THE INDUCTION OF THE ACROSOLE
REACTION IN GUINEA-PIG SPERM BY
THE DIVALENT METAL CATION
IONOPHORE A23187

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SUMMARY
The divalent metal cation ionophore A23187 induces an acrosome reaction in guinea-pig sperm which is dependent on external calcium. Examination of this acrosome reaction by electron microscopy shows that it is morphologically normal. The known properties of A23187 and the morphological similarity between the acrosome reaction and the secretory discharges of other secretory cells suggests that the immediate cause of the acrosome reaction is an increase in the cytoplasmic free calcium concentration.

INTRODUCTION
The mammalian egg at fertilization is surrounded by two concentric investments: the innermost is the amorphous mass of the zona pellucida; outside this lie the residual follicle cells of the corona radiata. Sperm are unable to penetrate the zona and subsequently fuse with the egg without first undergoing an acrosome reaction.

The acrosome is an intracellular membrane-bound organelle which forms a cap over the anterior two-thirds of the nucleus. The acrosome reaction is the fusion of the outer acrosomal membrane to the overlying plasma membrane: part of the outer and the inner acrosomal membrane are incorporated into the plasma membrane and become part of it; the remainder is lost as vesicles of plasma and acrosomal membrane (Barros, Bedford, Franklin & Austin, 1967; Bedford, 1970).

The natural stimulus for the acrosome reaction is unknown. It can, however, be induced by a number of artificial stimuli (Yanagimachi, 1969; Barros, Arrau & Herrera, 1972; Wooding, 1975; Yanagimachi, 1975) which include follicular fluid, serum and detergents and, in the case of guinea-pig sperm, extended incubation in simple medium (Barros, Berrios & Herrera, 1973; Yanagimachi & Usui, 1974). This spontaneous reaction is dependent on external calcium (Yanagimachi & Usui, 1974). An acrosome reaction can also be induced in guinea-pig sperm by the divalent metal cation ionophore A23187 (Green, 1976; Summers et al. 1976; Talbot et al. 1976).

This paper examines the ultrastructure of this A23187-induced reaction.
METHODS

Twenty-five per cent glutaraldehyde was purchased from Koch-Light, ethylene glycol tetra-acetic acid (EGTA) from Sigma, and Ficoll 400 from Pharmacia. All other reagents were A.R. grade. A23187 was a generous gift from Dr R. L. Hamill of Eli Lilly Laboratories, Indianapolis. Siliconized glassware was used throughout.

Two media were used throughout: calcium medium contained 140 mM NaCl, 4 mM KCl, 4 mM HEPES, 10 mM glucose and 2 mM CaCl₂, pH 7.4; magnesium medium was the same as calcium medium except that 2 mM MgCl₂ and 100 μM EGTA were substituted for the 2 mM CaCl₂. A23187 was used from a stock solution in dimethyl sulphoxide (DMSO) (2 mg/ml) kept at room temperature in the dark.

Epididymal sperm were obtained from adult guinea pigs (0.5–1 kg) killed by dislocation of the neck. The vas deferens and cauda epididymis were removed in one piece and sperm flushed out with magnesium medium containing 5% Ficoll 400 (w/v) and spun at 1500 g for 5 min. The sperm pellet was washed once in magnesium medium and centrifuged at 1500 g for a further 10 min. Sperm were then resuspended in either calcium or magnesium medium at a concentration of about 2 x 10⁹/ml.

A preliminary time course for the loss of the acrosome induced by A23187 was estimated from inspection by light microscopy of sperm fixed at suitable intervals after addition of A23187. The acrosome reaction was induced by addition of 50 μl of the stock solution of A23187 in DMSO to 5 ml of sperm suspension at 37 °C. Samples (0.5 ml) were pipetted into Karnovsky's fixative in which magnesium and 100 μM EGTA were substituted for calcium. 500 sperm were examined for the loss of the acrosome for each time point.

