POLYMERIZATION OF ACTIN

IV. Role of Ca^{++} and H^+ in the Assembly of Actin and in Membrane Fusion in the Acrosomal Reaction of Echinoderm Sperm

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ABSTRACT

When *Pisaster*, *Asterias*, or *Thyone* sperm are treated with the ionophore A23187 or X537A, an acrosomal reaction similar but not identical to a normal acrosomal reaction is induced in all the sperm. Based upon the response of the sperm, the acrosomal reaction consists of a series of temporally related steps. These include the fusion of the acrosomal vacuole with the cell surface, the polymerization of the actin, the alignment of the actin filaments, an increase in volume, an increase in the limiting membrane, and changes in the shape of the nucleus. In this report, we have concentrated on the first two steps in this sequence. Although fusion of the acrosomal vacuole with the cell surface requires Ca^{++}, we found that the polymerization of actin instead appears to be dependent upon an increase in intracellular pH. This conclusion was reached by applying to sperm A23187, X537A, or nigericin, ionophores which all carry H^+ at high affinity, yet vary in their affinity for other cations. When sperm are suspended in isotonic NaCl, isotonic KCl, calcium-free seawater, or seawater, all at pH 8.0, and the ionophore is added, the actin polymerizes explosively and an efflux of H^+ from the cell occurs. However, if the pH of the external medium is maintained at 6.5, the presumed intracellular pH, no effect is observed. And, finally, if egg jelly is added to sperm (the natural stimulus for the acrosomal reaction) at pH 8.0, H^+ is also released. On the basis of these observations and those presented in earlier papers in this series, we conclude that a rise in intracellular pH induces the actin to disassociate from its binding proteins. Now it can polymerize.

KEY WORDS actin polymerization · acrosomal reaction · H^+ · Ca^{++} · intracellular pH · sperm · exocytosis

In most nonmuscle cells, in contrast to skeletal muscle, organized arrays of actin filaments are transitory, appearing at the appropriate place and time only to disappear at a later developmental stage. Frequently, these stages are separated by only a minute or two as, for example, during cytokinesis in animal cells (see reference 25). Thus, the cell must be capable of controlling the rapid assembly and disassembly of actin. It must

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also regulate the formation of filament bundles and associations with membranes. Yet, purified actin from nonmuscle cells will polymerize explosively and completely at what are considered physiological pH's, physiological ionic strengths, and physiological temperatures and will remain as filaments until bacterial degradation begins (1, 11, 12, 17, 21, and 26). The disassembly of the actin filaments which must occur in vivo after cytokinesis, for example, and the failure of the bulk of the actin to be polymerized in vitro before purification (12, 17, 31, 32) indicate that other components in the cell must act to inhibit the polymerization of actin or to stimulate its depolymerization. Some success has been achieved in defining the components involved (2, 29, 31), but what must now be done is to isolate the inhibitory component or components, characterize them, and determine under what conditions the actin can be freed from these components enabling it to polymerize or depolymerize—in essence a "brute force and ignorance" approach. We thought that it might be more fun to try to determine what changes must take place in the cell itself in order to allow the actin to polymerize. If we were successful, we would then know what to look for in vitro.

Colwin and Colwin (6) were the first to demonstrate that the ammonium ion induced the acrosomal reaction in many echinoderm sperm, and Dan (8) has shown that Ca^{++} is required for the normal acrosomal reaction, a conclusion consistent with the later results of Takahashi and Sugiyama (27). Based on these and other observations, it seems reasonable to suspect that changes in the internal ion composition are responsible for triggering the polymerization of actin. Available now are a number of compounds called ionophores which are defined as "lipid soluble antibiotics that complex alkali cations and transport them across a variety of membranes" (22). We used these compounds as analytical tools with which to try to determine which ions must enter or exit from the cell in order to allow actin to polymerize. For this determination, we used three ionophores which differ in their selectivity for ions. If one regulates the ions outside the cell before the addition of the ionophore and then determines what comes out of the cell after the ionophore has been added, it is possible to ascertain which ions must be carried into or out of the cell before the actin can polymerize.

The ionophore A23187 catalyzes an electroneutral exchange of cations across lipid bilayers. For example, 2 H^+ can be exchanged for one divalent cation (Ca^{++}, Mg^{++}, etc.). Thus, Ca^{++} or Mg^{++} can be brought into a cell by being complexed to a dimer of A23187 which is present as the carboxylate anion, and 2 H^+ can be carried back across the membrane by the ionophore after it has released the divalent cation internally (3, 20, 22). K^+ can also be transported by A23187 although with lower affinity than Ca^{++}, Mg^{++}, or H^+ (20). X537A, whose mode of action is similar to that of A23187, is much less specific and will catalyze the electroneutral exchange of a variety of ions of high concentration outside the cell for those of high concentration inside the cell (3). Nigericin exchanges K^+ for H^+ (16).

In this report, we demonstrate that the acrosomal reaction in echinoderm sperm is very complex, involving a number of steps some of which may be controlled by one ion, others by different ions. For example, from our experiments it appears that a rise in internal pH triggers actin polymerization, whereas the fusion of the acrosomal vacuole with the cell surface requires Ca^{++}. Because we are dealing with an intact cell, the results presented in this report must be thought of as preliminary, but such an approach as that outlined here, coupled with the use of radioisotopes and substances that block or form ion channels specific to certain cations and anions, will allow us, during the next few years, to make quantitative statements about the species and amount of ions responsible for each step in the acrosomal reaction and as well will give us clues as to what to look for in other motile systems.

MATERIALS AND METHODS

Biological Material

This work was initiated on sperm from the starfish, *Marthasterias gracilis*, collected on mussel beds near the Station Zoologique (Villefranche-sur-Mer, France). *Pisaster ochraceus* were obtained from Pacific Bio-Marine Laboratories, Inc. (Venice, Calif.) and maintained in Instant Ocean tanks (Aquarium Systems, Inc., Eastlake, Ohio) in Philadelphia, courtesy of Drs. S. Inoué and H. Sato. They were fed *Mercenaria mercenaria*. *Thyone briareus* and *Asterias forbesi* were obtained from the supply department of the Marine Biological Laboratory (Woods Hole, Mass.) and maintained for short periods in Instant Ocean tanks.

Source and Method of Application of the Ionophores

A23187 was graciously supplied by Dr. R. Hammill,

Eli Lilly & Co. (Indianapolis, Ind.); X537A by Hoffmann-LaRoche, Inc. (Plainfield, N. J.); nigericin by Dr. A. Scarpa (Department of Biophysics, University of Pennsylvania Philadelphia, Pa.). To examine the effect of these ionophores on a dilute suspension of sperm, a 1-mg/ml stock was made of each ionophore using ethanol (nigericin or X537A) or a 1/1 mixture of ethanol and dimethyl sulfoxide (DMSO) (A23187) as the solvent. 10 μl of the ionophore solution were added to each milliliter of sperm suspension. (Thus, the final concentration of the ionophore in the sperm suspension was 20 μM. Below this, less than 100% of the sperm reacted.) The stocks were kept in the dark and refrigerated when not in use. For the experiments demonstrating the liberation of H^+ from the sperm, a 10-mg/ml stock of each ionophore was made since a much more concentrated suspension of sperm was used in these experiments.

