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Egg Activation

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Early in the fertilization process a series of events occurs, including depolarization of the egg plasma membrane, sperm penetration, release (exocytosis) of the contents of cortical vesicles (alveoli), formation of the perivitelline space, and resumption of meiotic division. Eggs undergoing these fertilization reactions, particularly exocytosis, are called activated eggs. Unfertilized eggs in most teleosts are activated not only by immersion in isotonic saline or freshwater resembling spawning environment, but also by chemical or physical stimulants (Yamamoto, 1958, 1961). In the egg activation, a number of functional proteins may be activated by proteolytic cleavage of a terminal peptide (Nakano, 1969) and by phosphorylation or dephosphorylation of functional proteins. Such phenomena induced by fertilization should also be observed in eggs parthenogenetically activated by chemical or physical stimuli, even though most of these eggs do not develop further. The present review deals with the cortical reaction events of egg activation, mainly in the medaka.

1. Responses of the egg to sperm stimulation

a. Morphological changes in the egg surface

External Ca^{2+} is necessary for intercellular fusion between the sperm and egg plasma membranes, as reported for the medaka (Yamamoto, 1944a; Iwamatsu *et al.*, 1985), *Oncrhynchus keta* (Hamano, 1949) and pacific herring (Yanagimachi and Kanoh, 1953). In the presence of Ca^{2+}, the first response of the egg to sperm-egg fusion is the change in membrane potential (Hori, 1958; Ito, 1962). The initial resting potential is greater than –20 mV (Nuccitelli, 1980). According to Nuccitelli (1980), this membrane depolarization occurs 5±2 sec (range 1–5 sec, N=4) after a spermatozoon enters the micropyle (Fig. 1). This time is in good agreement with the time required for the spermatozoon to stop moving as it attaches to the egg surface (Iwamatsu *et al.*, 1991). This event seems to mark initiation of intercellular membrane fusion of the gametes which is known to occur within 10 sec (Iwamatsu and Ohta, 1981). Five seconds after the cessation of sperm movement, the earliest exocytosis of small cortical alveoli takes place in a small area of the cortex in the vicinity of the inner orifice of the micropyle. The exocytosis (breakdown) of cortical alveoli in the medaka egg was first observed by Professor T. Yamamoto (1939). Before completion of exocytosis the contents of the cortical alveoli are dissolved by the enzyme N-glycosidase (Seko *et*

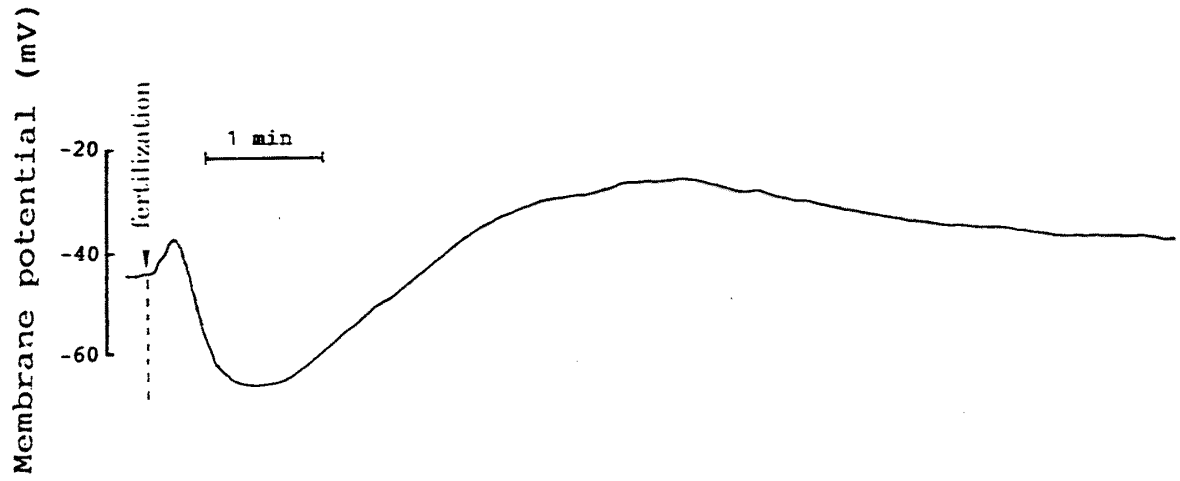

Fig. 1. Changes in membrane potential induced by fertilization in the medaka egg. Fertilization triggers a small depolarization, followed by a longer hyperpolarization phase in 10% Yamamoto's Ringer's solution (Nuccitelli, 1980).

al., 1991). The alveolar membrane adjoining the egg surface fuses with the plasma membrane to form a large aperture (Fig. 2) (Iwamatsu and Ohta, 1976, Iwamatsu and Keino, 1978). The alveolar contents, including spherical bodies and colloidal substances, are released through the aperture into the interstice between the egg plasma membrane and the chorion. During the release, the spherical bodies swell and the colloidal substances (including hyosophorins, Kitajima *et al.*, 1989) disperse. Microvilli arise on the membrane where the cortical alveoli have fused with the plasma membrane. The alveolar membrane area is thereby discernible from the rest of the egg surface during or shortly after exocytosis (Iwamatsu and Keino, 1978).

b. The "fertilization wave" and the wave of increase in cytoplasmic Ca^{2+}

Exocytosis begins at the point of sperm entry at the animal pole and spreads towards the antipode of the egg over the whole surface of the egg.

