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## [REVIEW]

# Mechanisms of Egg Activation and Polyspermy Block in Amphibians and Comparative Aspects with Fertilization in Other Vertebrates

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**ABSTRACT**—For precise temporal activation of the egg during amphibian fertilization, the sperm must provide a signal for egg activation at the time of membrane binding or fusion between sperm and eggs. A fertilizing sperm causes a  $\text{Ca}^{2+}$  wave which is both necessary and sufficient for egg activation at amphibian fertilization. The  $\text{Ca}^{2+}$  wave seems to be mediated by IP<sub>3</sub>-receptors on the endoplasmic reticulum and by IP<sub>3</sub> produced by hydrolysis of PLC activated by a Src-related protein tyrosine kinase (Xyk) in *Xenopus* eggs. We have proposed three different hypotheses for initiation of egg activation in amphibian eggs: the  $\text{Ca}^{2+}$ -influx model, the membrane receptor model, and the soluble factor model. The membrane receptor model and the soluble factor model seems to be applied to the monospermic *Xenopus* fertilization and the physiologically polyspermic *Cynops* fertilization, respectively. The  $\text{Ca}^{2+}$  wave at egg activation induces a positive fertilization potential which prevents entry of a second sperm in fertilization of monospermic species. In physiologically polyspermic urodele eggs, several sperm enter the egg at normal fertilization, but only one sperm nucleus with a centrosome participates in the embryonic development. The degeneration of accessory sperm nuclei is closely involved in differential distributions of both  $\gamma$ -tubulin and cyclin B in the egg cytoplasm, which causes developing a larger sperm aster and earlier entry into M phase in a zygote nucleus, respectively. We have discussed the molecular mechanisms of egg activation and polyspermy blocks in amphibians and make some comparisons with other vertebrates, such as fishes and mammals.

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## INTRODUCTION

Fertilization brings about at least three distinct reactions: restoration of the diploid configuration with mixing of the male and female genomes, introduction of the centriole necessary for cell division into the egg in most vertebrates, and activation of the process of cell division for development. Precise temporal activation of the egg during fertilization is essential for normal development, since activation before entry of the sperm nucleus can cause parthenogenesis. Conversely, a delay in activation can cause pathological polyspermy. Thus, the sperm must provide a signal for egg activation at the time of membrane binding or fusion between sperm and eggs.

Amphibians contain two groups exhibiting very different blocks to polyspermy (Elinson, 1986; Iwao, 2000). One is a block before sperm-egg fusion, operating in monospermic eggs

of anurans and some primitive urodeles, which is a species of the *Hynobius* genus (Iwao, 1989). Another is a block in egg cytoplasm after sperm entry, operating in physiologically polyspermic eggs in other urodeles. In monospermic species, development of an embryo with a diploid genome and a single centrosome (centriole) derived from a sperm is ensured by recruitment of a fast, electrical block to polyspermy on egg membrane, followed by a block at fertilization envelope formed by cortical granule exocytosis. The entry of more than two sperm causes abnormal development in monospermic species. In physiologically polyspermic species, several sperm enter an egg at normal fertilization, but only a single sperm nucleus with a single centrosome ultimately participates in embryonic development, while the other sperm nuclei and centrosomes degenerate before cleavage. Since faster activation seems to be necessary for the polyspermy block in monospermic species compared with physiologically polyspermic species, different mechanisms of egg activation may operate between these species. In this review we will discuss the molecular mechanisms of egg activation and polyspermy

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blocks in amphibians and will make comparisons with other vertebrates, such as fishes and mammals.

