermatozoa in ASW containing this concentration of Ca^{2+} was referred to as the control motility.

Text-figure 1 shows the motile activity of sea urchin spermatozoa suspended in bicarbonate-buffered ASW which had been brought to CaCl_2 concentrations ranging from 0 to 36 mmol/l. After incubation for 10 min in the medium with no added calcium, the swimming speed of the spermatozoa was half that of spermatozoa incubated in ASW containing 9 mmol CaCl_2 /l for an equal

period of time. As the concentration of Ca^{2+} was increased, the rate of movement increased and reached a maximum when the concentration of calcium was 9 mM. Further additions of CaCl_2 to the ASW resulted in a gradual decline in motility and in the presence of 36 mM Ca^{2+} , four times that of the control samples, the swimming speed fell to 55% of the control values. The pH ranged from 6.67 (in Ca^{2+} -free) to 6.74 in the presence of 36 mM- CaCl_2 .

The % control motility of spermatozoa exposed to various CaCl_2 concentrations did not materially alter when the period of incubation was increased from 10 to 25 min.

The motility of spermatozoa suspended in filtered sea water to which excess calcium ions were added was compared with that of spermatozoa in ASW (Text-fig. 1). Increasing the Ca^{2+} level of the 'natural' filtered sea water to final concentrations of 12 or 15 mmol/l had no significant effect on the motility of the spermatozoa which continued to swim at or near control rates. Although the swimming speed of the spermatozoa declined with further increases in the Ca^{2+} concentration of the filtered sea water, the motility declined less abruptly than when the sperm cells were suspended in ASW containing equivalent amounts of calcium. Doubling the Ca^{2+} ion concentration of filtered sea water to 18 mM caused a negligible decrease of 4% in sperm motility compared to an 18% decrease in ASW containing 18 mmol CaCl_2 /l. At the highest concentration of calcium chloride tested (36 mM), the swimming speed of spermatozoa in filtered sea water decreased by 20% while in ASW, the motility decreased by 45% below the control rate. (Filtered sea water varied by no more than ± 0.04 pH units on addition of the CaCl_2 .)

EGTA and EDTA

To determine the extent to which changes in sperm motility could be related directly to changes in environmental calcium, EGTA was used to lower the calcium ion concentration of filtered sea water. Text-figure 2 shows the response of the sea urchin spermatozoa in filtered sea water containing 0.1 to 4.5 mmol EGTA/l. In concentrations of EGTA up to 0.3 mM, motility declined relatively slowly, but increasing the concentration of EGTA from 0.3 mM to 0.5 mM decreased the swimming speed from 60% to 30% of the control motility. Further small increments of EGTA caused further decrease in swimming speed and motile activity had virtually ceased in 4.5 mM-EGTA. Examination of the *Arbacia* spermatozoa under phase-contrast microscopy revealed that the spermatozoa were completely immotile and the flagella were rigidly extended. There was no agglutination of the spermatozoa, but attempts to restore motility to the immobilized spermatozoa by the addition of calcium ions following treatment with EGTA were unsuccessful.

The motile activity of the spermatozoa remained consistently higher in EDTA than in EGTA at all the concentrations of the chelating agents tested at less than 1 mM (Text-fig. 2). In 0.5 mmol/l, the swimming speed was measured at 65% of the control values in EDTA while in EGTA, spermatozoa collected from the same sea urchin swam at only 13% of the control speed. Both EDTA and EGTA were equally effective in abolishing sperm motility in concentrations in excess of 1 mmol/l.

TEXT-FIG. 2. The effect of EGTA and EDTA on the swimming speed of *Arbacia punctulata* spermatozoa. The EGTA (■) or EDTA (●) was added to filtered Woods Hole sea water containing $10^7 \pm 10\%$ spermatozoa/ml. The broken line represents the Ca^{2+} content of Woods Hole sea water. The points and ranges represent the mean and standard deviations of separate determinations made on spermatozoa collected from at least three individual sea urchins. The temperature was 22 to 23°C.

TEXT-FIG. 3. The effect of LaCl_3 on the swimming speed of *Arbacia punctulata* spermatozoa. The LaCl_3 was added to artificial sea water (Marine Biological Laboratory, Woods Hole) containing 9 mM- CaCl_2 and $10^7 \pm 10\%$ spermatozoa/ml. The broken line represents the Ca^{2+} content of Woods Hole sea water. The points and ranges represent the mean and standard deviations of separate determinations made on spermatozoa collected from a minimum of four individual sea urchins. The temperature was 22 to 23°C.

Lanthanum chloride

To determine the extent to which calcium conductance may be involved in regulation of sperm motility, spermatozoa were suspended in solutions of unbuffered ASW containing 9 mM-CaCl₂ and various concentrations of LaCl₃ from 50 nM to 100 mM. No bicarbonate was added to the ASW in these experiments in order to avoid precipitation of the lanthanum. (The pH varied between 6.44 and 6.36 over a concentration range of 10⁻⁴ to 10⁻⁸ M-LaCl₃.) Text-figure 3 shows the % control swimming speed plotted against lanthanum ion concentration. Swimming speed decreased with increased La³⁺ concentration. In 50 nM-LaCl₃, the treated sperm cells and the control sperm cells swam at the same speed, while 10 μM-La³⁺ decreased motility to 52% of the control value. At a lanthanum concentration of 20 μmol/l, motility ceased entirely. Microscopic examination of the immotile *Arbacia* spermatozoa showed large numbers of spermatozoa clumped together tail-to-tail with many of the flagella coiled around one another.