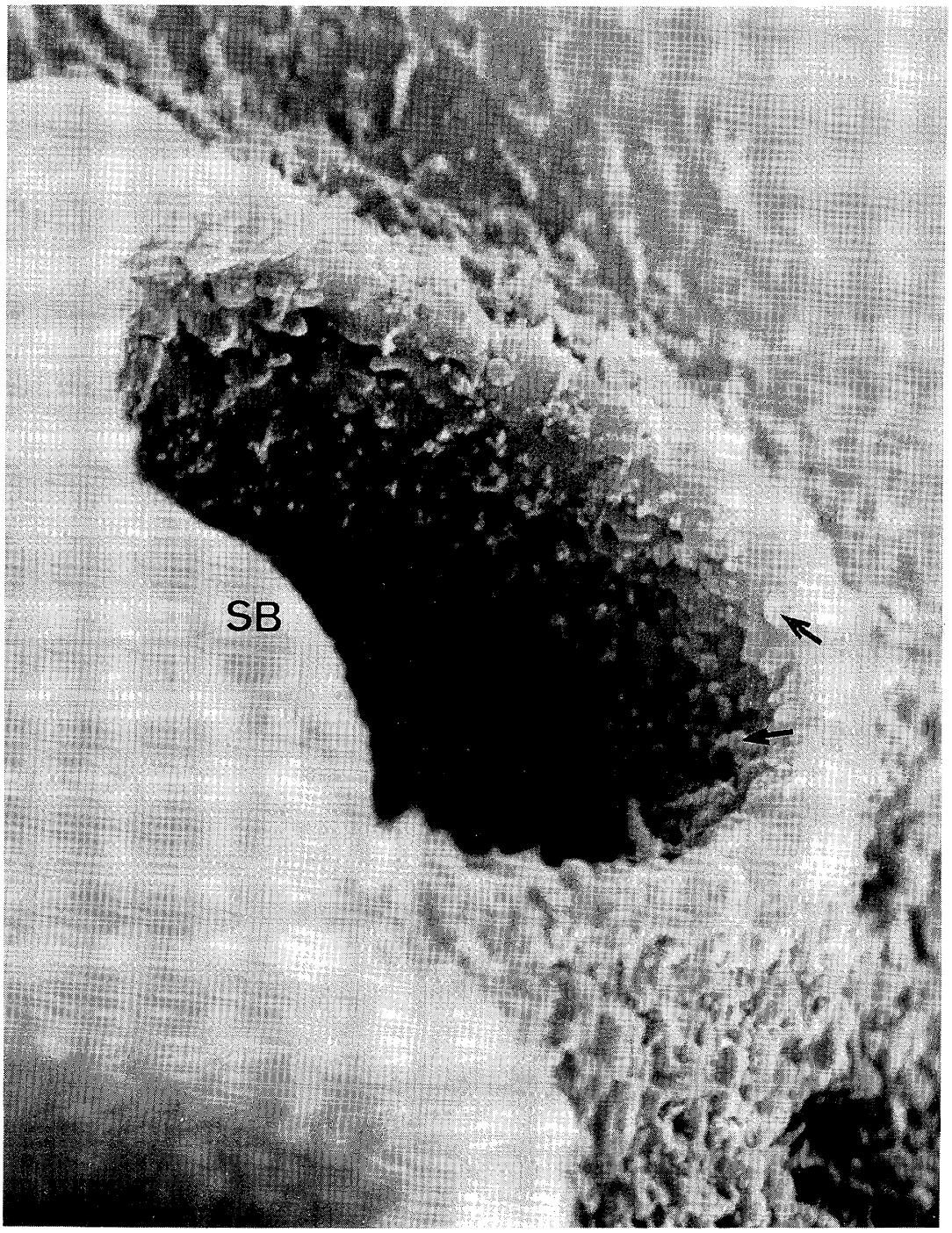

Fig. 2. A large aperture in a cortical alveolus of a medaka egg during exocytosis. Microvilli (arrows) are produced beginning at the aperture and continuing towards the bottom of the inner wall of the cortical alveolus. SB, spherical body. ×4,840 (Iwamatsu and Keino, 1978)

Yamamoto (1958) proposed three of possible mechanisms by which the wave of exocytosis might occur: (1) domino fashion, (2) dispersion of a substance that induces exocytosis and (3) protoplasm propagation. When unfertilized eggs were centrifuged, and the cortical alveoli were dislocated to the equatorial side of the egg. When the alveolus-free cortex in the animal hemisphere of the centrifuged eggs can be stimulated by pricking, cortical alveoli that had been dislocated to the equator broke down. This result implies that the invisible change or impulse that induces exocytosis is propagated in the cortical layer. Thus, the concept of a "fertilization wave" as the impulse wave was offered by Yamamoto (1943).