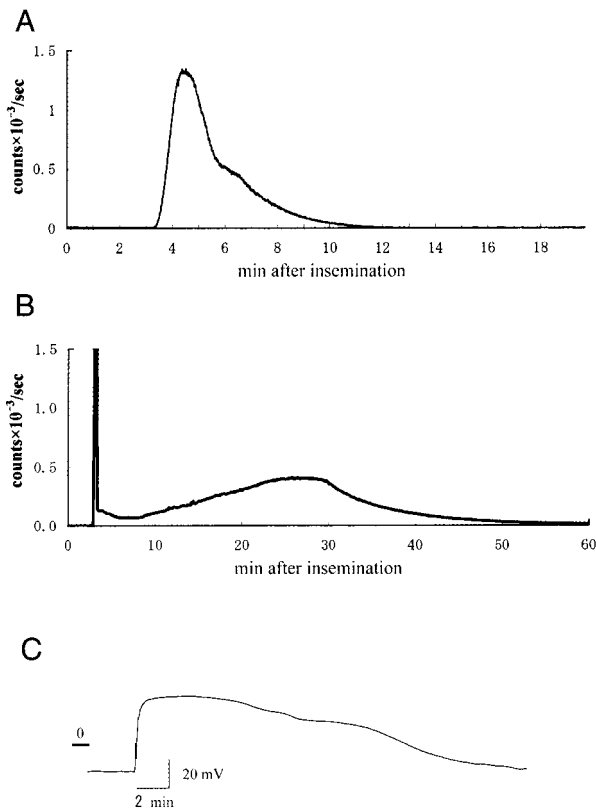
### Primary role of $\text{Ca}^{2+}$ in amphibian egg activation

A fertilizing sperm causes an increase in intracellular free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) in the eggs of both the anuran *Xenopus laevis* (Fig. 1A) (Busa and Nuccitelli, 1985; Nuccitelli *et al.*, 1993; Iwao and Fujimura, 1996; Fontanilla and Nuccitelli, 1998), and the urodeles *Pleurodeles waltl* (Gradin and Charbonneau, 1992) and *Cynops pyrrhogaster* (Fig. 1B) (Yamamoto *et al.*, 1999a), as well as in other vertebrates (Stricker, 1999). In *Xenopus* eggs, an initial  $\text{Ca}^{2+}$  increase occurs near the sperm entry site, followed by a propagative  $\text{Ca}^{2+}$  wave spreading towards the opposite side of the egg (Nuccitelli *et al.*, 1993; Fontanilla and Nuccitelli, 1998). The peak level of  $[\text{Ca}^{2+}]_i$  is estimated to be about  $1.2 \mu\text{M}$  in the cortex and  $0.7 \mu\text{M}$  in the center of the egg (Fontanilla and Nuccitelli, 1998). The  $\text{Ca}^{2+}$  increase in *Xenopus* eggs is similar to that observed in  $\text{Ca}^{2+}$  oscillation of mammalian eggs ( $0.5\text{--}2.5 \mu\text{M}$ , Miyazaki *et al.*, 1993), but lower than that in the eggs of *Oryzias latipes* eggs ( $30 \mu\text{M}$ , Gilkey *et al.*, 1978), which is a fish. The  $\text{Ca}^{2+}$  increase ( $0.15 \mu\text{M}$ ) in *Pleurodeles* is less than those in other vertebrates (Gradin and Charbonneau, 1992). Since the velocity of the  $\text{Ca}^{2+}$  wave in *Xenopus* is somewhat greater in the cortex ( $8.9 \mu\text{m/sec}$ ) than in the center of the egg ( $5.7 \mu\text{m/sec}$ )

(Fontanilla and Nuccitelli, 1998), the  $\text{Ca}^{2+}$  wave reaches the opposite side of the egg about 10 min after initiation. At *Cynops* fertilization, after an initial spike-like  $\text{Ca}^{2+}$  increase lasting about 30 sec, a  $\text{Ca}^{2+}$  wave spreads with a velocity of  $5.0\text{--}6.0 \mu\text{m/sec}$  for about 40 min (Fig. 1B) (Yamamoto *et al.*, 1999a,b). The velocity of  $\text{Ca}^{2+}$  waves in amphibians is somewhat slower than that in fishes ( $9\text{--}12 \mu\text{m/sec}$ , Gilkey *et al.*, 1978; Lee *et al.*, 1999) or mammals ( $16\text{--}28 \mu\text{m/sec}$ , Miyazaki *et al.*, 1993). Since the single  $\text{Ca}^{2+}$  increase occurs at amphibian fertilization, a relatively high  $[\text{Ca}^{2+}]_i$  continues for 10–15 min in *Xenopus* eggs (Fig. 1A) and for 30–40 min in *Cynops* eggs (Fig. 1B). *Oryzias* eggs exhibit a single  $\text{Ca}^{2+}$  wave lasting about 15 min (Gilkey *et al.*, 1978), but a repetitive  $\text{Ca}^{2+}$  increase ( $\text{Ca}^{2+}$  oscillation) occurs in mammalian eggs, each lasting 0.5–4 min for about 2 hor (Miyazaki *et al.*, 1993).

