

CYTOPLASMIC ACTIVATION OF STARFISH OOCYTES BY SPERM AND DIVALENT IONOPHORE A-23187

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ABSTRACT

The relationship between onset of the early cytoplasmic stages of oocyte activation (vitelline membrane separation and elevation) and nuclear meiotic maturation was investigated in starfish oocytes after their exposure to divalent ionophore (A-23187) or sperm. Meiotically mature oocytes, isolated in calcium-free seawater, underwent activation in response to sperm or ionophore as previously reported. Large, immature starfish oocytes, arrested in prophase I of meiosis (germinal vesicle stage), underwent vitelline membrane elevation when treated with divalent ionophore A-23187 or starfish sperm. Histological studies demonstrated that cortical granule breakdown in the oocyte cortex was associated with vitelline membrane elevation after these treatments. Activation of oocytes by sperm occurred only in response to starfish sperm. Sea urchin, sand dollar, surf clam, or marine worm sperm did not induce vitelline membrane elevation of either immature or mature starfish oocytes. Sperm- or ionophore-activated immature oocytes underwent nuclear maturation after addition of the meiosis-inducing hormone, *l*-methyladenine; however, parthenogenetic development did not occur and embryonic development was markedly inhibited. In contrast to previous studies, the present results indicate that cytoplasmic activation can be initiated before and without hormone induction of the nuclear maturation process. Differentiation of the oocyte cell surface or cortex reactivity therefore appears to occur during oogenesis rather than as a consequence of maturation. The data further support the view that divalent ions mediate certain of the early activation responses initiated by sperm at the time of fertilization and that synchronization of fertilization to the meiotic process in the oocyte is important for the occurrence of normal development.

In female gametes, a complicated process of cytoplasmic and nuclear differentiation occurs in preparation for possible fertilization and embryonic development (19). In starfish, gametes remain in arrested meiosis (germinal vesicle stage) within the gonad until just before the time of spawning. At this time, nuclear breakdown and reinitiation of

meiosis are induced as a result of the action of a gonadotrophic-like polypeptide (radial nerve factor [RNF]) released from the radial nerve (2, 3, 10, 11). The RNF appears to act indirectly to initiate these processes by stimulating the production and/or release from the ovary of a secondary material which has been identified as *l*-methylade-

nine (12, 13, 21). Spawned meiotically, mature oocytes can be fertilized and subsequently undergo a process of cytoplasmic and nuclear activation which is important for establishing the block to polyspermy, for fusion of male and female pronuclei, and for the initiation of embryonic development. Discharge of cortical granules as well as elevation of the extracellular vitelline membrane and its subsequent transformation into a fertilization membrane are integral parts of the oocyte cytoplasmic activation process in *Asterias* as well as many other species (1).

Classical studies have suggested that a necessary prerequisite for the occurrence of the cytoplasmic activation response is the initiation of nuclear breakdown or the actual dispersal of nuclear contents into the cytoplasm (4, 6). Recent studies, however, indicate that cytoplasmic maturation in starfish oocytes can be instituted by *l*-methyladenine even in the absence of the germinal vesicle and its contents (9). It is not clear, however, whether the activation processes at the oocyte surface (cytoplasm) are correlated with the nuclear events and to what extent they are dependent on exposure of oocytes to *l*-methyladenine.

Activation response is normally initiated in mature oocytes by sperm at fertilization, and recent studies indicate that divalent ions may be directly involved in this process. Addition of ionophore A-23187 to mature sea urchin or starfish oocytes was shown to induce most aspects of activation independent of sperm (25, 26).

In the present experiments, the relationship between cytoplasmic and nuclear (meiotic) maturation has been investigated by studying the cytoplasmic response of oocytes to sperm and the divalent ionophore (A-23187) at varying stages of nuclear maturation. Observations reported here demonstrate for the first time that induction of cytoplasmic aspects of the activation process can precede and be separated from the nuclear maturation process in the starfish. Additional experiments designed to analyze the nature of the immature oocyte activation response to ionophore and sperm and to explore the consequences of precocious activation to the developmental potential of the gametes are described.