Sperm in either magnesium or calcium medium were fixed for electron microscopy by addition of an equal volume of 0.15 M sodium cacodylate buffer, pH 7.2, containing 2% glutaraldehyde. After 1 h at room temperature the fixed suspensions were pelleted in Eppendorf tubes at 14,000 g for 10 min. Pellets were detached from the bottom of the tube with the aid of a needle and given three 10-min washes in 0.1 M sodium cacodylate buffer, pH 7.4, post-fixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature and given a further three 10-min washes in 0.1 M sodium acetate buffer, pH 5.1. The pellets were then block-stained with 1% uranyl acetate in 0.1 M sodium acetate buffer, pH 5.1, overnight at room temperature before dehydration in graded ethanols and propylene oxide and embedding in Spurr (Spurr, 1969). Silver sections were cut on a Reichert microtome, post-stained with Reynold's lead citrate and examined in a Philips 300 electron microscope.

Fig. 1. A stack of guinea-pig sperm heads in head-to-tail section fixed 30 s after addition of A23187 to sperm in calcium medium (A, acrosome; N, nucleus). In the centre are 2 sperm which have not yet undergone an acrosome reaction: b is an approximately elliptical region containing denser, round material within it, c is the demarcation line between 2 distinct areas of the acrosome (seen more clearly once the acrosome reaction has taken place) and d and d' are 2 lighter areas close to the sites of initial membrane fusion. Immediately above and below the intact sperm are 2 which have undergone an acrosome reaction. The areas b and d have become devoid of material and occupied the area now occupied by b', membrane fusion has occurred at a and a', close to the areas d and d' in the intact acrosome, the demarcation line c has become much clearer, and approximately half-way between this line and the concave edge of the acrosome has appeared a large cavity, e. For the material lying between c and the concave edge, the cavity represents an approximately 85% increase in total area for the loss of no more than 10% of the material (assessed by measuring the areas of electron-dense material before and after the acrosome reaction). x 30400.
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RESULTS

The results in this paper are designed to provide a foundation for the three papers which follow (Green, 1978a, b; Green & Hockaday, 1978) by, firstly, showing that the acrosome reaction induced in guinea-pig sperm by A23187 in calcium medium is morphologically normal and, secondly, by establishing the changes which occur in the morphology of the acrosomal contents during the acrosome reaction.

Guinea-pig sperm agglutinate spontaneously to form well ordered stacks by head-to-head association. Fig. 1 shows a head-to-tail section through such a stack fixed 30 s after addition of A23187. The 2 sperm in the centre still have intact acrosomes but they are flanked above and below by 2 sperm which have undergone the early stages of the acrosome reaction, including cavitation of the acrosomal matrix.

The acrosomal contents of the intact acrosome have a very characteristic morphology: there is the elliptical area b, often containing circular bodies; there is the line c, which marks the division of the acrosomal contents into 2 further regions, and there are the 2 areas d and d', identified by Friend, Orci, Perrelet & Yanagimachi (1977) as areas of initial membrane fusion during the acrosome reaction.

The sperm which have undergone the acrosome reaction show the 2 areas a and a' of initial membrane fusion, corresponding to the 2 light areas d and d' in the intact sperm; the total disappearance of the area b and its conversion into the swollen space b'; the increased clarity of the boundary c; and the appearance of a cavity e within the area delimited by the boundary at c and the concave surface of the acrosome.

The sequence of events which lead from the intact acrosome to the early stage of the acrosome shown in Fig. 1 is outlined diagrammatically in Fig. 7 (p. 148). Membrane fusion occurs initially at the tip, and possibly on the convex surface of the acrosome; this leads to the immediate loss of the area b; at this stage cavitation begins in the acrosomal matrix delimited by c and the concave surface of the acrosome to give the cavity e, and the appearance of sperm immediately after the early stages of the acrosome reaction shown in Fig. 1.

Some of the evidence for this mechanism is shown in Figs. 2 and 3; the remainder is dealt with in Green (1978b). Fig. 2 shows a sperm after membrane fusion and the loss of the area b, but before cavitation has begun: Fig. 3 shows a sperm at the next stage; cavitation has just begun and the matrix has the appearance of being torn apart. Cavitation in this matrix causes an approximately 85% increase in area for less than 10% loss of material as assessed by area.