Measurement of External pH.

A stock suspension of sperm was made by suspending one part of a sperm pellet with two parts of seawater or, in place of seawater, calcium-free seawater or isotonic NaCl or KCl; 1 mM buffer was also added (see the Results for the solution used in each experiment). Moore's calcium-free seawater, Shapiro's calcium-free seawater, or artificial seawater was made up after the formulae in Cavanaugh (4). 0.5 milliliters of this suspension was then diluted to 1 milliliter and placed in a small test tube containing a tiny stirring flea. The pH measurement was made with a Radiometer (Radiometer Co., Copenhagen, Denmark), using a small combination electrode. The pH was recorded at 10-s intervals. After several intervals, the ionophore (10 λ) was added to the text tube. As a control, ethanol or the DMSO/ethanol mixture (10 λ) was added to the sperm suspension and the pH measured at 10-s intervals as before.

In order to easily compare results from experiments in which the ionophores were added to different media, i.e. seawater, calcium-free seawater, NaCl isoosmotic with seawater, or either of the latter with ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetate (EGTA) added, we carried out a titration curve for each solution by the addition of known amounts of HCl. This is important as different buffers with different pK's (EGTA, Tris, seawater, etc.) are present in each solution. We then measured the rate of decrease in pH vs. time for sperm incubated with and without ionophores in the various media. We plotted mequivalents of H^+ vs. time, using the above data and the titration curves. Finally, we substracted the control curve from the experimental curve and replotted all the data on one graph (Fig. 8).

Preparation of Egg Jelly

A dense suspension of eggs was obtained from the ovaries of *Asterias forbesi* by the addition of 1 methyl adenine (10^{-5} M) to the seawater in which the ovaries were suspended. These eggs were then washed in seawater, concentrated by gravity, and the pH was lowered to 5.0 with gentle stirring. The eggs were removed, and the pH of the supernate was adjusted to 8.0.

Light Microscope Techniques

1.5 min after the application of the ionophore, 0.2 milliliters of a solution of 8% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) was added to each milliliter of sperm suspension. Fixation is necessary here because, if the slides and coverslips are not absolutely clean, some of the sperm will react to substances on these glass surfaces or, on rare occasions, to compression by the cover slip. The sperm were examined with a Zeiss phase-contrast microscope using oil immersion (100 × objective) or under low power (16 × brightfield objective using the no. 3 phase ring; the latter gives an image that resembles a darkfield image although actually forming an image with diffracted light). Some photographs were taken with a Leitz phase-contrast microscope. For comparison, a normal acrosomal reaction was induced by adding a dense suspension of sperm to eggs of the same species. The eggs were fixed after 1 min and examined by phase-contrast microscopy.

Electron Microscope Techniques

PREPARATION OF MATERIAL FOR THIN SECTIONS: Suspensions of sperm which had been treated with ionophores (or ethanol or DMSO/ethanol as controls) were fixed by the addition of sufficient glutaraldehyde (8% stock from Electron Microscopy Sciences) to bring the suspension to 1.25% glutaraldehyde. Fixation was carried out for 30 min. The sperm were pelleted by centrifugation (1,000 *g* for 5 min), washed briefly in the fluid minus glutaraldehyde, then postfixed in 1% OsO_4 in 0.1 M phosphate buffer at pH 6.0 for 45 min at 0°C. The preparation was then washed three times with cold (0°C) distilled water and stained en bloc in 0.5% uranyl acetate for 2–3 h at 0°C, rapidly dehydrated in acetone, and embedded in Araldite or Epon. Thin sections were cut with a diamond knife on a Sorvall-Porter Blum MT2 ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.), stained in uranyl acetate and lead citrate and examined with a Philips 200, Hitachi 12, or Hitachi II electron microscope.

The fixation method described above is similar to that described by Tilney (28). We should emphasize that the temperature, the time of glutaraldehyde and osmium fixation, and the pH of the osmication are all critical; otherwise, the filaments are not preserved. It is also crucial to have a good batch of glutaraldehyde. Although we have found that the 8% glutaraldehyde from Electron Microscopy Sciences is considerably better than that from other suppliers, we have received bad batches from them. Thus, each batch must be assayed on a

system in which the morphology of the actin filaments is known, i.e., microvilli (18) or sperm (28). For example, during the course of these experiments, we finished one batch and ordered another. The new batch did not preserve actin filaments even though filaments in sperm from the same animal that had been fixed at the same time in an old batch of glutaraldehyde were excellently preserved. Why different batches of glutaraldehyde vary so much can only be speculated upon, but in our hands it is the purest glutaraldehyde that generally gives the poorest fixation, indicating that it is either an impurity in the glutaraldehyde that is the important ingredient in fixation or the selection of a short oligomer of glutaraldehyde rather than the monomer that is the key element for good preservation.

FREEZE-FRACTURE TECHNIQUES: Sperm suspensions after treatment with ionophores or controls were fixed for 15 min in 1% glutaraldehyde, as outlined above, and glycerinated (10, 20, and 30% at 0°C); the sperm were concentrated by centrifugation after each step. In 30% glycerol, they were concentrated at 20,000 *g* for 10 min. The sperm were then freeze fractured at −110°C in a Balzers apparatus (Balzers AG, Balzers, Liechtenstein) in the laboratory of Dr. Baccio Baccetti in Siena, Italy. The replicas were examined in a Philips 300 electron microscope in Siena.

RESULTS

The morphology of *Marthasterias* and *Pisaster* sperm, although not previously described, is not appreciably different from that of other starfish, e.g. *Asterias amurensis* (32), sea cucumbers (*Thyone briareus;* references 7 and 28), or brittle stars (*Ophiocoma;* reference 14). We will summarize briefly the morphology of these sperm as a basis for our description of the effect of the ionophore. Located within a cup-shaped depression in the anterior end of the nucleus is the acrosomal vacuole. Between this vacuole and the nucleus is an amorphous material which has been referred to as profilactin (28, 29), based upon the fact that it is composed of actin in a nonfilamentous state. The most basal part of the cup is denser than the rest (see Fig. 3 in reference 7 and Fig. 1 in reference 30). Along the posterior margin of the nucleus are the mitochondrion and the basal body of the flagellar axoneme.

When sperm are induced to undergo the acrosomal reaction either by treatment with ammonia (14, 32) or by contact with eggs (7), the acrosomal vacuole fuses with the cell surface thereby liberating its contents, and a needlelike process, the acrosomal process, is formed. Within the process is a bundle of parallel actin filaments. The length of the process is species dependent, being up to 90 μm in length for *Thyone*, 25 μm in *Asterias*, 18 μm in *Marthasterias*, and 15 μm in *Pisaster*.