MATERIALS AND METHODS

Preparation of Immature Gametes

Immature oocytes of *Asterias forbesi* were obtained from dissected ovaries after artificial induction of spawn-

ing. All experiments were carried out under *in vitro* conditions with filtered seawater (FSW) or artificial seawater (18). After their removal from the coelomic cavity, gonads were placed in calcium-free seawater for several hours or until the follicle cells surrounding each oocyte were dispersed within the ovary (22). Gonads were then retransferred back to FSW or artificial seawater containing calcium. Such treatment resulted in an immediate contraction of the ovary and the extrusion of immature oocytes denuded of follicle cells (5). Immature oocytes were identified under a dissecting microscope by the presence of the large nucleus (germinal vesicle) and the single prominent nucleolus (Fig. 1 a). The absence of the nucleolus and germinal vesicle in mature oocytes (Fig. 1 e) was utilized to assess the incidence of spontaneous or induced nuclear maturation.

Collection of Sperm

Dry preparations of sperm were obtained from starfish *Asterias forbesi*, sea urchin *Arbacia punctulata*, sand dollar *Echinarachnius parma*, marine worm *Hydroides hexagonus*, and surf clam *Spisula solidissima*, and used immediately after being diluted with filtered seawater to a concentration of 0.2%. Starfish sperm in some cases were stored dry in the refrigerator at 4°C for 12–24 h before use. Viability of the different sperm preparations was confirmed at the time of utilization by testing their capacity to fertilize eggs obtained from the same species.

Hormones and Ionophore

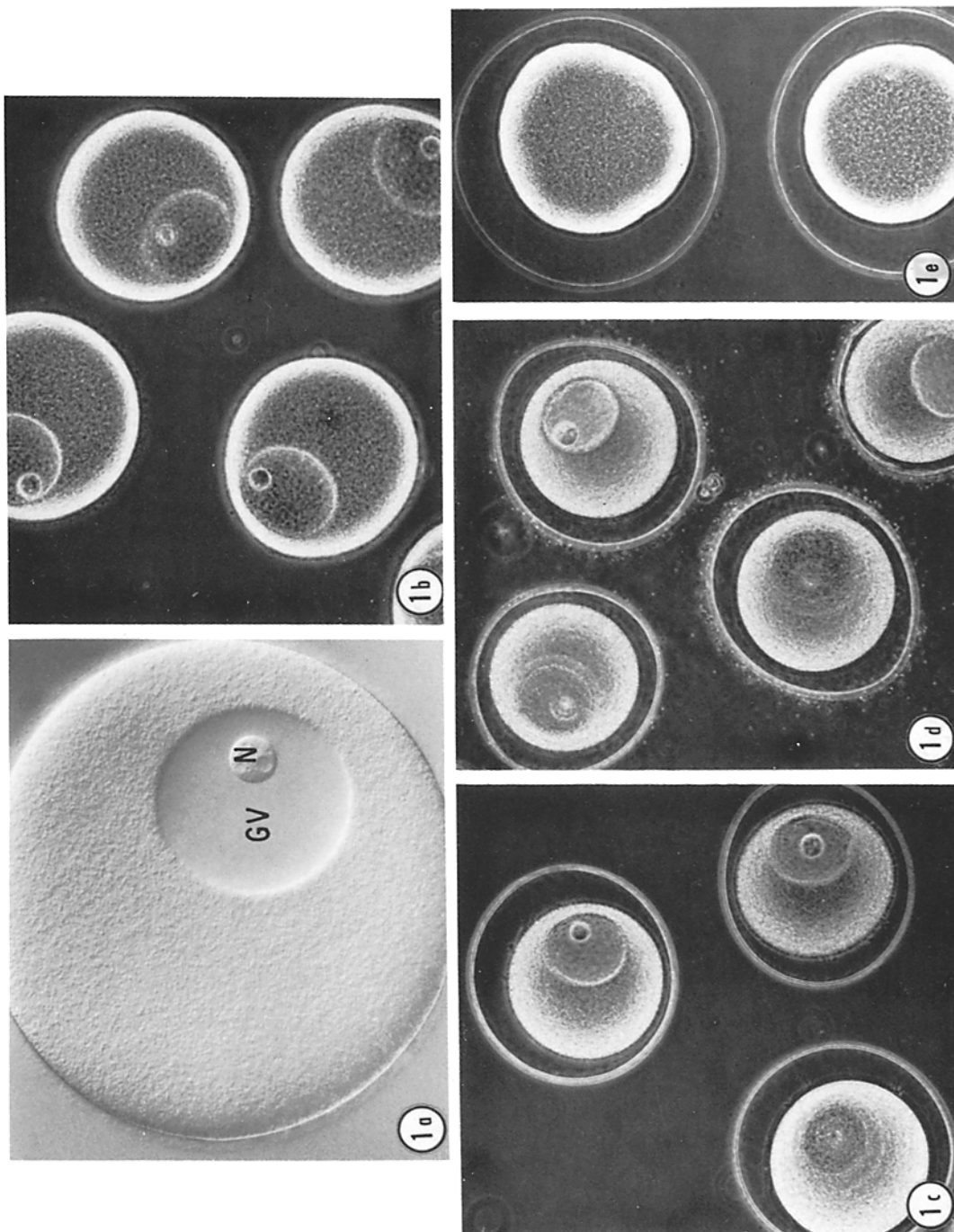
Starfish ovarian hormone, *l*-methyladenine (Sigma Chemical Co., St. Louis, Mo.), was dissolved in distilled H₂O and stored in the dark at a concentration of 1 mg/ml. The ionophore A-23187 was obtained from R. L. Hamill, Eli Lilly Co., Indianapolis, Ind. Ionophore was dissolved in dimethyl sulfoxide (Me₂So) at a concentration of 2 mg/ml and stored in the dark. When diluted in seawater, the solution was continuously stirred to insure good mixing and to minimize precipitation. 10–20 µl were normally added to oocytes to induce activation.