The increase in  $[\text{Ca}^{2+}]_i$  is both necessary and sufficient for egg activation in amphibians. Prevention of this increase at fertilization by injection of the  $\text{Ca}^{2+}$  chelator, BAPTA, inhibits all events in egg activation, including elicitation of the fertilization potential, cortical granule exocytosis, cortical contraction in *Xenopus* (Kline, 1988), and resumption of meiosis in both *Xenopus* (Kline, 1988) and *Cynops* (Yamamoto *et al.*, 1999a). A  $[\text{Ca}^{2+}]_i$  increase induced by the  $\text{Ca}^{2+}$  ionophore A23187 causes egg activation in both anurans (Steinhardt *et al.*, 1974; Iwao, 1982) and urodeles (Charbonneau and Picheral, 1983; Iwao and Masui, 1995). Anurans eggs can be activated by introduction of  $\text{Ca}^{2+}$  into the egg cytoplasm by injection (Cross, 1981) or by pricking with a fine needle (Goldenberg and Elinson, 1980; Iwao *et al.*, 1981). Longer treatment with a higher concentration of ionophore A23187 is necessary for activation of *Cynops* eggs in comparison with anuran eggs (Iwao and Masui, 1995). While pricking can cause egg activation in *Pleurodeles* (Aimar and Larousse, 1975) and *Hynobius nebulosus* (Iwao, 1989), the eggs of most urodeles are relatively insensitive to pricking (Fankhauser, 1967; Iwao and Masui, 1995). Injection of  $\text{Ca}^{2+}$  into *Oryzias* eggs induces egg activation with a  $\text{Ca}^{2+}$  wave (Iwamoto *et al.*, 1988a,b). The introduction of  $\text{Ca}^{2+}$  into mammalian eggs does not appear to be sufficient to induce sustained  $\text{Ca}^{2+}$  oscillation (Swann and Ozil, 1994).

One  $\text{Ca}^{2+}$  store in amphibian eggs seems to be the endoplasmic reticulum, which is abundant in the egg cortex (Gardiner and Grey, 1983; Campanella *et al.*, 1988). Inositol-1,4,5-trisphosphate (IP3)-receptors preferentially localized in the egg cortex (Kume *et al.*, 1993) are likely involved in the  $\text{Ca}^{2+}$  increase at amphibian fertilization. The amount of IP3 in *Xenopus* egg cytoplasm increases 3- to 5-fold at fertilization (Stith *et al.*, 1993, 1994; Snow *et al.*, 1996). Injection of IP3 into the eggs of both *Xenopus* (Busa *et al.*, 1985; Larabell and Nuccitelli, 1992) and *Cynops* (Yamamoto *et al.*, 1999b) causes an increase in  $\text{Ca}^{2+}$ . Injection of heparin, an inhibitor of IP3-receptors, prevents  $\text{Ca}^{2+}$  waves at fertilization in both *Xenopus* (Nuccitelli *et al.*, 1993) and *Cynops* (Yamamoto *et al.*, 1999b) eggs. Injection of an antibody against type 1 IP3-receptor into *Xenopus* eggs reduces the  $\text{Ca}^{2+}$  increase at fertilization (Runft *et al.*, 1999). The  $\text{Ca}^{2+}$  wave in *Xenopus* eggs



**Fig. 1.**  $\text{Ca}^{2+}$  increases at fertilization of *Xenopus* (A) and *Cynops* (B) eggs. The level of  $[\text{Ca}^{2+}]_i$  was monitored by aequorin injected into the eggs. A positive-going fertilization potential at fertilization of a *Xenopus* egg (C).