Furthermore, Yamamoto (1954) suggested that calcium ions are released from the protoplasm as a result of external stimulation. In order to demonstrate that free Ca^{2+} is released from the cortex, an egg was pricked with a needle the tip of which was large enough to trigger progressive exocytosis of cortical alveoli in Ca-free saline containing 0.3% sodium alizarin sulfonate. The saline immediately turned deep red due to formation of Ca-alizarin sulfonate when the contents of the cortical alveoli were released into it (Yamamoto, 1956). When the same experiment was performed on unfertilized eggs anesthetized with either phenylurethane or chloretone, only a slight red exudate formed, indicating that Ca-alizarin sulfonate was formed in only small amounts. On the basis of this experiment, the idea was promoted that free Ca^{2+} is released from the cortical cytoplasm when unfertilized eggs are stimulated by pricking. That is, cytoplasmic Ca^{2+} is necessary or at least has a favorable effect on fertilization or exocytosis of cortical alveoli, or both. Later, the excellent experiments of Ridgway *et al.* (1977) and Gilkey *et al.* (1978) used aequorin, which releases light energy upon binding with free Ca^{2+}, to demonstrate that the wave of increase in cytoplasmic free Ca^{2+} is propagated from the point of sperm entry toward the antipode of the egg (Fig. 3). This Ca^{2+} wave was an invisible cortical reaction and was considered to correspond to the fertilization wave (Gilkey *et al.*, 1978). The wave of increased free Ca^{2+} passed over the egg with the same time course as the fertilization current wave reported (Nuccitelli, 1987). The increase in intracellular Ca^{2+} can occur following Ca-ionophore treatment in the absence of external free Ca^{2+}. Furthermore, no distinct increase in uptake of external $^{45}Ca^{2+}$ has been measured (Fig. 4 in Iwamatsu *et al.*, 1985), though a possible Ca^{2+} influx has been suggested by Nuccitelli (1987). Moreover, we have recently found that the amount of $^{45}Ca^{2+}$ previously incorporated in the microsomal fraction decreases after fertilization (unpublished data by Iwamatsu and

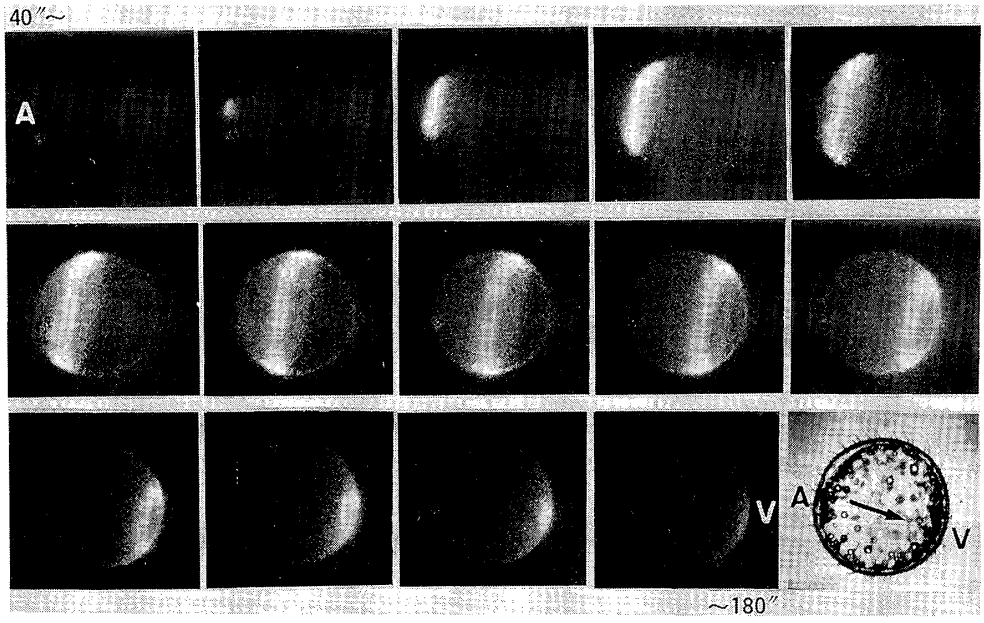

Fig. 3. A Ca^{2+} wave induced by fertilization and visualized by the luminescence of aequorin previously loaded into the medaka egg. Photographs were taken at 10 sec intervals beginning 40 sec after insemination. Each photograph is a 5 sec exposure of the videotape image of the egg played back on the monitor screen. The last photograph is a transmitted light image of the egg (1.2 mm in diameter) in which an arrow indicates the direction of propagation of the Ca^{2+} wave from the animal pole (A) to the vegetal pole (V).