Experimental Conditions

Induction of meiotic maturation in immature oocytes was accomplished by addition of *l*-methyladenine (1 µg/ml) to small Stender dishes containing oocytes in 5 ml of seawater. One to three drops of the oocyte suspension (500–1,000 oocytes) were added to each dish and the progress of meiosis was assessed under magnification with a Wild dissecting microscope. Separation and elevation of the vitelline membrane from the oocyte surface was distinguished under the dissecting microscope and was utilized as a primary criterion of cytoplasmic activation.

Histological Procedures

Oocytes were fixed in Karnovsky's paraformaldehyde-glutaraldehyde (14) mixture in phosphate buffer, post-



fixed in 1% OsO₄ in phosphate buffer, dehydrated in acetone, and embedded in Spurr's (24). Sections of oocytes were cut on a Porter-Blum MT2-B ultramicrotome, stained with toluidine blue, and examined for changes in the oocyte cortex. Photographs were taken with Zeiss Aristophot or Universal microscopes.

RESULTS

Vitelline Membrane Elevation

Exposure of large immature oocytes to ionophore produces an almost immediate separation and partial elevation of the vitelline envelope from the oocyte membrane. These events are accompanied by concomitant formation of the perivitelline space (Table I, Fig. 1 c). Completion of vitelline membrane elevation, however, was a gradual process and in some cases continued over a period of some 30 min or more. A partial localized separation of the vitelline membrane was noted in some cases. Evident vitelline membrane activation was restricted to larger immature oocytes. In the few smaller more darkly colored oocytes, one typically could not distinguish a clear separation of the vitelline membrane from the oocyte. The effects of the ionophore were essentially the same as those produced by sperm fertilizing mature oocytes (Fig. 1 e). These results clearly demonstrate that vitelline membrane separation could be induced without nuclear maturation. Addition of ionophore vehicle, dimethyl sulfoxide (DMSO), to immature oocytes freed of follicle cells was never observed to result in membrane elevation. Likewise, when treated with sperm or ionophore, small or large immature oocytes, surrounded by follicle cells, did not exhibit membrane elevation if they were released from ovaries not preincubated in calcium-free seawater.

Induction of vitelline membrane elevation in immature oocytes was clearly contrary to previous observations concerning the relationship of maturation to the fertilization process. Since activation is normally induced at the time of fertilization,

TABLE I
Ionophore (A23187)- and Sperm-Induced Activation of Immature Oocytes of the Starfish*

Treatment	Fertilization membrane elevation (activation)	
	Animals	
	No.	%
Ionophore†	7	90-100
Sperm	7	88-100
Controls (vehicle)	7	0-1
Control	7	0

1974, Woods Hole, *Asterias forbesi*.

* Immature oocytes collected by artificial spawning CaFSW → normal seawater.