Addition of X537A or A23187 to a Sperm Suspension in Sea Water

When *Thyone*, *Pisaster*, and *Marthasterias* sperm are treated with X537A, or *Pisaster* and *Thyone* sperm with A23187 in seawater at pH 8.0, all the sperm undergo an acrosomal reaction. However, instead of a needlelike process extending from the anterior end, most of the sperm produce a mushroomlike growth (Fig. 1 *a*) which may be pointed (Fig. 1 *c*). Sometimes in *Marthasterias* sperm and more often in *Thyone* sperm, an abnormally short process is generated. This process seldom exceeds 2 μm in length in *Marthasterias* (Fig. 1 *b*) and 6 μm in *Thyone*, in contrast to the 18 μm (*Marthasterias*) or 90 μm (*Thyone*) long acrosomal process formed when these sperm are activated by eggs of the same species. The reacted sperm often stick to one another at their apical ends, forming clumps as has recently been described for sea urchin sperm by Collins (5).

When thin sections of *Marthasterias* or *Pisaster* sperm are examined after treatment with X537A, the nucleus, mitochondrion, and flagellum remain unchanged. In most sperm the acrosomal vacuole is absent, having fused completely with the cell surface, and in place of the profilactin are filaments (Figs. 2, 4, 5, and 6). Near the apicolateral margins of the nuclear envelope are some dense granules each about 250 Å in diameter (Figs. 4, 5, and 6) often in association with small vesicles (Fig. 6). In a few sperm the acrosomal vacuole appears to be intact as it preserves its normal density (Fig. 2), but a careful examination of the membrane limiting this vacuole shows it to be broken in one or more places (Figs. 2 and 4). These interruptions in the continuity of the membrane are frequently seen in untreated sperm as well and may be an artifact of osmication, dehydration, or embedding. When freeze-fracture preparations of ionophore-treated sperm are examined, it can be seen that the acrosomal vacuole membrane is indeed intact in some cells (Fig. 3). The particles in the plane of the membrane appear randomly distributed, as expected from what we know of untreated sperm (our unpublished observations). The bulge in the plasma membrane anterior to the acrosomal vacuole is due to the accumulation of filaments, as can be seen in the thin sections (Fig. 2). These filaments are gener-

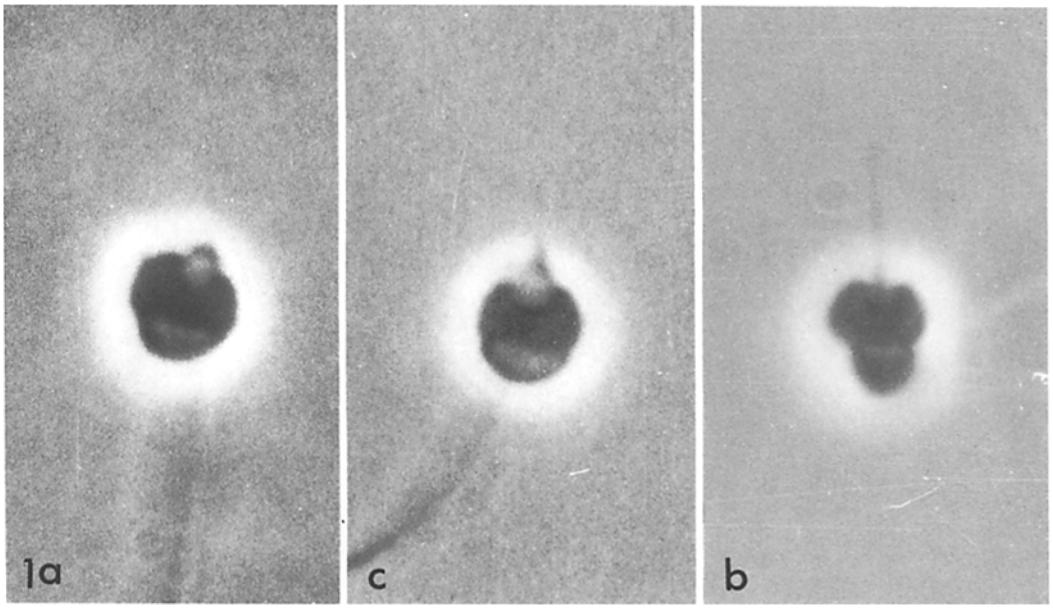

FIGURE 1 Phase-contrast micrographs of three *Marthasterias* sperm treated with X537A. × 4,000.

ally randomly oriented (Figs. 2 and 4). In some sperm (Fig. 4), the density of the contents of acrosomal vacuole is reduced. In these, sperm fusion of the acrosomal vacuole with the cell surface must have occurred at a point out of the plane of section.

In sperm where the mushroomlike growth is pointed at its apical end (Fig. 1*c*), the filaments, rather than being randomly oriented, tend to lie nearly parallel to one another (Fig. 5). Similarly, in sperm which form short processes (Fig. 1*c*), most of the actin filaments are aligned parallel to one another and extend from the tip of the process to the dense amorphous material at the base of the cup-shaped indentation in the nucleus (Fig. 6). However, there is always a small number of randomly oriented filaments within the nuclear indentation. This can best be seen in a transverse section through the filament bundles.

When *Pisaster* sperm are treated with A23187, they all react. In most cases, the acrosomal vacuole fuses with the cell surface, and a short process forms. The nucleus remains indented, and the region formerly occupied by the acrosomal vacuole is filled with filaments. In this case, small dense granules with their associated vesicles may be randomly located in this region instead of clustering at the apicolateral surface of the nucleus. If the acrosomal vacuole does not fuse with the cell surface, a tiny mushroom forms above the vacuole. The periacrosomal region and the tiny mushrooms are filled with filaments (randomly oriented), some profilactin, and some dense granules.

Effect of the External pH on the Action of X537A and A23187

The action of X537A and A23187 upon the acrosomal reaction in sperm appears to be dependent on the pH of the seawater surrounding the sperm. At pH 7.8–8.2, the profilactin is transformed into filaments and exocytosis of the acrosomal vacuole generally occurs. At pH 7.0, however, there is only a partial polymerization of the actin and exocytosis does not usually occur. At pH 6.0–6.5 (the range here takes in species differences), no change is seen. We presume that no effect is seen at pH 6.5, as we have approximated the external pH with the internal pH (9, 10, 13, 15, 23).