Nakashima). These facts support the idea (Gilkey *et al*, 1978) that Ca^{2+} is stored in the microsomal fraction. Thus, sperm stimulation seems to release Ca^{2+} from microsomes into cytosol, and the wave of increase in cytoplasmic free Ca^{2+} is propagated to the whole egg surface prior to the wave of exocytosis.

c. The relationship between the fertilization wave and the exocytosis wave

As described above, exocytosis is propagated from the stimulation point toward the antipode of the egg. In some inseminated eggs, exocytosis in almost all areas of the surface is complete, but some alveoli at the vegetal pole region remain intact. The latter alveoli exhibit complete breakdown when the egg is pricked (Yamamoto, 1956, 1958). A similar phenomenon has been observed in oocytes during the maturation period (Iwamatsu, 1965). Yamamoto (1944b) proposed that the fertilization wave or impulse is propagated with diminishing intensity and velocity and that the impulse elicited by sperm stimulation is weaker than that elicited by pricking the egg with a glass needle. In incompletely anesthetized eggs, cortical alveoli break down in the animal hemisphere, while those in the vegetal hemisphere remain unchanged. This suggests that in these eggs the impulse elicited by sperm stimulation is so weak in intensity that it can break down cortical alveoli only in the animal hemisphere, not in the vegetal hemisphere, due to the decreasing intensity of the impulse. If the wave of increase in cytoplasmic Ca^{2+} corresponds to the fertilization wave, the former should diminish as it is propagated. However, the wave of increase in cytoplasmic Ca^{2+} has the same velocity in the animal hemisphere as in the vegetal hemisphere and is propagated without diminishing in intensity or velocity (Yoshimoto *et al.*, 1986).

A decrease in the rate of conduction of the fertilization wave in the direction from the animal pole to the vegetal pole was prepared to explain the observation that when propagation was induced from the vegetal pole (V) to the animal pole (A), the velocity of the wave in the area (VE) between the vegetal pole and the equator (E) was almost the same as that in the area from the equator to the animal pole (AE). The velocity in the area AE was slower than would be expected if the rate of propagation was constant. If the fertilization wave travels from the stimulation point to the antipode of the egg without decreasing against a gradient of increasing sensitivity, the velocity in the area AE would increase compared with that in the area VE. In order to ascertain the decrease in propagation velocity of exocytosis, we recently re-examined the velocity of the exocytosis wave. As shown in Fig. 5, the velocity of the wave of exocytosis as well as the wave of increase in cytoplasmic free

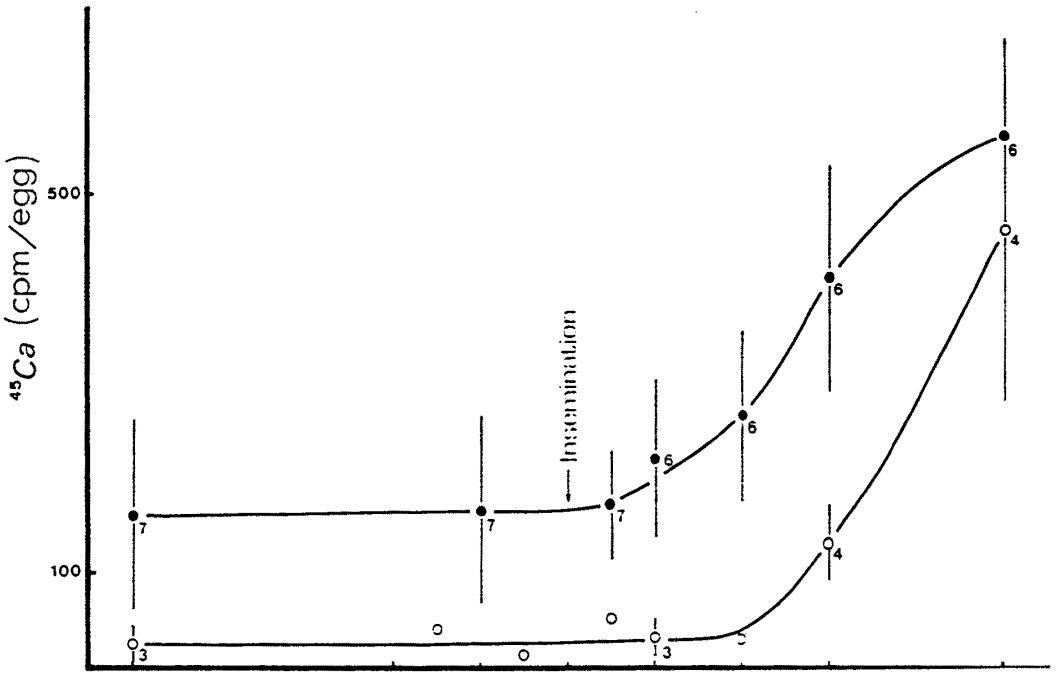

Fig. 4. ^{45}Ca uptake by medaka eggs before and after fertilization. –○–, Intact eggs; –●–, Naked eggs. Numbers indicate the number of experiments for each point (Iwamatsu *et al.*, 1985).

Ca^{2+} (Fig. 3) did not diminish between the starting point of exocytosis and the end point in the egg cortex, though the velocity of exocytosis in the animal hemisphere was faster than that in the vegetal hemisphere, as found by Yamamoto (1943). The velocity of the wave in the area AE was 17.1 μm/sec, while that in the area EV was 12.1 μm/sec. This difference in the velocity of the wave in the animal and vegetal hemispheres seems to be a function of egg polarity.