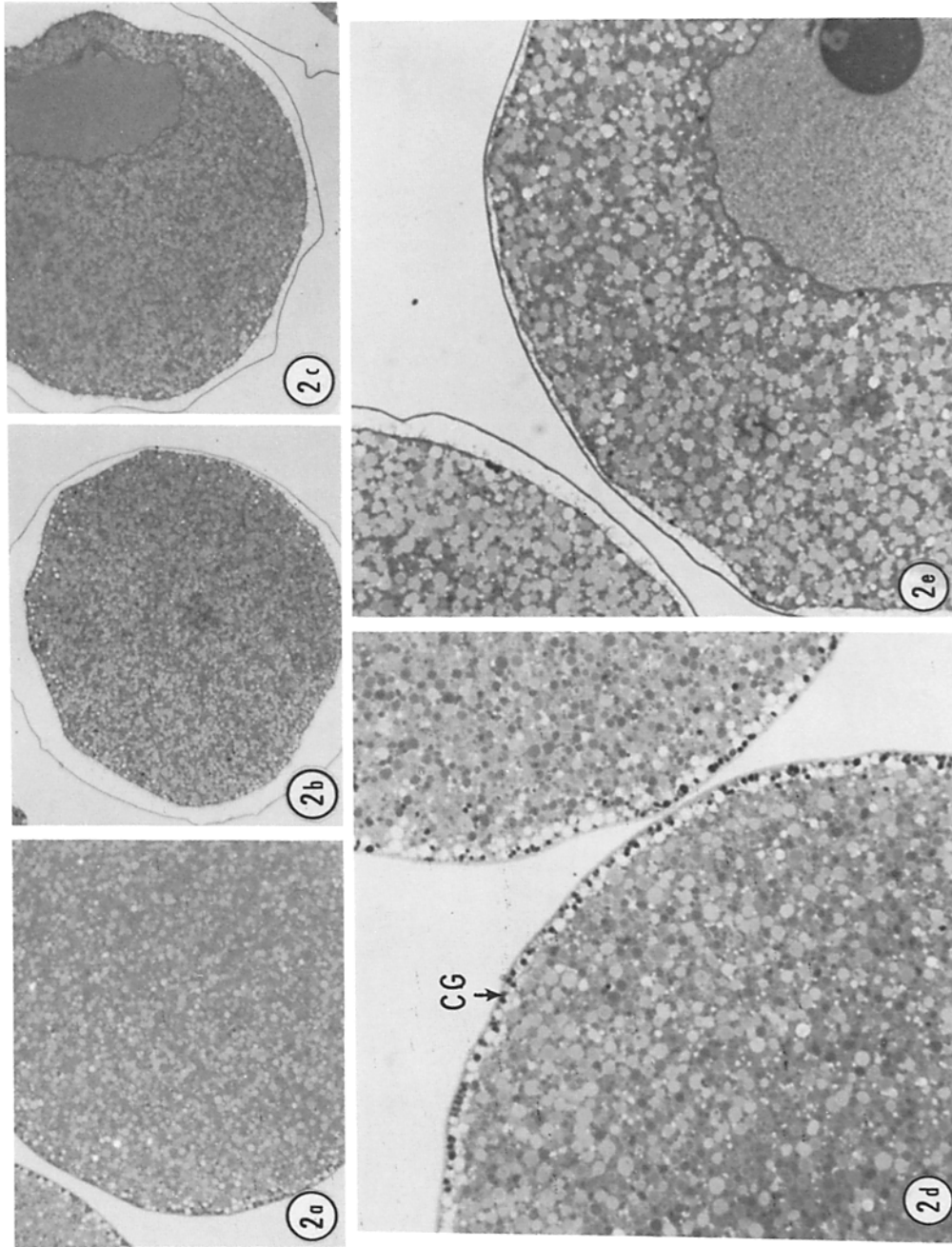
† Ionophore concentration (4 µg/ml).

studies were carried out to assess whether starfish sperm could also initiate activation of immature oocytes. The results of these experiments, presented in Table I and Fig. 1 d, demonstrate that starfish sperm have the capacity to induce the separation and elevation of the vitelline membrane from the immature oocyte. To determine whether the reaction to sperm was unique, sperm from a variety of invertebrate species were collected, and fertilization and activation of immature and mature oocytes were attempted. Starfish oocytes were exposed to viable preparations of *Echinarachnius*, *Arbacia*, *Spisula*, and *Hydroides* sperm. In no case was membrane elevation induced in either immature or mature starfish oocytes with other than starfish sperm. The data indicate that the mechanisms of oocyte vitelline membrane elevation result from sperm interaction with specific sites on the female gamete and that these sites are present in the immature as well as mature oocytes.