seems to be induced by  $\text{Ca}^{2+}$  that promotes  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) acting on IP<sub>3</sub>-receptors directly or through IP<sub>3</sub> production (Nuccitelli *et al.*, 1993). The faster  $\text{Ca}^{2+}$  wave in the cortex may be due to abundant endoplasmic reticulum with IP<sub>3</sub>-receptors in the cortex. However, local and transient  $\text{Ca}^{2+}$  increases, known as “hot spots”, have been observed at fertilization of *Xenopus* eggs injected with heparin (Nuccitelli *et al.*, 1993; Fontanilla and Nuccitelli, 1998) or anti-IP<sub>3</sub> receptor antibody (Runft *et al.*, 1999). Since each hot spot probably represents a  $\text{Ca}^{2+}$  increase at each sperm entry site, another mechanism that is not mediated by IP<sub>3</sub> receptors may operate in the initial phase of the  $\text{Ca}^{2+}$  increase in *Xenopus* eggs. IP<sub>3</sub>-injection causes a  $\text{Ca}^{2+}$  increase in *Oryzias* eggs (Nuccitelli *et al.*, 1987; Iwamatsu *et al.*, 1988a). Injection of an antibody against IP<sub>3</sub>-receptors into hamster eggs completely inhibits the  $\text{Ca}^{2+}$  increase at fertilization (Miyazaki *et al.*, 1992). However, IP<sub>3</sub> can not fully mimic a  $\text{Ca}^{2+}$  oscillation in mammalian eggs (Swann and Ozil, 1994). Although ryanodine receptors also seem to function in the  $\text{Ca}^{2+}$  oscillation in mammalian eggs (Stricker, 1999), they are unlikely to be involved in the  $\text{Ca}^{2+}$  increase in amphibian eggs, since they have not been observed in *Xenopus* eggs (Parys *et al.*, 1992) and injection of cyclic-ADP ribose does not cause a  $\text{Ca}^{2+}$  increase in *Xenopus* egg homogenate (Whitaker and Swann, 1993) or in *Cynops* eggs (Yamamoto S and Iwao Y, unpublished data).