When Ca^{2+} or inositol 1,4,5-trisphosphate (IP_3) is microinjected into different regions of the cortical cytoplasm of unfertilized eggs, exocytosis begins earlier at the animal pole than at other regions (Fig. 6); exocytosis is observed 7 sec after Ca^{2+}-microinjection at the animal pole, 10 sec at the equator and 14 sec at the vegetal pole. As seen in Fig. 6, following IP_3-microinjection exocytosis begins after 5 sec at the animal pole, 6 sec at the equator and 9 sec at the vegetal pole. The time (s_{AE}) required for exocytosis to travel from the animal pole (A) to the equator (E) is 53.4 sec.

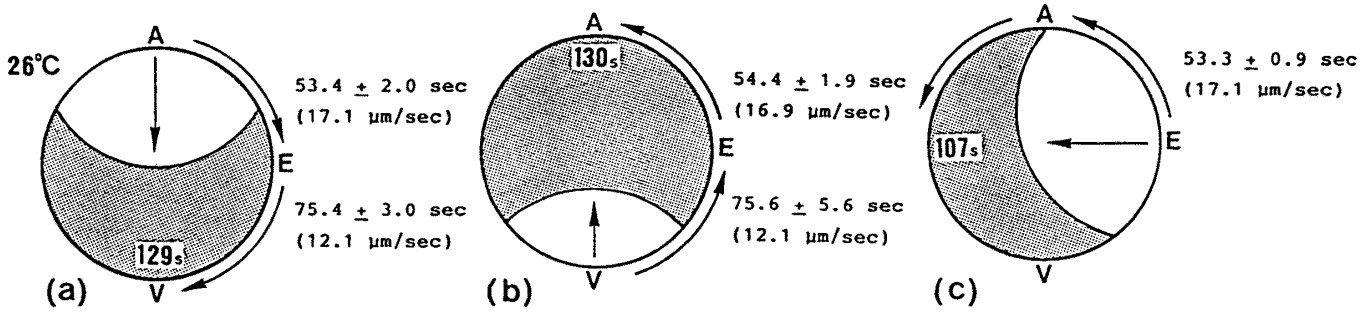

Fig. 5. Diagrams illustrating the propagation of exocytosis of cortical alveoli in the animal and vegetal hemispheres of the medaka egg. Unfertilized eggs were stimulated by pricking them with a glass needle at the animal pole (a), the vegetal pole (b), or the equatorial region (c). The time required for exocytosis to travel from a pole to the equator or from the equator to each pole and the calculated velocity of the wave are listed next to each diagram (Iwamatsu *et al.*, 1992).