Histological Examination

Histological examination of activated immature oocytes revealed that cortical granules at the oocyte surface were markedly reduced in number after exposure to either the ionophore or starfish

FIGURE 1 Activation response in immature starfish oocytes. Oocytes approximately 150 µm in diameter. (a) Immature starfish oocyte with large germinal vesicle (GV) or nucleus containing a single nucleolus (N). The vitelline membrane is closely attached to the oocyte surface. (b) Immature starfish oocytes treated with ionophore vehicle (DMSO) resemble control oocytes. (c) Vitelline membrane separation and elevation (activation) after ionophore A-23187 treatment of immature oocytes. (d) Vitelline membrane separation and elevation (activation) after treatment of immature starfish oocytes with starfish sperm. (e) Activation response normally seen when oocytes are fertilized or treated with ionophore after maturation (nuclear breakdown) induced by 1-methyladenine.



sperm (Fig. 2 *a-e*). These changes are similar to those seen in mature activated oocytes. The results indicate that ionophore- and sperm-induced vitelline membrane elevations are linked to the cortical granule discharge process, rather than being just a separation of the vitelline membrane from the oocyte surface. Thus, it appears that a true activation response has been elicited at the surface and cortex in immature oocytes.

Maturation and Development of Activated Immature Oocytes

Activation of immature oocytes by sperm or ionophore results in asynchrony of the normal fertilization process. The question of whether such immature activated oocytes were viable and capable of undergoing maturation was examined. Immature oocytes that had been treated with sperm (Fig. 3 *a*) or ionophore within 5 min of spawning were exposed 1 h later to *l*-methyladenine. Examination of these oocytes within 1 h after *l*-methyladenine treatment indicated that 96% or more of all activated oocytes underwent nuclear breakdown (Table II). In two separate experiments when sperm-preactivated oocytes were again examined 9 or 24 h after induced maturation, less than 1% of the total preactivated oocyte population formed normal embryos (Fig. 3 *b*). More than 95% of control unactivated oocytes, fertilized 1 h after *l*-methyladenine-induced maturation, developed to the blastula stage within 24 h. No embryonic development occurred in ionophore-activated oocytes after the induction of nuclear maturation with *l*-methyladenine. Oocytes were not examined for the presence of incorporated sperm in these studies. However, preliminary attempts to refertilize sperm- or ionophore-activated immature oocytes after induction of nuclear maturation were unsuccessful in producing cleavage or embryonic development. This result suggests that discharge of the cortical granules probably occurred at the time of initial cytoplasmic activation and resulted in establishment of a block to polyspermy.

DISCUSSION

The results presented here demonstrate for the first time that certain aspects of the cytoplasmic activation process, specifically, vitelline membrane elevation and cortical granule discharge, can be initiated while oocytes remain in the immature, germinal vesicle stage or without exposure to sufficient *l*-methyladenine to induce nuclear maturation. Previous studies of starfish oocytes have consistently indicated that meiotically immature oocytes are not activated by sperm, whereas those oocytes undergoing spontaneous (4, 6) or *l*-methyladenine-induced nuclear maturation can be activated at about the time of nuclear breakdown (9, 27).

Several factors appear to be of importance in attempting to explain the apparent discrepancy between present and previous results. In some early studies, cleavage rather than fertilization membrane elevation was utilized as a criterion of cytoplasmic activation. The present data indicate that fertilization membrane elevation can be induced without any evidence of cleavage even though the oocytes are capable of undergoing development if properly matured before being fertilized. Thus, the absence of cleavage does not necessarily mean the absence of cortical changes and fertilization membrane formation. In addition, until the discovery of the inducers of maturation (RNF and *l*-methyladenine), oocyte maturity and/or immaturity were defined on the basis of an oocyte's capacity to undergo spontaneous maturation when released from the ovary. It is now apparent that many oocytes which do not undergo spontaneous nuclear maturation can readily be induced to do so.