### Signaling pathways in the $\text{Ca}^{2+}$ increase at egg activation

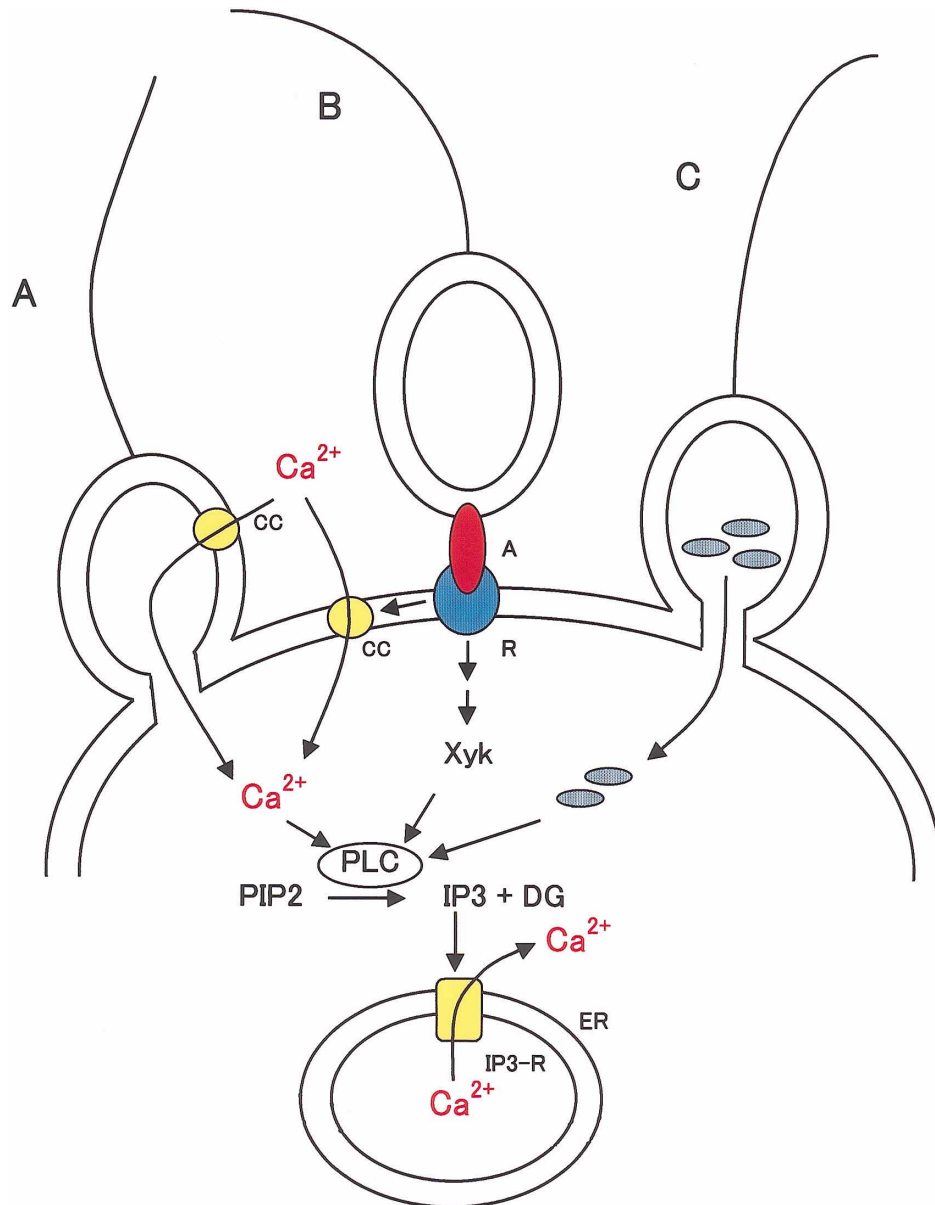
IP<sub>3</sub> is produced by hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) into IP<sub>3</sub> and diacylglycerol (DG) with the phospholipase C (PLC) enzymes which include three subgroups: PLC- $\beta$ , PLC- $\gamma$ , and PLC- $\delta$ , (Fig. 2). Both a functional G-protein/ PLC- $\beta$  pathway (Kline *et al.*, 1988) and a PLC- $\gamma$  (Yim *et al.*, 1994) are present in *Xenopus* eggs, which are responsive to exogenously expressed receptors for serotonin 1C and epidermal growth factor, respectively. However, the  $\text{Ca}^{2+}$  increase in *Xenopus* eggs is not inhibited by pertussis toxin, which inhibits a Gi family of G-proteins (Kline *et al.*, 1991), or by an antibody against a Gq family G-protein (Runft *et al.*, 1999). There is no direct evidence in favor of the involvement of a G-protein/ PLC- $\beta$  pathway in egg activation at amphibian fertilization. The  $\text{Ca}^{2+}$  increase is not inhibited by injection of the SH2-domain of PLC- $\gamma$ 1 in which tyrosine kinases bind to activate PLC- $\gamma$  (Runft *et al.*, 1999). No PLC- $\gamma$ 2 is detected in *Xenopus* eggs (Runft *et al.*, 1999), although some inhibitors of tyrosine kinases block a  $\text{Ca}^{2+}$  increase and egg activation in *Xenopus* eggs (Glahn *et al.*, 1998; Sato *et al.*, 1998; 1999). It has been shown that a Src-related protein tyrosine kinase (Xyk) localized in the egg cortex is activated and translocated to egg cytoplasm (a soluble fraction) at fertilization in *Xenopus* (Sato *et al.*, 1996; 1999). Both activation and translocation are induced by a fertilizing sperm, but not by an artificial  $\text{Ca}^{2+}$  increase induce by an ionophore or electric shock (Sato *et al.*, 1999), while injection of a peptide that inhibits Xyk does block egg activation in *Xenopus* (Sato *et al.*, 1999). These results suggest that Xyk plays a role in the cascade between sperm-egg binding/fusion and the  $\text{Ca}^{2+}$  increase. Xyk seems

to stimulate PLC- $\gamma$  through a SH2-domain-independent mechanism, such as a partial proteolysis or noncatalytic interaction with other molecules, since Xyk is associated with PLC- $\gamma$  and activation of PLC- $\gamma$  is inhibited by a specific inhibitor of Src-related kinases (Sato K, *et al.*, 2000). However, since  $\text{Ca}^{2+}$  hot spots are seen at fertilization of eggs in which the  $\text{Ca}^{2+}$  waves were inhibited by a tyrosine kinase inhibitor (Glahn *et al.*, 1998), a pathway that is different from the Xyk cascade may be involved in the initial and local  $\text{Ca}^{2+}$  increase at the sperm entry site. The role of PLC- $\delta$  in the  $\text{Ca}^{2+}$  increase remains to be investigated in amphibian fertilization. Among mammals, injection of GDP- $\beta$ -S inhibits G-proteins and blocks the  $\text{Ca}^{2+}$  increase in hamster eggs (Miyazaki *et al.*, 1993), while injection of an antibody against the Gq family G-protein does not inhibit activation of mouse eggs (Williams *et al.*, 1998). Furthermore, a  $\text{Ca}^{2+}$  increase in mouse eggs is not blocked by injection of the SH2-domain of PLC- $\gamma$  (Mehlmann *et al.*, 1998).