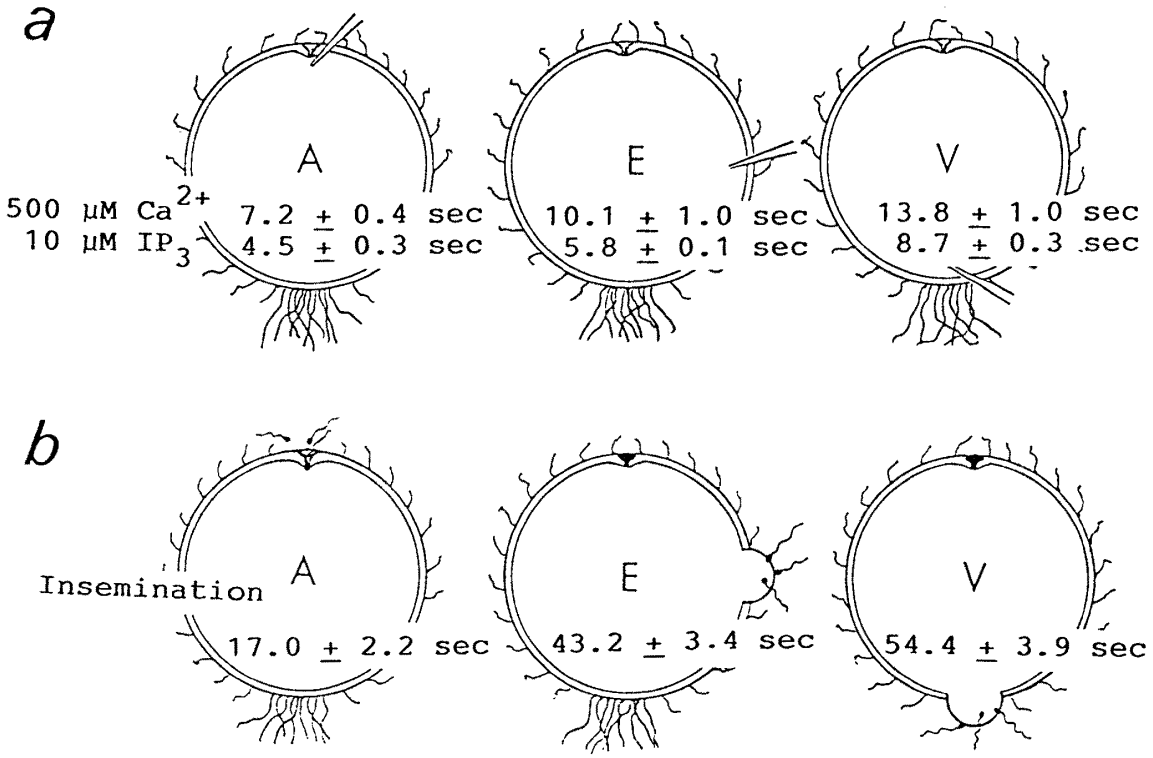

Fig. 6. The initiation times for cortical exocytosis following sperm stimulation and microinjection of Ca^{2+} and IP_3. a: $CaCl_2$ or IP_3 was injected into the cortical cytoplasm through a micropipette. b: Partially dechorionated eggs were inseminated after the micropyle was closed (black) by filling it with paraffin oil using micropipette. The spermatozoon directly attached to the egg surface at the animal pole (A), the equatorial region (E) or the vegetal pole region (V) of unfertilized eggs (Iwamatsu *et al.*, 1992).

When this time is divided by the average time (s_{A-E}, 5.2 sec) between both times (s_A 4.5 sec, s_E 5.8 sec) required for the beginning of exocytosis at the animal pole and at the equator respectively by IP_3 microinjection, a quotient 10.3 sec is obtained. When the value of this quotient is multiplied by the average time (s_{E-V}, 7.3 sec) between both times (s_E 5.8 sec, s_V 8.7 sec) required for the beginning of exocytosis at the equator and at the vegetal pole, the result almost agrees with the time (s_{EV}, 75 sec) required for exocytosis to travel from the equator (E) to the vegetal pole (V). Since the Ca^{2+}-wave is propagated from the starting point to the antipode of the egg without a delay, the propagation velocity of exocytosis seems to result from a difference in the time (sec) required for the beginning of Ca^{2+}- or IP_3-induced exocytosis in different areas. The difference in the propagation velocity of exocytosis in AE and EV areas indicates that the exocytosis reaction begins at different speeds in response to stimulation by increased intracellular Ca^{2+} or IP_3 in different regions of the egg.

2. The propagation of increase in cytoplasmic Ca^{2+} and pH in the process of activation

Gilkey *et al.* (1978), as described above, believed that the increase in cytoplasmic Ca^{2+} released from stores is a direct result of an initial increase in Ca^{2+} levels. However, if the increased cytoplasmic Ca^{2+} directly induces the release of Ca^{2+} into the cytoplasm, the Ca^{2+} release must originate in the region of the injection point where Ca^{2+} levels are locally increased. According to our observation (Iwamatsu *et al.*, 1988a,b), release of Ca^{2+} into the cytoplasm actually begins in the vicinity of the plasma membrane a few seconds after microinjection, but does not originate around the Ca^{2+}-injection point. Depolarization of the membrane potential does take place upon Ca^{2+} microinjection, however (Iwamatsu and Ito, 1986). The amount of injected Ca^{2+} is sufficient to immediately induce the propagative Ca^{2+} release (Fig. 7). Therefore, the delay in triggering release of Ca^{2+} is not caused to a low concentration of Ca^{2+}. The delay in release decreases as the amount of injected Ca^{2+} increases, because the injected Ca^{2+} is initially sequestered. Thus, it is suggested that the injected Ca^{2+} may cause release of Ca^{2+} into the cytoplasm, but may not act directly on the store. Instead the increase of stored Ca^{2+} is due to action of a membrane factor (Fig. 8). This interpretation is supported by Nuccitelli's (1987) experiment using IP_3 iontophoresis and by our experiments on microinjection of varioius agents