Transportation of H^+ out of the Sperm by X537A and A23187

To test whether the pH dependence of the ionophore-induced reaction is due to the transport H^+ out of the sperm by the ionophore in exchange

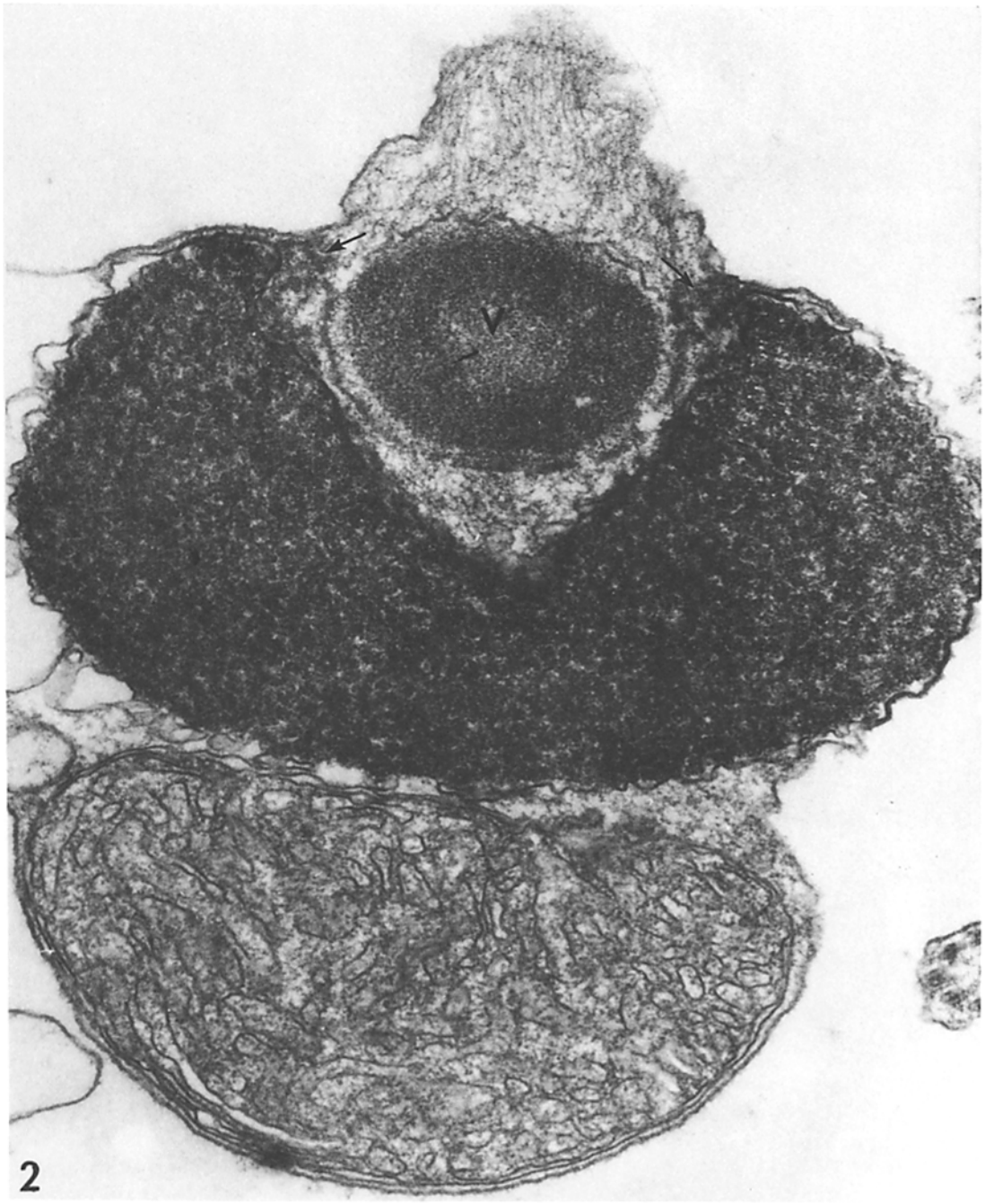

FIGURE 2 Thin section of a *Marthasterias* sperm which had been treated with X537A. Lying within an indentation in the nucleus is the acrosomal vacuole (*V*). The electron density and the differentiation of the material is the same as that of an untreated sperm. Note the large number of filaments in the periacrosomal region. Near the apicolateral surfaces of the nucleus (see arrows) are some small dense granules. × 67,000.

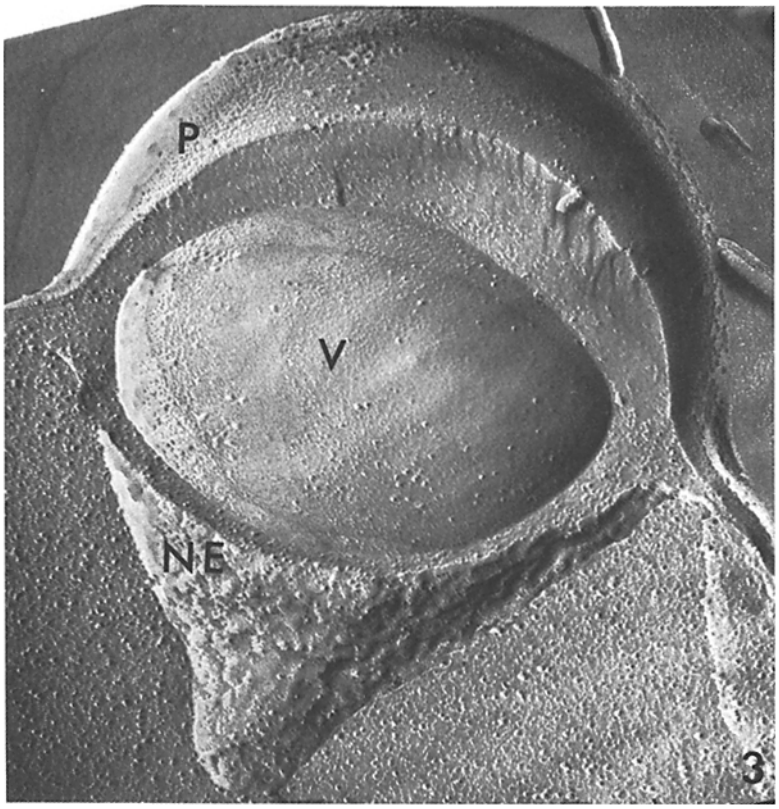

FIGURE 3 Freeze-fracture preparation of the anterior end of a *Marthasterias* sperm treated with X537A. In this sperm, as in Fig. 2, the acrosomal vacuole (*V*) has not fused with the plasma membrane (*P*). A portion of the nuclear envelope (*NE*) is visible as well. × 70,000.

for another ion which is carried in, we attempted to measure this presumed flux by following the pH of the medium. When A23187 (Fig. 7) or X537A (Fig. 8) is added to a suspension of *Pisaster* sperm at pH 7.8, all the sperm react and there is a rapid decrease in pH; within 1.5 min, the pH has decreased by 1 log unit (pH 7.8–6.9; Fig. 7). The extent of this change is dependent on the concentration of sperm and of the ionophore.

The minimum time for the production of the acrosomal process in an individual sperm is about 10 s. Yet, if one adds ionophores, or, as will be seen later, jelly, all the sperm do not undergo the acrosomal reaction simultaneously. Instead, it takes about 25 s for half the sperm to react. Thus, the time-course is roughly comparable to the rate of release of H^+.

In controls where the ionophore solvent alone is added to the sperm suspension, there is a small decrease in pH (Figs. 7 and 8) which may be due to respiration of these motile cells (19). Ohtake's values (19) and ours concur. The decrease in pH of the control suspension is less than 20% of that of the ionophore-treated cells regardless of the density of the sperm in the preparation.