Within a few minutes of addition of A23187, all sperm have the appearance shown in Fig. 4. Vestigial remnants of acrosomal material remain attached at the sperm tip,

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Fig. 2. Two sperm heads in approximately the same section as Fig. 1. The upper is still intact but the lower shows an early stage of the acrosome reaction immediately after membrane fusion at the tip of the acrosome (arrow). The material in area b of Fig. 1 has already disappeared but cavitation has not begun. × 34 800.

Fig. 3. The stage immediately after that shown in Fig. 2. Membrane fusion has occurred at the tip of the acrosome (arrow), the area b of Fig. 1 has more completely disappeared and cavitation has begun. × 39 000.
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Fig. 4. The completed A23187-induced acrosome reaction. The bulk of the acrosomal material has detached but residual vesicles of plasma and outer acrosomal membrane remain attached to the inner acrosomal membrane. × 40,500.
Fig. 5. Close-up of the area of membrane fusion between plasma and outer acrosomal membrane of the centre sperm in Fig. 4. \( \times 191200 \).

Fig. 6. The area immediately posterior to the base of the acrosome showing the additional bilaminar material extending under the membrane of the equatorial segment and running from it at a distance of 10–20 nm. It appears to bifurcate when it meets the acrosomal membrane, become amorphous and then rapidly stop. \( \times 138800 \).
and fusion of acrosomal and plasma membrane to form vesicles and cisternae has extended over the sperm surface to give the characteristic double hairpin profile, shown in greater detail in Fig. 5, where the rump of the outer acrosomal membrane fuses with the plasma membrane.

Block-staining with uranyl acetate also reveals very clearly the material which lies caudal to the posterior tip of the acrosome (Fig. 6). The precise form of this material has been the subject of some controversy (Fawcett, 1965; Fawcett & Ito, 1965; Gordon, 1972). In the guinea pig, it appears as a bilaminar structure, marginally wider than the plasma membrane, from which it runs at a distance of 10-20 nm. It appears to bifurcate at the tip of the acrosome before becoming amorphous and rapidly petering out. There is no periodic structure holding it to the plasma membrane or the nuclear membrane.

The criteria by which an acrosome reaction can be judged to be morphologically normal are very limited: they are basically the occurrence of proper vesiculation and the formation of the double hairpin structure at the base of the acrosome (Bedford, 1968, 1970; Stefaniini, Oura & Zamboni, 1969; Yanagimachi & Noda, 1970). By these criteria, the acrosome reaction induced in guinea-pig sperm by A23187 is normal.

The time course for the acrosome reaction is dealt with in the following paper (Green, 1978a). However, a few general points will be made here. External calcium is essential; A23187 in magnesium medium has no effect. Neither does DMSO in calcium medium without A23187. A23187 in calcium medium, in addition to inducing an acrosome reaction, also produces immotility; in magnesium medium, A23187 has no effect on motility.

The acrosome reaction induced in guinea-pig sperm after extended incubation in simple media described by Yanagimachi & Usui (1974) was examined using the media described in Methods. Sperm incubated in calcium medium for 16 h at 37 °C did not undergo an acrosome reaction but subsequent addition of A23187 caused one within a few minutes. Incubation in magnesium medium for 16 h followed by resuspension in calcium medium also produced no acrosome reaction. The origin of the difference between these results and those of Yanagimachi & Usui (1974) is unknown but on their part it may be due to the fluctuations in the pH of the incubation media: both media use bicarbonate but it is titrated off as CO₂ by acid neutralization before the incubation begins.

DISCUSSION

There is now increasing evidence that in a wide variety of secretory cells, secretion is a consequence of calcium-mediated stimulus-secretion coupling (Douglas, 1968; Rubin, 1970; Baker, 1974). It is the conclusion of this paper that the acrosome reaction is caused by an increase in cytoplasmic free calcium and that this, taken with its morphology, strongly suggests that it, too, is an example of stimulus-secretion coupling.