DISCUSSION

Calcium is essential for the maintenance of normal membrane permeability of a variety of cells (Manery, 1969; Miledi, 1971), is intimately involved in the control of contractile processes (Heilbrunn & Wiercinski, 1947; Ebashi, 1972) and is important in the regulation of cell metabolism (Lardy & Phillips, 1943; Quinn *et al.*, 1970). Extracellular calcium is responsible for modulation of ciliary movement in *Paramecium aurelia* (Kung & Naitoh, 1973) and plays a similar rôle for the ciliated epithelial cells in the oviduct of *Necturus maculosus* (Murakami & Eckert, 1972). These investigators suggest that calcium acts indirectly on one or more steps in the pathway of energy metabolism; membrane stimulation increases the intracellular calcium concentration.

The motile activity of sea urchin spermatozoa also depends critically on the concentration of environmental calcium ions. In these experiments on motility effects alone, we have not attempted to specify directly whether calcium exerts its influence at the membrane, on the glycolytic enzymes, or on the contractile apparatus itself. Since Mg²⁺ and ATP will reactivate 'demembranated' spermatozoa (Brokaw & Benedict, 1968) it seems fair to conclude that the contractile apparatus cannot by itself respond to the calcium treatments in the manner observed. The cell membrane (Manery, 1969; Miledi, 1971) or the metabolic machinery (Lardy & Phillips, 1943; Quinn *et al.*, 1970) of the cell must also contribute to these results.

Sea urchin spermatozoa exhibit a monophasic response to increases in the calcium ion above the usual environmental concentration while sperm cells suspended in calcium-free ASW swim at approximately half the rate of control spermatozoa. This may be partly attributed to the fact that dilution into a calcium-free medium provokes the loss of calcium ions from sites on the surface of the spermatozoa which are essential not only for membrane integrity but for maintenance of calcium gradients between the sperm cell surface and the interior. When calcium is added to calcium-free ASW, motility increases to a maximum in 9 mM-CaCl₂ and then begins to decline as the calcium ion con-

centration is further increased above normal in either artificial or natural sea water.

The addition of excess Ca^{2+} to filtered 'natural' sea water has a less adverse effect on sperm motility than does the addition of excess calcium to ASW, but even in filtered sea water motility begins to decline when the calcium ion concentration is increased to twice its normal value (Text-fig. 1). Barron, Gasvoda & Flood (1949) reported that ASW does not support sea urchin sperm motility as well as does 'natural' sea water which may contain protective substances in trace amounts.

The ability of EGTA drastically to reduce the swimming speed of dilute suspensions of *Arbacia* spermatozoa indicates that a critical amount of calcium must be necessary for optimum motility. Whether calcium exerts control of the contractile process through sites at the membrane or by its effects on cell metabolism, e.g. ATP synthesis, has not yet been determined.

The chelator, EDTA, sustains the motility of *Arbacia* spermatozoa at somewhat higher rates than equimolar concentrations of EGTA. This is probably due to the fact that EDTA interacts with magnesium as well as with calcium while EGTA interacts preferentially with calcium and there is approximately five times as much Mg^{2+} as Ca^{2+} in Woods Hole sea water (Cavanaugh, 1956). More EDTA than EGTA may therefore be required to reduce the calcium ion concentration to a level below that critical for sustained motility. Reduction of the Ca^{2+} content about tenfold below normal either by washing the spermatozoa in Ca-free sea water or by titration in EDTA or EGTA (about 1 mmol/l) almost abolishes sperm motility.

The lanthanum ion, which acts by interfering with calcium movements across cell membranes that normally undergo increased calcium conductance during excitation (Van Breeman & De Weer, 1970; Miledi, 1971; Van Breemen, Farinas, Gerba & McNaughton, 1972), is an even more potent inhibitor of sea urchin sperm motility than EGTA and EDTA. Concentrations of lanthanum as low as 100 nM adversely affect *Arbacia* sperm swimming speed (Text-fig. 3). At a concentration of 20 μM La^{3+} , the spermatozoa become immotile and their flagella can be seen microscopically to be in a highly coiled state intertwined in large clumps. Lanthanum begins to exert its effects at concentrations about three orders of magnitude lower than either EDTA or EGTA. We may speculate that lanthanum competes with calcium for attachment to specific membrane sites or that lanthanum itself binds allosterically at membrane sites altering the physical or chemical characteristics of the membrane. Reversible release of calcium from sequestration in membrane loci appears to be responsible for the control of enzyme activity or for the initiation and control of the interaction of the contractile components of the sea urchin sperm flagellum. By blocking either Ca^{2+} -release or -binding, lanthanum acts more powerfully than the chelating agents which appear to combine with Ca^{2+} stoichiometrically, the possibility that La^{3+} may prevent ATP depletion is suggested by the 'relaxed' appearance of the sperm flagella.

Using motility as an index of the metabolic and structural status of *Arbacia punctulata* spermatozoa, the present report shows that critical levels of calcium ions must be maintained and that intracellular levels apparently depend

critically on the calcium ions present in the environment for optimum motility. In contrast, however, to the biphasic dose-dependent effects of other divalent cations (Young & Nelson, 1974) and of neurochemical agents (Nelson, 1972), changing the Ca^{2+} concentration does not increase sperm motility above the control level.

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