### Mechanisms of sperm-induced initiation of $\text{Ca}^{2+}$ increase

While it is unknown just how a fertilizing sperm transmits the initial signal for the  $\text{Ca}^{2+}$  increase at fertilization, there are at least three different hypotheses for initiation of egg activation in animal eggs: the  $\text{Ca}^{2+}$ -influx model, the membrane receptor model, and the soluble factor model (Fig. 2). In any case, a fertilizing sperm must stimulate IP<sub>3</sub> production in the egg cytoplasm to potentiate the  $\text{Ca}^{2+}$  wave.

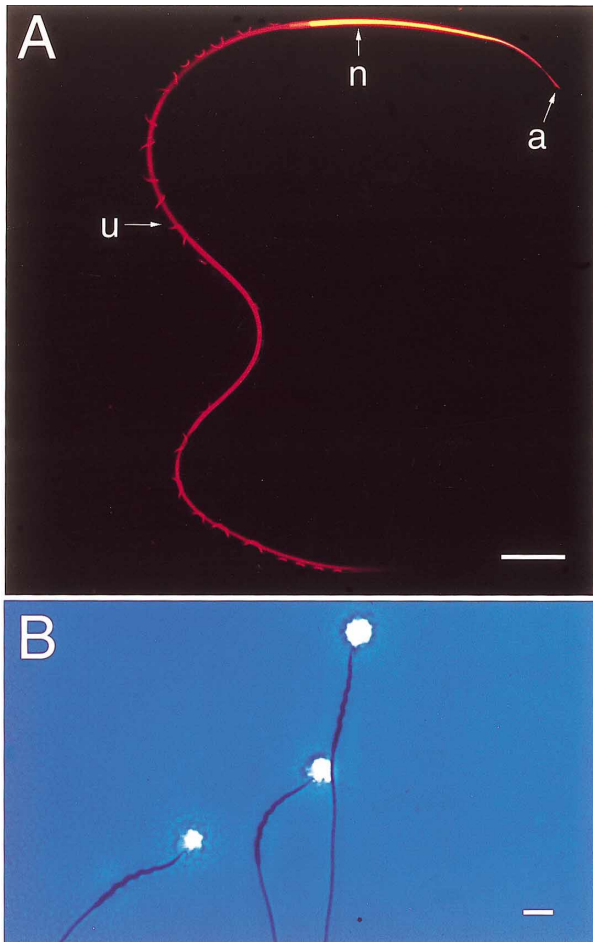
(A) In the  $\text{Ca}^{2+}$  influx model (Fig. 2A), a fertilizing sperm induces an influx of external  $\text{Ca}^{2+}$  required for egg activation either through  $\text{Ca}^{2+}$  channels on the sperm membrane following sperm-egg fusion or on the egg membrane at the sperm-egg binding/fusion. Amphibian eggs can be artificially activated by a  $\text{Ca}^{2+}$  release from internal stores in the absence of external  $\text{Ca}^{2+}$  (Steinhardt *et al.*, 1974; Yamamoto *et al.*, 1999b). The progression of the  $\text{Ca}^{2+}$  wave is not affected by depletion of external  $\text{Ca}^{2+}$  (Fontanilla and Nuccitelli, 1998; Yamamoto, 1999b). No cortical flush of the  $\text{Ca}^{2+}$  increase is seen at the initial phase of the  $\text{Ca}^{2+}$  increase. The  $\text{Ca}^{2+}$  increase from internal stores seems to be sufficient for egg activation of amphibian eggs. Since CICR is induced by  $\text{Ca}^{2+}$  injection (Cross, 1981) and PLCs can be stimulated by 1–10  $\mu$  M  $\text{Ca}^{2+}$  (Hwang *et al.*, 1996), it remains to be determined whether the initial  $\text{Ca}^{2+}$  increase around the sperm entry site is dependent upon external  $\text{Ca}^{2+}$ . In order to determine a role of the  $\text{Ca}^{2+}$  influx at fertilization of amphibians, it should be determine whether amphibian eggs can be fertilized and are normally activated in the absence of external  $\text{Ca}^{2+}$  ions. *Oryzias* eggs can be fertilized, and the  $\text{Ca}^{2+}$  wave is not affected in the absence of external  $\text{Ca}^{2+}$  (Gilkey *et al.*, 1978). In zebrafish eggs, the external spawning medium triggers an activating  $\text{Ca}^{2+}$  wave without sperm-egg fusion, but neither sperm nor external  $\text{Ca}^{2+}$  is required to initiate the  $\text{Ca}^{2+}$  wave (Lee *et al.*, 1999). The exact mechanism of egg activation in fishes remains to be investigated. In mouse eggs, a fertilizing sperm can induce the  $\text{Ca}^{2+}$  increase even where there is a very low concentration of external  $\text{Ca}^{2+}$  (13 nM) (Jones *et al.*, 1998b).