The concept of stimulus-secretion coupling rests on the following propositions: that secretion is the result of the exocytosis of the contents of intracellular secretory
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granules, that exocytosis is caused by fusion of the secretory granule membrane with the plasma membrane, that cytoplasmic free calcium is normally low, that it rises on the receipt by the cell of the stimulus to secretion, at least in the region between the secretory granules and the plasma membrane, and that it is the increase in cytoplasmic free calcium which causes the fusion of the secretory granule membrane to the plasma membrane with exocytosis of the granule contents.

The evidence that the acrosome reaction is an example of stimulus-secretion coupling can be divided into 2 parts: there is the evidence that the acrosome is a secretory organelle whose fusion to the plasma membrane results in exocytosis of the acrosomal contents, and secondly, there is the evidence that this fusion is the consequence of an increase in cytoplasmic free calcium.

The origins and contents of the acrosome are very similar to those of the zymogen granules of the exocrine pancreas (Burgos & Fawcett, 1955; Palade, Siekevitz & Caro, 1962; Jamieson & Palade, 1971a, b); both contain zymogens (Meizel & Mukerji, 1976), both are formed by budding off of the rough endoplasmic reticulum, both undergo condensation in conjunction with the Golgi apparatus (the proacrosomal granules also fuse at this stage to form a single granule) and both move into apposition to the plasma membrane. (In the case of the acinar cells of the pancreas, the geometry of the cell remains fixed and the granules move to the plasma membrane; in the developing sperm, the whole geometry of the cell is changing.) There is one important difference between the pancreatic exocrine cell and sperm and that is that sperm are terminal cells: the rough endoplasmic reticulum and Golgi apparatus are lost early in sperm development and with them, the ability to synthesize the acrosome. The acrosome reaction, therefore, commits the cell irreversibly.

The morphology of the acrosome reaction is well established for a number of species which have either undergone an acrosome reaction in the course of fertilization (Bedford, 1968, 1970; Stefanini et al. 1969; Yanagimachi & Noda, 1970) or in response to one of a number of artificial stimuli (Yanagimachi & Usui, 1974; Wooding, 1975; Yanagimachi, 1975). The principle morphological features of the acrosome reaction are the inclusion of the inner acrosomal membrane and a small part of the outer into the plasma membrane of the head and the fusion of the remainder of the outer acrosomal membrane with the overlying plasma membrane with the formation of vesicles (Barros et al. 1967) which are subsequently lost during penetration of the zona (Bedford, 1968). The acrosome reaction induced by A23187 is morphologically normal when judged by these criteria (Figs. 1, 4, 5). With the exception of the incubation technique of Yanagimachi & Usui (1974), which was found to be irreproducible, the use of A23187 is the only means available for inducing an acrosome reaction rapidly in a whole population of sperm. Using the A23187-induced reaction, it has been possible to show that proacrosin is activated and that acrosin, the putative zona lysin, is secreted as a consequence of the acrosome reaction (Green, 1978a); there is separate evidence that the 2 of them are constituents of the acrosome (Green & Hockaday, 1978).

The ability of A23187 to induce an acrosome reaction in calcium medium suggests that the reaction is a consequence of an increase in intracellular calcium, and if the
acrosome reaction is to be an example of stimulus–secretion coupling, a consequence of an increase in cytoplasmic free calcium. In those cells where it has been measured, cytoplasmic free calcium is low, possibly no more than 100 nM (Harrison & Long, 1968; Ashley & Ridgway, 1970; Baker, 1972; Ridgway, Gilkey & Jaffe, 1977; Steinhardt, Zucker & Schatten, 1977). Extracellular free calcium, on the other hand, is often about 1 mM, i.e. 4 orders of magnitude greater. There is, therefore, a steep electrochemical gradient for inward calcium movement at membrane potentials which are either negative or moderately positive: in guinea-pig sperm it is about +10 mV (Rink, 1977).