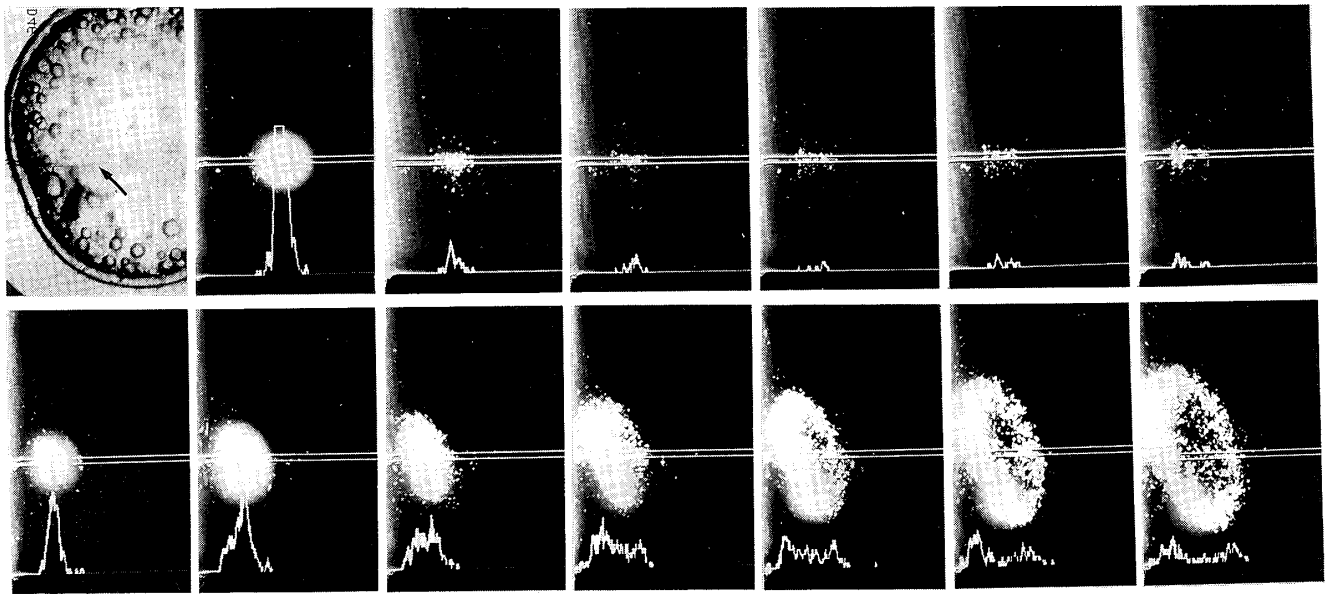

Fig. 7. Sequential accumulation (2 sec) and intensity distribution of aequorin luminescence in the medaka egg at time intervals of 4 sec after Ca^{2+} microinjection. Thirteen photographs (B-N) indicate changes in the distribution of light intensity (relative values) from the time of microinjection up to 52 sec. The light sampling was made in a restricted area (ca. 30 μm between two scanning lines) of the egg for 2 sec. The first figure shows the tip of a micropipette inserted in the animal pole region (arrow) and photographed by transmitted light just before injection (Iwamatsu *et al.*, 1988a).

such as IP_3 and cGMP. Sperm-egg interaction in the sea urchin stimulates the metabolism of polyphosphoinositides to IP_3 and diacylglycerol (DG) (Ciapa and Whitaker, 1986). IP_3 then stimulates the release of Ca^{2+} from intracellular stores (Clapper and Lee, 1985; Oberdorf *et al.*, 1986; Swann and Whitaker, 1986). Our results suggest that the same sequence of events occurs in the medaka egg. Binding of the spermatozoon to its receptor on the egg plasma membrane causes activation of phosphatidyl-inositol 4,5-diphosphate (PIP_2) phosphodiesterase (phospholipase C, PLC) via a G-protein (Gp). PLC cleaves inositol-diphospholipids into IP_3 and DG. The release of Ca^{2+} from cytoplasmic Ca^{2+}-storage is then triggered by IP_3. It has been found in the sea urchin egg that DG derived from polyphosphoinositides stimulates Na^+-H^+ exchange, (Lau *et al.*, 1986; Shen and Burgart, 1986). A propagative rise in intracellular pH is also observed in the cytoplasm of the activated medaka egg. In addition, propagation of the Ca^{2+} wave is slowed by the injected buffers of pH 6.9 or below (Gilkey, 1983) and progressive exocytosis is prevented by holding the eggs in acidic saline (Iwamatsu, 1984). The prevention of exocytosis may indicate (1) that Na^+-H^+ exchange stimulated by DG is inhibited in acidic medium, or/and (2) that the thresholds for the induction of Ca^{2+} release by IP_3 rises.

A close correlation between the increase in intracellular free Ca^{2+} and the inward activation current was observed by Nuccitelli (1987). He considered it a possibility that the current wave reflects an increase in the number of activated channels resulting from a localized increase in intracellular free Ca^{2+}. The results of his experiments, in which the ionic composition of the external medium was varied, indicated that a Na^+ influx and K^+ efflux was involved in the current wave. Furthermore, 5 min after fertilization, the outward current was 7-fold larger on the centripetal side of the egg where the membrane of most cortical alveoli had fused with the plasma membrane. This suggests that the increase in K^+ permeability requires the fusion of alveolar membranes and the plasma membrane (Kiyohara and Ito, 1968). Nuccitelli confirmed that K^+ is the most likely candidate ion for carrying outward current and obtained good evidence that the permeability of K^+ increases more than that of any other ions during the short period following fertilization (Nuccitelli, 1980). The K^+ channel participating in the outward current is distinct from those channels present in the plasma membrane prior to exocytosis. Thus, it was suggested that the localized addition of the membrane of the cortical alveolus enhances the local K^+ permeability by introducing new channels to the plasma membrane (Nuccitelli, 1980).