**Fig. 2.** Models for the mechanism of  $\text{Ca}^{2+}$  increase at fertilization.  $\text{Ca}^{2+}$  influx model (A), membrane-receptor model (B), and sperm factor model (C). A, sperm agonist; CC,  $\text{Ca}^{2+}$  channel; IP3-R, ER, endoplasmic reticulum; IP3-receptor; R, receptor; Xyk, Src-related tyrosine kinase. Blue oval symbols indicating the sperm factor. See text for detail.

(B) The membrane receptor model proposes that an agonist (ligand) on the sperm membrane binds to a receptor on the egg membrane to cause IP3 production in the egg cytoplasm (Fig. 2B). We have suggested that a positively charged molecule(s) on the sperm membrane is involved in sperm-egg binding and fusion, based on the analysis of cross-fertilization between voltage-sensitive and voltage-insensitive species (see below). In *Xenopus* eggs, a  $\text{Ca}^{2+}$  increase is induced by external treatment with peptides containing an RGD sequence (Iwao and Fujimura, 1996), which is well known as a ligand for integrins. RGD-containing peptides can induce a  $\text{Ca}^{2+}$  increase in the absence of external  $\text{Ca}^{2+}$ . The treatment with RGD-containing peptides causes activation in *Hynobius* eggs (Fujimura and Iwao, 1997), but does not in *Cynops* eggs

(Iwao Y, unpublished data). *Xenopus* sperm contain a protein of the metalloprotease/disintegrin/cysteine-rich (MDC) family (xMDC16) (Shilling *et al.*, 1997). Peptides containing a sequence (KTE) of its disintegrin domain inhibit fertilization (Shilling *et al.*, 1997). Treatment with a high concentration of these peptides causes a  $\text{Ca}^{2+}$  increase and activation in *Xenopus* eggs (Shilling *et al.*, 1998). These results indicate that the sperm protein binds to a receptor, probably an integrin(s), on the egg membrane and transmits a signal for the  $\text{Ca}^{2+}$  increase at fertilization. However, no receptor has been found for either the RGD-containing peptides or the xMDC16. Another potential candidate for the sperm agonist is a sperm acrosomal protease purified from *Cynops* sperm (Fig. 3) (Iwao *et al.*, 1994; Mizote *et al.*, 1999). *Xenopus* eggs can be fertilized by



**Fig. 3.** (A) A confocal fluorescence image of the newt *Cynops pyrhogaster* sperm stained by acridine orange and neutral red, showing a nucleus (n) with an arrow head-like acrosome (a) in the head region and a tail with an undulating membrane (u). (B) A protease activity in *Cynops* sperm, showing a halo in each acrosome by digestion of a gelatin film. Bars, 20  $\mu$  m.