The level of cytoplasmic free calcium which normally exists in cells is the result of 3 fluxes: an influx due to calcium leaking through the plasma membrane down its electrochemical gradient, an efflux due either to calcium pumping or coupled exchange with another moving into the cell down its own electrochemical gradient, e.g. sodium, or both, and calcium uptake into intracellular organelles. The stimulus to secretion normally increases cytoplasmic free calcium by increasing the calcium permeability of the plasma membrane, thereby allowing external calcium to move into the cell down its electrochemical gradient (Douglas, 1968; Rubin, 1970; Baker, 1974) although in some cases, calcium is subsequently released from intracellular stores (Steinhardt et al. 1977).

The divalent metal cation ionophore A23187 can increase cytoplasmic free calcium either by producing an increase in the inward calcium leak (Babcock, First & Lardy, 1976; Desmedt & Hainaut, 1976) or by releasing calcium from intracellular stores (Steinhardt et al. 1977). A23187 causes electroneutral exchange of a number of divalent metal cations and protons across membranes (Reed & Lardy, 1972; Babcock et al. 1976); for practical purposes, the only 2 metal cations which need be considered are magnesium and calcium. Because ion movement is tightly coupled (Kafka & Holz, 1976) the exchange which A23187 induces is not equivalent to the introduction of a calcium permeability into the membrane: net calcium movement across a membrane in one direction occurs only as long as net movement of magnesium and protons occurs in the opposite direction. It stops when the activities of the 3 ions collectively move into equilibrium on each side of the membrane and this, of course, need not be when the calcium activities on either side are themselves in electrochemical equilibrium. Although the effects of A23187 are theoretically so complicated, it is known empirically that it increases cytoplasmic free calcium (Babcock et al. 1976; Desmedt & Hainaut, 1976; Steinhardt et al. 1977) as well as stimulating secretion in a wide variety of secretory cells (Foreman, Mongar & Gomperts, 1973; Prince, Rasmussen & Berridge, 1973; Chambers, Pressman & Rose, 1974; Cochrane & Douglas, 1974; Plattner, 1974; Steinhardt & Epel, 1974; Steinhardt, Epel, Carroll & Yanagimachi, 1974) for which there is evidence of secretion as a concomitant of increased cytoplasmic free calcium (Kanno, Cochrane & Douglas, 1973; Ridgway et al. 1977; Steinhardt et al. 1977) or calcium uptake (Foreman et al. 1973).

The natural stimulus for the acrosome reaction is unknown but whatever it is, it will be assumed that its effect is to increase cytoplasmic free calcium, i.e. that A23187 is a surrogate stimulus but the increase in calcium is not. The evidence suggests that
any natural stimulus must increase the calcium permeability of the plasma membrane, since A23187 does not induce an acrosome reaction in the absence of external calcium (cf. the sea-urchin egg (Steinhardt et al. 1974)), and that calcium moves into the cytoplasm from the external medium down its electrochemical gradient to cause membrane fusion and exocytosis of the acrosomal contents. There is no need to postulate metabolically dependent calcium uptake into the cell, i.e. inward calcium pumping, as suggested by Gordon (1973): all the evidence suggests quite the contrary, that inward calcium movement is down the electrochemical gradient for calcium and that calcium pumping is outwards (Babcock et al. 1976). Rink (1977) has effectively eliminated a change in membrane potential as the stimulus for calcium entry in the acrosome reaction. This leaves a ligand as the most likely stimulus, acting on receptors in the plasma membrane of the head. Provided that they are restricted to the plasma membrane overlying the acrosome, they are lost as the result of the vesiculation which follows their occupancy: continued occupation would not, therefore, lead to problems of calcium regulation for the rest of the cell, assuming that desensitization did not rapidly curtail calcium influx immediately after ligand binding.