3. Participation of G-proteins in activation process

In order to analyse the mechanism of exocytosis of cortical alveoli, the effects of cGMP, GTP and Co^{2+} were examined. When cGMP or GTP was injected into the cortical cytoplasm of unfertilized medaka eggs, it caused exocytosis (Iwamatsu, 1989). The initiation of exocytosis by cGMP microinjection was accompanied by an increase in intracellular Ca^{2+} (Iwamatsu *et al.*, 1988b). In contrast, exocytosis was blocked in the region where Co^{2+} was preinjected, though it had no inhibitory effect when co-injected with Ca^{2+} or IP_3. These results suggest that Co^{2+} interfered with the process prior to the production of IP_3 or the release of Ca^{2+} (Fig. 8). On the other hand, the preinjection of GDP-β-S, which acts as a competitor of GTP at the regulatory site on the G-protein, did not prevent the egg from undergoing exocytosis. According to Shimamoto and Ito (personal communication), who performed an experiment on the change in membrane potential using chorela toxin (CTX), the G-protein may participate in the step in which the egg activation is triggered. Recently, it has been shown in the sea urchin egg that cGMP activates eggs by stimulating the release of Ca^{2+} from IP_3-sensitive Ca-storage, independent of an IP_3-receptor (Whalley *et al.*, 1992).

4. Activation without increase in intracellular Ca^{2+} and exocytosis

When unfertilized eggs which had been either anesthetized with chloletone or phenylurethane or pretreated with acetone were inseminated, cortical reactions such as the increase in intracellular Ca^{2+} and exocytosis of cortical alveoli failed to occur. These eggs underwent other fertilization reactions following sperm penetration and started to develop. In the absence of a transient increase in cytoplasmic Ca^{2+}, normal formation and migration of the pronuclei occurred. This suggests that the transient increase in intracellular Ca^{2+} and exocytosis are not only separable from the factor(s) responsible for nuclear behaviour, but are also unnecessary for initiation of development. A small amount of Ca^{2+} may be required by a protein involved in metabolic changes in the egg, because

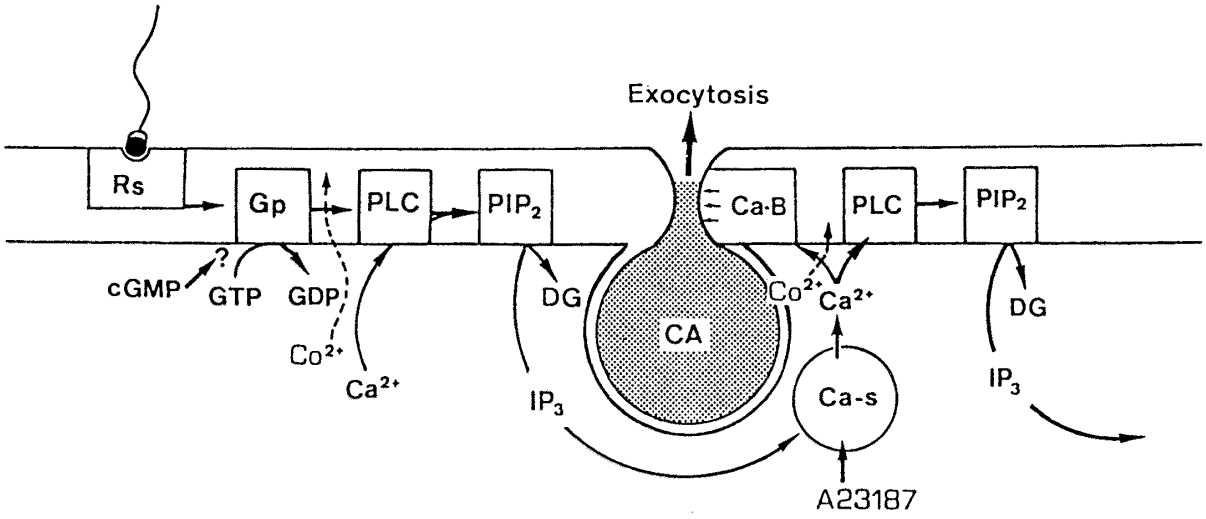

Fig. 8. Schematic diagram of the events leading to Ca^{2+} release and cortical alveolus exocytosis in the medaka egg. Binding of a spermatozoon to its receptor (Rs) leads to activation of phospholipase C (PLC) mediated by a guanine nucleotide-dependent regulatory protein (Gp). PLC catalyzes breakdown of phosphatidyl-inositol 4,5-diphosphate (PIP_2) into diacylglycerol (DG) and inositol 1,4,5-trisphospahte (IP_3). IP_3 interacts with a specific receptor (Ri) on the vesicles storing cytoplasmic Ca stores (Ca-s) adjacent to the egg surface causing release of free Ca^{2+} into the cytosol. The cytosolic Ca^{2+} activates PLC and Ca-binding substance(s) Ca-B), which participates in the exocytosis of cortical alveoli (CA) (Iwamatsu, 1989).

all the fertilization reactions are completely blocked when the Ca-chelator EGTA is microinjected.

The effects of the calmodulin inhibitors W-5, W-7 and W-13 on exocytosis were examined by microinjecting the inhibitors into the cortical cytoplasm of unfertilized eggs. This treatment failed to inhibit exocytosis, suggesting that a Ca-dependent protein other than calmodulin is involved in exocytosis.

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