Role of Calcium in the Acrosomal Reaction

Since X537A and A23187 induce the rapid efflux of H^+ from sperm cells, we carried out experiments in various external salt solutions to determine which cationic species outside the cell had to be exchanged for H^+ in order to induce polymerization. The results are consistent with the hypothesis that any of a variety of cations can be exchanged for H^+, that the H^+ efflux is sufficient for actin polymerization, and that Ca^{++} influx promotes secretion of the acrosomal vacuole.

The efflux of H^+ induced by X537A in 0.5 M NaCl is similar in the presence and absence of external Ca^{++} (Fig. 8). Since the only cation

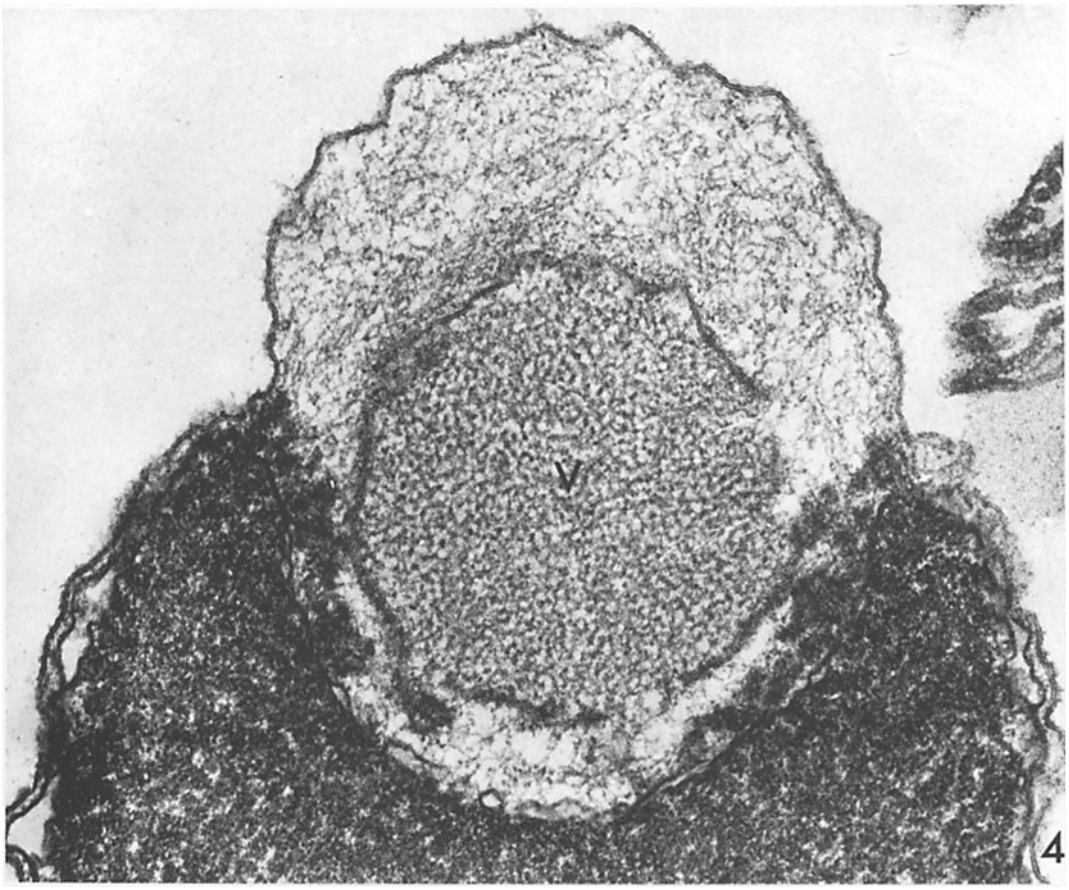

FIGURE 4 Apical end of a *Marthasterias* sperm treated with X537A. The acrosomal vacuole (*V*) has lost much of its electron density. Of greatest interest is the randomly oriented population of filaments above and below the acrosomal vacuole. × 89,000.

outside the sperm is Na^+ and since the concentration of Na^+ outside the sperm is at least 10 times that inside, Na^+ will be exchanged for H^+, particularly since X537A carries Na^+ and H^+ with high affinity. This pure H^+-Na^+ exchange activated 100% of the sperm. These sperm formed tiny apical mushrooms containing enormous numbers of filaments (Fig. 9) which tended to be aligned parallel to any asymmetry in the mushroomlike growth. Many filaments fanned out in all directions from a dense material in the nuclear indentation. The profilactin had disappeared. However, in all cases the acrosomal vacuole remained intact, having failed to fuse with the cell surface (Fig. 9). Not only was the membrane of the acrosomal vacuole not interrupted, but the density and substructure remained completely unaltered.

Since A23187 carried monovalent cations (with the exception of H^+) with low affinity, we measured the amount of H^+ released by this ionophore when sperm were suspended in Ca^{++}-free seawater or isotonic NaCl. In both cases, Na^+ would have to be exchanged for H^+ (Mg^{++} would not be exchanged for H^+ as it is in approximately equal concentrations inside and outside the cell). The amount of H^+ released from sperm suspended in NaCl or in calcium-free seawater is much less than that released from sperm which are suspended in seawater containing A23187, as would be expected from the low affinity of this ionophore for Na^+ (Fig. 8). However, the amounts of H^+ released in NaCl and in calcium-free seawater are almost identical. When we examined sperm treated with A23187 in NaCl and in calcium-free seawater, we found that only about 50% of the sperm had reacted to form small mushrooms. In