Follicle cells also appear to be a complicating factor with regard to the induction of cytoplasmic activation in immature oocytes. A single layer of follicle cells characteristically encompasses each oocyte when the ovary is minced or torn to release meiotically immature eggs. In *Asterias*, follicle cells typically remain attached to the oocytes until the time at which nuclear breakdown occurs

FIGURE 2 Structural alterations at the cell surface after activation of immature starfish oocytes (cross section). (*a*) Immature starfish oocyte, cortical granules intact, and vitelline membrane attached to oocyte. (*b*) Sperm-activated immature oocyte. (*c*) Ionophore (A-23187)-activated immature oocyte. (*d*) Ionophore vehicle (DMSO)-treated immature oocytes. CG, cortical granules. Magnification approximately $\times 1,100$. (*e*) Ionophore-activated (A-23187) immature oocyte. Magnification approximately $\times 1,100$.

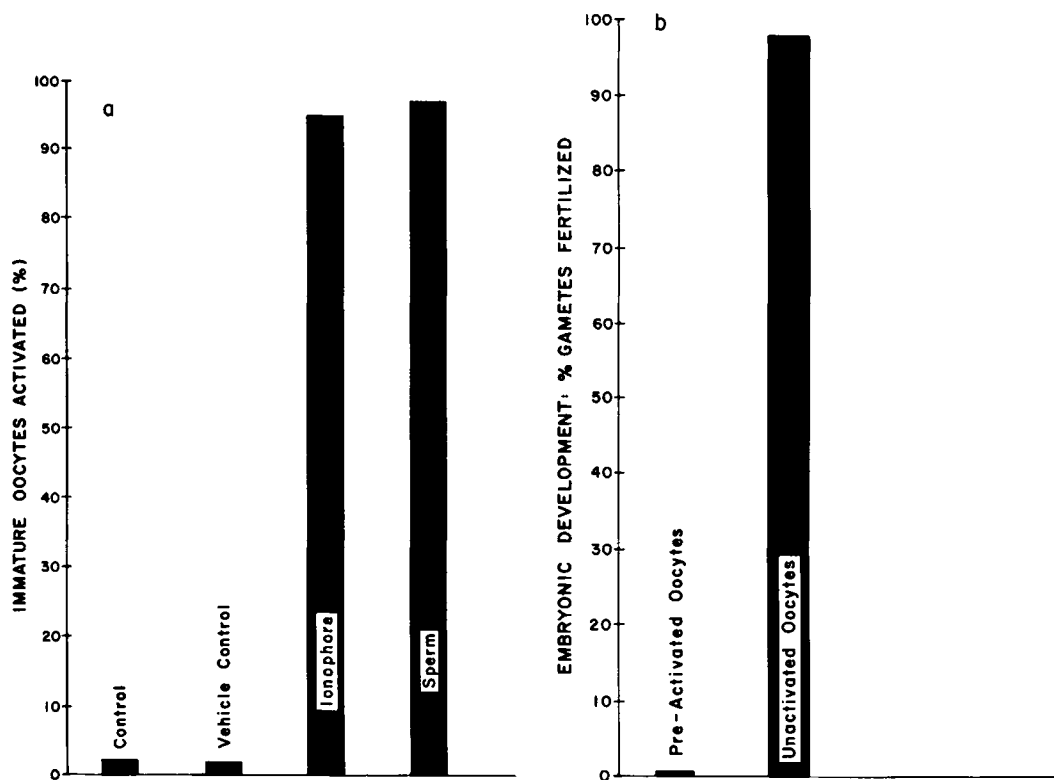


FIGURE 3 (a) Incidence of activation in immature oocytes exposed to divalent ionophore or starfish sperm. (b) Incidence of embryonic development in prematuration sperm-activated oocytes (preactivated oocytes) after induction of maturation with *l*-methyladenine. Normal controls (unactivated oocytes) were fertilized after *l*-methyladenine-induced maturation.

TABLE II
Induction of Oocyte Maturation by *l*-Methyladenine
in Activated Immature Starfish Oocytes

Type of activation	Activation range	Animals	IMA*	Response†
	%	No.		
Sperm	96-100	3	+	96-100
Sperm	96-100	3	-	4-12
Ionophore	92-100	4	+	96-100
Ionophore	96-100	4	-	8-20

* *l*-Methyladenine (0.5 μ g/ml).