A23187 is not alone in inducing an acrosome reaction artificially; it can also be induced by serum, follicular fluid, detergents, etc. (Yanagimachi, 1969; Yanagimachi & Usui, 1974; Wooding, 1975; Yanagimachi, 1975). The incubation media invariably contain calcium, inter alia, and since the evidence suggests that a rise in intracellular calcium is the cause of the acrosome reaction, it is likely that these agents all act by somehow increasing the calcium permeability of the plasma membrane, thereby allowing calcium in and initiating the acrosome reaction. Cells which, for whatever reason, have an increased calcium permeability will, in the presence of external calcium, expend increasing quantities of metabolic energy, in the form of ATP, in maintaining a low cytoplasmic free-calcium level: reducing the level of intracellular ATP will impair motility, since flagellar movement employs a magnesium-dependent ATPase: at the same time, the increasing levels of cytoplasmic free calcium will induce the acrosome reaction. This may well explain why the substantial fraction (25-30%) of epididymal sperm which have undergone an acrosome reaction by the time of isolation (Green, 1978a) are also immotile since, in these circumstances, both immotility and the occurrence of the acrosome reaction represent a collapse in the ability of the cell to maintain low cytoplasmic free calcium. Depletion of intracellular ATP due to pumping would also explain why A23187 at high concentrations causes immotility as well as inducing an acrosome reaction, although it may be due to depletion of intracellular free magnesium. The effect of A23187 in causing immotility is, however, one of dosage and at lower concentrations an acrosome reaction can be induced, albeit much more slowly, without impairing motility (Talbot et al. 1976).

The mechanism by which an increase in cytoplasmic free calcium causes fusion of plasma and acrosomal membranes is unknown. Yanagimachi & Usui (1974) suggested that calcium entering the cell causes the acrosome to swell and that this swelling, by pushing the acrosomal membrane against the plasma membrane, causes fusion. The mechanism proposed for the origin of the swelling has been examined elsewhere (Green, 1978b); however, the evidence suggests that swelling (or more strictly,
Fig. 7. A schematic representation of the stages in the acrosome reaction of guinea-pig sperm: A, normal acrosome before reaction; B, reaction starts through initial membrane fusion at the leading edge of the acrosome; C, elliptical area (b in Fig. 1) disappears; D, cavitation begins in area adjacent to concave edge of acrosome; E, cavitation complete; and F, acrosomal contents have detached except for vesicles attached to inner acrosomal membrane.
cavitation of the acrosomal contents) is the consequence, not the cause, of membrane fusion and this is confirmed by electron micrographs (Figs. 2, 3) which show membrane fusion preceding cavitation. The sequence of events suggested by the evidence is shown diagrammatically in Fig. 7. One further argument against a mechanism which relies on acrosomal swelling as the cause of membrane fusion is that it would be a mechanism peculiar to sperm, because only in sperm is the geometry of the secretory granule such that swelling would bring it automatically against the plasma membrane. Membrane fusion does not depend on proacrosin activation or acrosin activity (the latter a suggestion of Gordon (1973)) since the acrosome reaction takes place in the presence of a membrane-permeable acrosin inhibitor (Green, 1978a).

Finally, there is the question of what prevents a premature acrosome reaction. One answer is, of course, the absence of the stimulus. But the evidence suggests that something more is involved. Sperm are incapable of undergoing an acrosome reaction until they have been capacitated (Bedford, 1970). One explanation of the need for capacitation is that it represents the removal of an inhibitor from the receptor for the stimulus ligand or the calcium channel which it opens. The possibility that the acrosome reaction can be prevented during sperm storage in the epididymis and vas deferens because of the absence of external free calcium can, at least in the case of the latter, be ruled out: measurement of the free calcium in the guinea-pig vas deferens using a calcium electrode shows it to be about 300 μM (T. J. Rink & R. Y. Tsien, unpublished observations). It must fall at the time of ejaculation because of the large injection of citrate, which is more than sufficient to chelate it, from the seminal vesicles (Mann, 1964) but it almost certainly rises in the female reproductive tract as sperm progressively free themselves from the seminal plasma: the free calcium level in the tract is about 2-4 mM (Olds & Van Demark, 1957; Hamner & Williams, 1965; Holmdahl & Mastroianni, 1965; Restell & Wales, 1966). The evidence suggests, therefore, that a low cytoplasmic free calcium is maintained in sperm by active calcium efflux and a low resting permeability to calcium and that this, possibly together with a high threshold to calcium activation of membrane fusion, prevents a premature acrosome reaction.

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