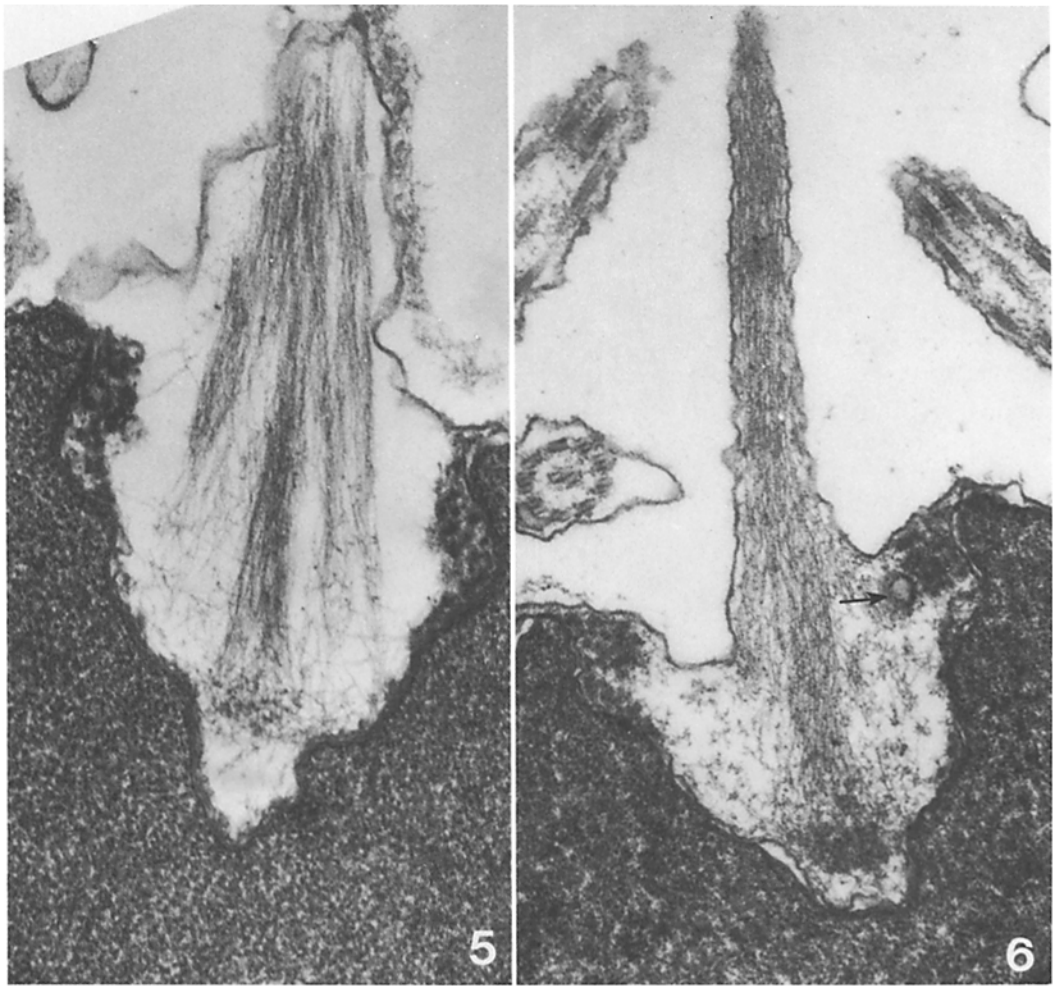

FIGURE 5 Thin section through a *Marthasterias* sperm treated with X537A. This sperm has formed a short acrosomal process. Of particular interest is the large population of filaments which are aligned parallel to one another. Note the small dense granules at the apicolateral margins of the nucleus. × 76,000.

FIGURE 6 Thin section through a *Marthasterias* sperm treated with X537A. Note the slender acrosomal process within which is a large population of filaments. These filaments insert in some dense material at the base of the indentation in the nucleus. A small vacuole (arrow) lies near some dense granules. × 64,000.

those that had reacted, the acrosomal vacuole in general had failed to fuse with the cell surface although the profilactin was transformed into filaments. When 10 mM Ca^{++} was added to the calcium-free seawater so that the resultant seawater contained roughly the same ions in the same concentration as those present in natural seawater, then A23187 was added, mushrooms or short processes developed in almost all the sperm. In most cases, the acrosomal vacuole fused with the cell surface and the actin polymerized completely.

In order to demonstrate that other cations besides Na^+ and Ca^{++} could be exchanged for H^+, we suspended sperm in 0.5 M KCl and added nigericin. Under these conditions, there was a release of H^+ and a partial polymerization of actin, but seldom did the acrosomal vacuole fuse with the cell surface (Fig. 10).

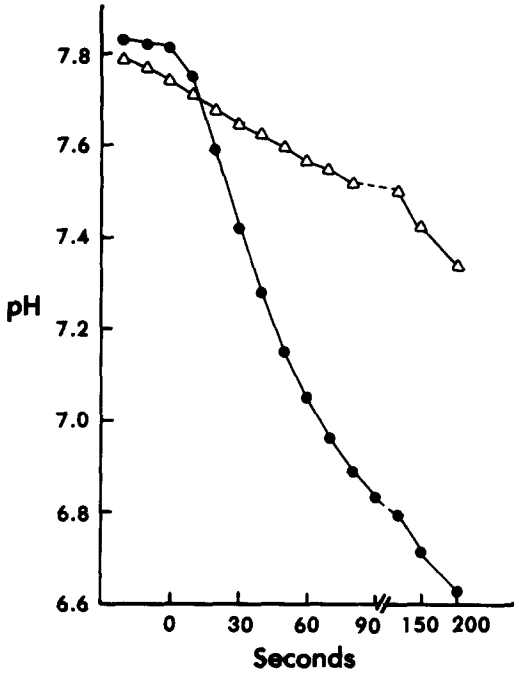

FIGURE 7 Graph illustrating the change in pH of the medium outside a suspension of *Pisaster* sperm plotted against time after the addition of A23187 to the sperm. At zero time the ionophore was added. (●–●–●) A23187 was added to *Pisaster* sperm in artificial seawater. (△–△–△) the control with an ethanol/DMSO mixture added at time zero.

Release of H^+ from Sperm Induced to Undergo the Acrosomal Reaction with Egg Jelly

When a solution of egg jelly is added to a dense suspension of sperm, essentially all the sperm undergo a normal acrosomal reaction, producing long processes. If the pH of the solution is measured, we can show that there is a rapid release of H^+ (Fig. 11). For the control, 0.5 M NaCl instead of egg jelly was added to a dense suspension of sperm and the pH also measured. We could not calculate the amount of H^+ released with the ionophores, as no titration curve was run on the jelly before the breeding season was over.

DISCUSSION

This study with ionophores has provided us with two important pieces of information. First, these experiments with ionophores have enabled us to dissect the acrosomal reaction into several discrete steps which, for a normal reaction, must be coupled spatially and temporally. These events include: (*a*) fusion and exocytosis of the acrosomal vacuole; (*b*) polymerization of the actin; (*c*) alignment of the actin filaments; (*d*) increase in cell surface; (*e*) increase in cell volume; and (*f*) change in the shape of the nucleus. We recognize that these events must take place because, when

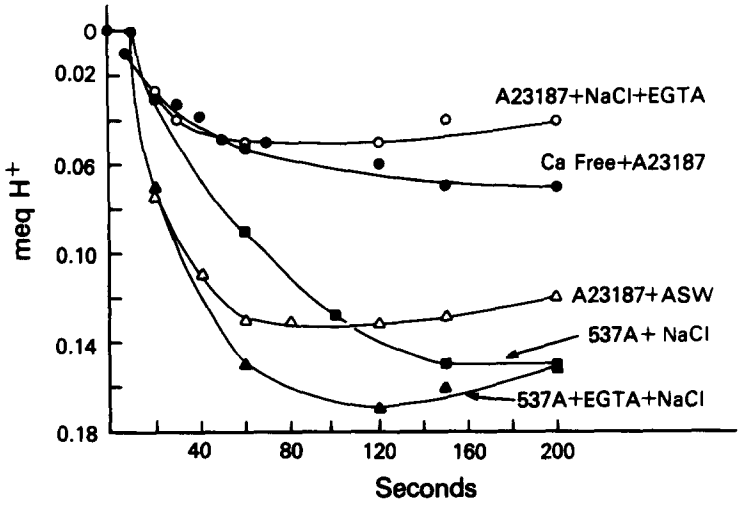

FIGURE 8 The change in pH of the medium outside a suspension of *Pisaster* sperm was measured at 10-s intervals for each condition seen in Fig. 8. A titration curve of the medium used for each experiment was run, and the data are expressed as meq. of H^+ released per unit of time. The control curves were then subtracted from the experimental curve and the data were plotted. This allows all the data in this figure to be compared. ASW = artificial seawater (MBL formula, reference 4).