† Germinal vesicle breakdown.

spontaneously, possibly as a result of the release of *l*-methyladenine from these cells, or after treatment with *l*-methyladenine (5). Oocytes which do not undergo spontaneous maturation retain their follicular envelope for extended periods of time. Thus, follicle cells, in fact, may cover the oocyte surface and vitelline membrane and interfere with the cytoplasmic activation. This idea is supported

by the fact that fertilization membrane elevation in response to either ionophore or starfish sperm was not observed when immature oocytes were still surrounded by follicle cells. In the present experiments, follicle cells were not a problem since they were removed from oocytes by treatment of ovaries in calcium-free seawater before being used in activation experiments. Prewashing of oocytes in Ca-FSW may, however, sensitize the oocyte cortex to sperm and/or ionophore induction of activation. However, since such prewashed oocytes still undergo normal maturation, fertilization, and early development (Fig. 3), this treatment clearly does not appear to have detrimental effects on the oocytes. Possible species variations in the loss of follicle cells from oocytes or in oocyte responsiveness to activating stimuli may also explain some of these discrepancies.

Therefore, it appears that activation processes which occur in the cytoplasm can proceed separately from nuclear events rather than being a necessary consequence of physiological changes

associated with or after induced or spontaneous nuclear breakdown. It might be argued that cytoplasmic activation of immature oocytes simply delays the inevitable nuclear maturation process. This seems highly unlikely, however, since the incidence of nuclear maturation in immature control oocyte preparations did not increase, even after 24 h of additional culture; and yet these oocytes retained their responsiveness to *l*-methyladenine. Although the present results demonstrate that cytoplasmic and nuclear events can be dissociated, it is not clear whether cytoplasmic maturation occurs completely independently of *l*-methyladenine. The possibility exists that the amount of *l*-methyladenine required for cytoplasmic maturation is less than that required for the induction of nuclear breakdown. Release of small amounts of *l*-methyladenine from the ovary, during artificial spawning of immature oocytes, may be a possible source of such amounts of purine (5, 8). A direct effect of *l*-methyladenine on oocyte cytoplasm has been demonstrated after enucleation of starfish oocytes (9).

Induction of cytoplasmic maturation without hormone-induced nuclear maturation in the starfish oocyte is in contrast to experimental results on amphibian oocytes in which cytoplasmic and nuclear maturation are initiated by steroid hormones (19, 23; A. Belanger and A. W. Schuetz, unpublished observations). Cytoplasmic factors produced in response to the steroid hormones, furthermore, appear to act as secondary intermediates in producing nuclear breakdown and cytoplasmic activation in the amphibian oocytes (16). Since, in the starfish, these two processes can be dissociated, it would appear that if cytoplasmic factors are involved in these events, they may play different roles or may be regulated by different mechanisms. Thus, there may be fundamental differences in the way oocytes of different species mediate various aspects of the maturation process (20).

Important questions concerning the mechanism of sperm and ionophore activation in immature oocytes are also raised by these observations. In particular, is it necessary for the starfish sperm to penetrate the vitelline membrane and oocyte membrane to induce vitelline membrane separation in the immature oocytes, and what role do divalent ions play? Definitive data on the cytoplasmic incorporation of sperm were not obtained in these studies. However, the fact that over 95% of the control fertilized mature oocytes developed,

whereas only 1% of the sperm-preactivated oocytes developed (Fig. 3), may indicate that sperm can initiate vitelline membrane separation and elevation without entering the oocyte. This seems unlikely however, since the sperm penetrate the vitelline membrane and enter the cytoplasm of immature sea urchin oocytes (7) or rabbit oocytes (17). Thus, if sperm are entering the oocyte, the absence of embryonic development in such oocytes after the induction of maturation by *l*-methyladenine is remarkable in view of the short period of time between addition of sperm and induction of maturation. Clearly, these results demonstrate that the stage of meiotic progression in the oocyte at the time of fertilization plays an important role in determining the nature of the activation response and the subsequent fate of the oocyte (15). In essence, it appears that exposure of oocytes to sperm at other than particular stages of meiosis and/or physiological maturation can prevent the utilization of these gametes for embryogenesis.

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