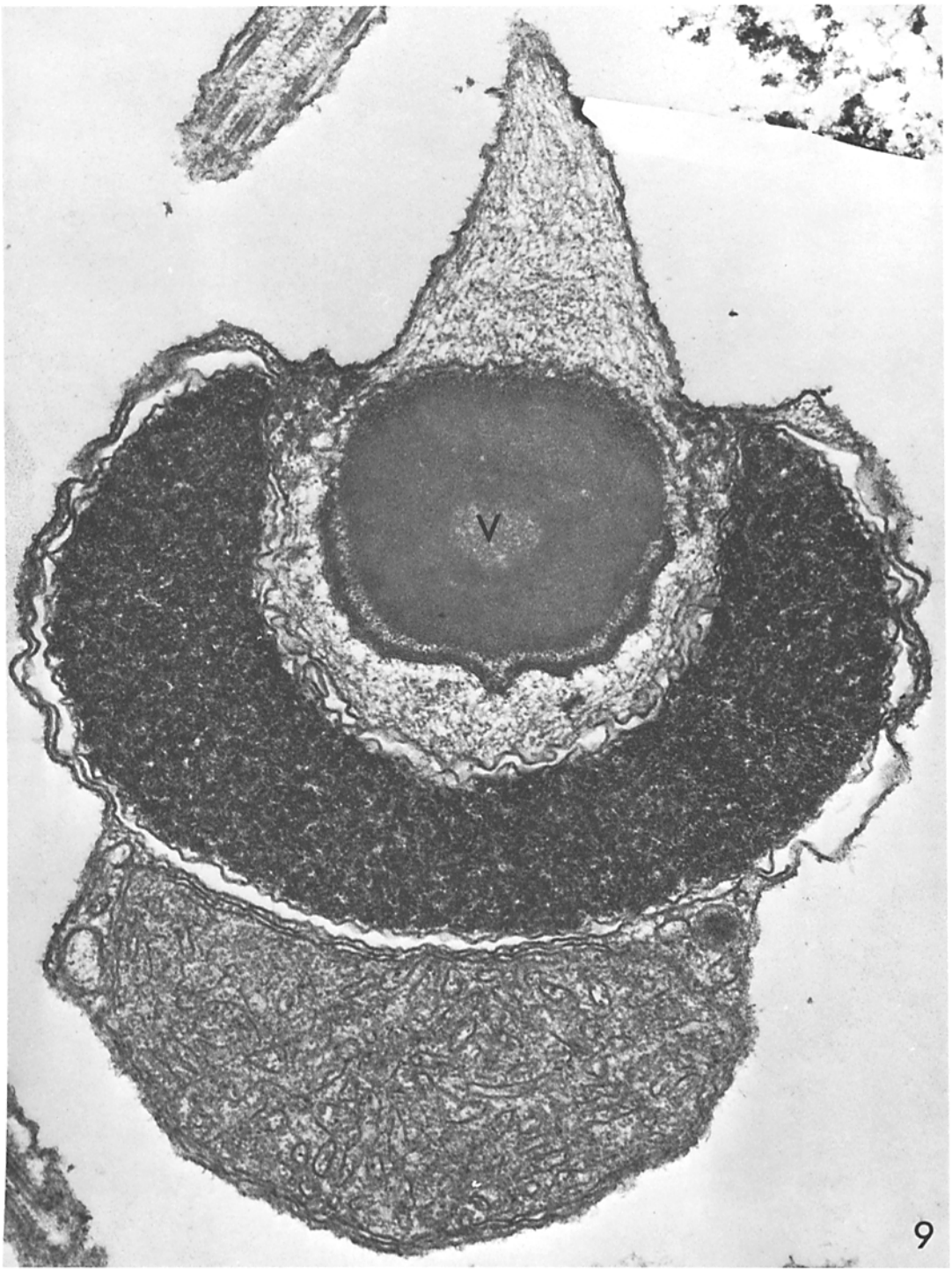

FIGURE 9 Thin section through a *Pisaster* sperm which had been washed twice in 0.5 M NaCl containing 1 mM EGTA, then fixed 200 s after the addition of X537A. It is of particular interest that the profilactin has been converted into filaments, but the acrosomal vacuole (*V*) remains intact. The rest of the sperm appears unaltered. × 74,000.

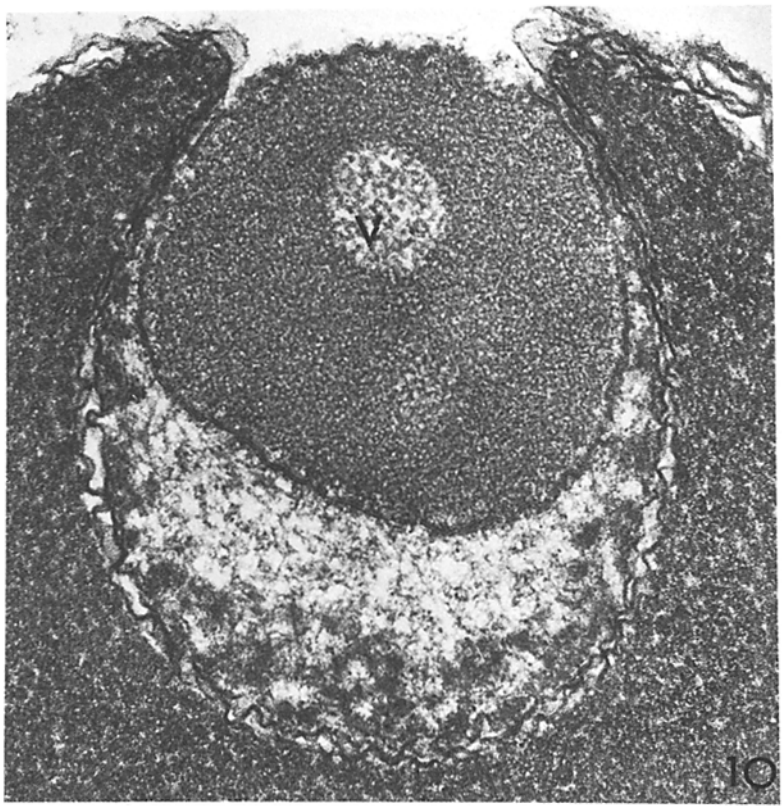

FIGURE 10 Thin section through a *Thyone* sperm which had been washed in 0.5 M KCL, then fixed 90 s after the addition of nigericin. It is of particular interest that the acrosomal vacuole (*V*) remains intact and that much, but not all, of the actin has been converted into a randomly oriented population of filaments. The black dots are actin filaments cut in transverse section. × 100,000.

an ionophore is added to a sperm suspension under one set of conditions, some of these changes do not occur, yet under other conditions different events may not occur. In this study, we have concentrated on steps 1 and 2. More information on steps 2 and 3 is presented in the following paper (30), and step 4 has been described in a preliminary way in another publication (24). Secondly, the effects of the ionophores on sperm suspended in various media has led us to the hypothesis that ion fluxes control exocytosis and actin polymerization. More specifically, we propose that calcium entry is required for exocytosis and that H^+ exist is essential for actin polymerization.

All of the evidence is consistent with the above, but in the absence of data on direct changes in the internal ion concentrations the hypothesis remains unproven. Nevertheless, these are good postulates because they not only suggest further experimentation but also focus our attention on how changes in the intracellular concentration of ions might regulate what a cell does—this is, after all, one area that a cell is capable of regulating with great efficiency. Since the ion-exchange induced by ionophores probably alters the internal ion composition of the sperm and since many of the events during the acrosomal reaction proceed only when specific ions are present in the external medium, we presume that most of these events are either directly or indirectly related to ion fluxes.

For step 1, fusion of the acrosomal vacuole with the cell surface, it seems probable that Ca^{++} must enter the sperm cell because in Ca^{++}-free media fusion is not induced by any of the ionophores or by egg jelly, yet those ionophores (X537A and A23187) which carry Ca^{++} induce fusion in a Ca^{++}-containing medium. Since we are dealing with a whole cell involving interactions across membrane compartments, we do not know whether the calcium acts directly or indirectly. The speed of the reaction, however, suggests that

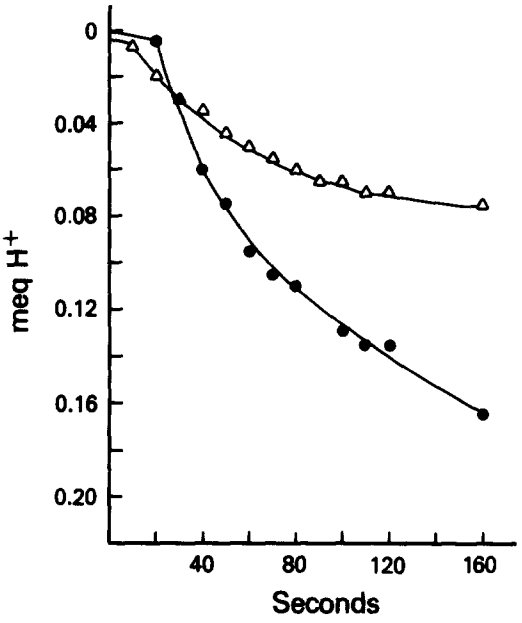

FIGURE 11 Egg jelly was added to *Asterias* sperm and the drop in pH measured relative to time. A titration curve of the buffer used (1 mM Tris) was run, and the data are expressed as meq of H^+ released relative to time. (●–●–●) Egg jelly added to the sperm. (△–△–△) Control curve in which NaCl isotonic with seawater was added to sperm rather than jelly.

a large number of intermediates are not involved. Furthermore, since it would take time for the ionophore to partition itself within an internal membrane system and since the reaction fails to proceed in calcium-free media, it seems unlikely that the ionophore acts by releasing an ion from an internal membrane system, i.e., the mitochondrion, the nucleus, or the acrosomal vacuole.

Five different observations indicate that step 2, actin polymerization, is triggered by a rise in the intracellular pH. First, the ionophores will induce filament formation irrespective of the species of cation outside the sperm cell, albeit Na^+, K^+, or Ca^{++}, provided the pH of the external medium is high, i.e., above 6.5. Thus, if the external pH is 8.0, essentially all of the profilactin is transformed into filaments. However, if the pH is 7.0, only a portion of the actin polymerizes. Secondly, the time-course of H^+ release from the cell and that of actin polymerization roughly coincide. Thirdly, when the actin is induced to polymerize either by ionophores or more normally by egg jelly, H^+ is released from the sperm cell. Fourthly, ammonia, which induces the actin to polymerize in vivo, appears to act by raising the internal pH (32). Finally, isolated cups of profilactin dissolve when the pH is raised from 6.4 to 8.0 (29). On the other hand, changing the concentration of Ca^{++}, Mg^{++}, or Na^+ has no effect on the solubilization of the profilactin.

On the basis of these in vitro observations, it was suggested in an earlier paper in this series (29) that the following reaction probably occurs in the cell:

$$\text{Profilactin} \xrightarrow{\uparrow \text{pH}} \text{G-actin} + \text{Binding proteins} \longrightarrow \text{F-actin.} \quad (1)$$

We now know by column chromatography (our unpublished observations) that at pH 8.0 the actin is indeed no longer bound to the other proteins present. Thus, from the data summarized above, it appears that the first step in the reaction (profilactin to G-actin) requires a rise in pH. We do not know what the second step (G-actin to F-actin) entails; the substance or substances may already be in the sperm cell, or it may be a cation that is exchanged for H^+. If the latter is true, all we can say is that the reaction does not require external Ca^{++} specifically since the event takes place in calcium-free media such as NaCl or KCl.

Since little is known about the buffers that might be present inside the sperm cell in vivo, no statements can be made about what the actual in vivo change in pH might be. Furthermore, we do not know where the H^+ is coming from in the sperm. It is unlikely that it is coming from the secretion of the acrosomal vacuole contents because the drop in pH of the media around the sperm does not vary in amount or rate if one compares the efflux of H^+ from sperm which discharge their acrosomal vacuole, i.e., when ionophores are added to sperm in seawater at pH 8.0, or from sperm in which the acrosomal vacuole remains intact.

We should put in a word of caution about interpreting the experiments with egg jelly even though the observations are consistent with the results obtained with ionophores, for it is possible that the H^+ release is due, at least in part, to increases in the metabolism of the sperm when they come into contact with egg jelly (19). Further experimentation is necessary.

A23187 Carries Na^+

Recently, Pfeiffer and Lardy (20) presented evidence that A23187 carries K^+, although with low affinity. From our study, it appears that A23187 carries Na^+ as well. Our evidence consists of the observation that when A23187 is added to sperm suspended in isotonic NaCl with EGTA, or in calcium-free seawater, H^+ is carried out of the sperm and the actin polymerizes (Fig. 8). A23187 is probably not carrying Mg^{++} to any extent in calcium-free seawater, as the release of H^+ is the same as when the ionophore is added to a solution of sperm in NaCl. This suggests also that the Mg^{++} concentrations inside and outside the sperm cell are nearly equivalent. Alternatively, the concentration gradient for Na^+ in both isotonic NaCl and calcium-free seawater is 10-fold higher outside the sperm as compared to inside. Thus, Na^+ will be exchanged for H^+. One could argue that traces of Ca^{++} are carried in in order to bring the H^+ out, but this is improbable as the acrosomal vacuole does not fuse with the cell surface. It is also unreasonable to suggest that the exodus of H^+ is brought about by the release of Ca^{++} from internal stores. If this were the case, H^+ should change intracellular compartments, not be carried extracellularly, a possibility, but unlikely due to the time-course. Furthermore, if the external pH is dropped to 6.5, H^+ is not carried out, nor is the actin polymerized. The most likely interpretation is that A23187 carries Na^+, but with less affinity than it does Ca^{++}.

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