*Cynops* sperm and are completely activated by the external application of the protease purified from *Cynops* sperm. The sperm protease causing a  $Ca^{2+}$  wave in *Xenopus* eggs (Iwao *et al.*, 1995) is a high-molecular weight complex with a unique tryptic protease that efficiently hydrolyzes the C-terminus of double arginine protein residues (Mizote *et al.*, 1999). Homologous *Xenopus* fertilization is inhibited by inhibitors for the sperm protease (Mizote *et al.*, 1999) and a similar protease is localized on the *Xenopus* sperm membrane (Mizote *et al.*, 1999; Iwao Y, unpublished data). The sperm protease might cleave a receptor on the egg membrane, as does a protease-activating receptor for the thrombin system (Vu *et al.*, 1991). Thus, there are strong evidences in support of the membrane receptor model for the  $Ca^{2+}$  increase in *Xenopus* eggs, although the target of the sperm protease on the egg membrane is still unknown. In mammals, sperm-egg binding is mediated by the interaction between the fertilin  $\alpha/\beta$  MDC proteins on the sperm membrane and the integrin  $\alpha_6/\beta_1$  on the egg membrane (Almeida *et al.*, 1995). Since mouse sperm that lack the fertilin

subunit can fertilize the eggs and cause activation (Cho *et al.*, 1998), fertilin is probably not involved in egg activation in mammals. Since CD9, which is a member of the transmembrane-4 superfamily, is found on the egg membrane and is required for membrane fusion (Miyado *et al.*, 2000; Le Naour *et al.*, 2000), its role in  $Ca^{2+}$  signaling in mammalian eggs should be investigated further.

(C) The soluble factor model proposes that a soluble component(s) in sperm cytoplasm is transmitted to egg cytoplasm after sperm-egg fusion, which then causes the  $Ca^{2+}$  increase. This model, based on the latent period between sperm-egg membrane fusion and the onset of the  $Ca^{2+}$  increase in egg cytoplasm (Whitaker and Swann, 1993), may apply to *Cynops* egg activation. Injection of sperm soluble components into *Cynops* eggs causes a  $Ca^{2+}$  increase and complete egg activation (Yamamoto *et al.*, 1999b). This appears to be consistent with the finding that *Cynops* eggs are resistant to a transient  $Ca^{2+}$  increase by pricking and to the treatment with RGD-containing peptides, as discussed above. In support of this, only a small percentage of *Cynops* eggs are activated by external treatment with the sperm protease (Iwao *et al.*, 1994). The sperm factor in *Cynops* is known to be a heat-labile and proteinous molecule(s). However, further investigation is necessary to determine whether *Cynops* sperm contains a sufficient amount of sperm factor to activate an egg. Recent studies on mammals strongly support the soluble factor model for  $Ca^{2+}$  oscillation (Swann and Parrington, 1999). Injection of a soluble sperm extract into mammalian eggs is known to trigger a  $Ca^{2+}$  oscillation (Parrington *et al.*, 1996; Swann and Parrington, 1999). While a 33-kDa protein (oscillin) has been proposed as the sperm factor responsible for the  $Ca^{2+}$  oscillation (Parrington *et al.*, 1996), other recent candidates include a PLC (Dupont *et al.*, 1996; Jones *et al.*, 1998a), a truncated c-kit (Sette *et al.*, 1997), or various perinuclear substances (Perry *et al.*, 2000).

### Fast polyspermy block at the egg membrane in monospermic amphibians

The unfertilized amphibian eggs are surrounded with several jelly layers and a vitelline envelope which play important roles in polyspermy block (Iwao, 2000). In monospermic amphibian species, the eggs from which external coats have been removed and surrounded with only egg membrane exhibit monospermy, indicating a polyspermy block at the level of the sperm-egg binding or fusion (Elinson, 1973; Katagiri, 1974). The propagative  $Ca^{2+}$  wave at egg activation induces a propagative opening of  $Cl^-$  channels (halide ion channels) on the egg membrane (Kline and Nuccitelli, 1985), which causes a positive shift in the potential of the egg membrane (fertilization potential) in conditions of low external  $Cl^-$  such as fresh water (Fig.1C). While the level of fertilization potential is species specific, the eggs of most species have positive potentials of about +10~+40 mV (Iwao, 2000). The positive fertilization potential prevents entry of a second sperm for 10–15 min after fertilization. When the membrane potential of unfertilized eggs remains higher than 0 mV under voltage-clamp

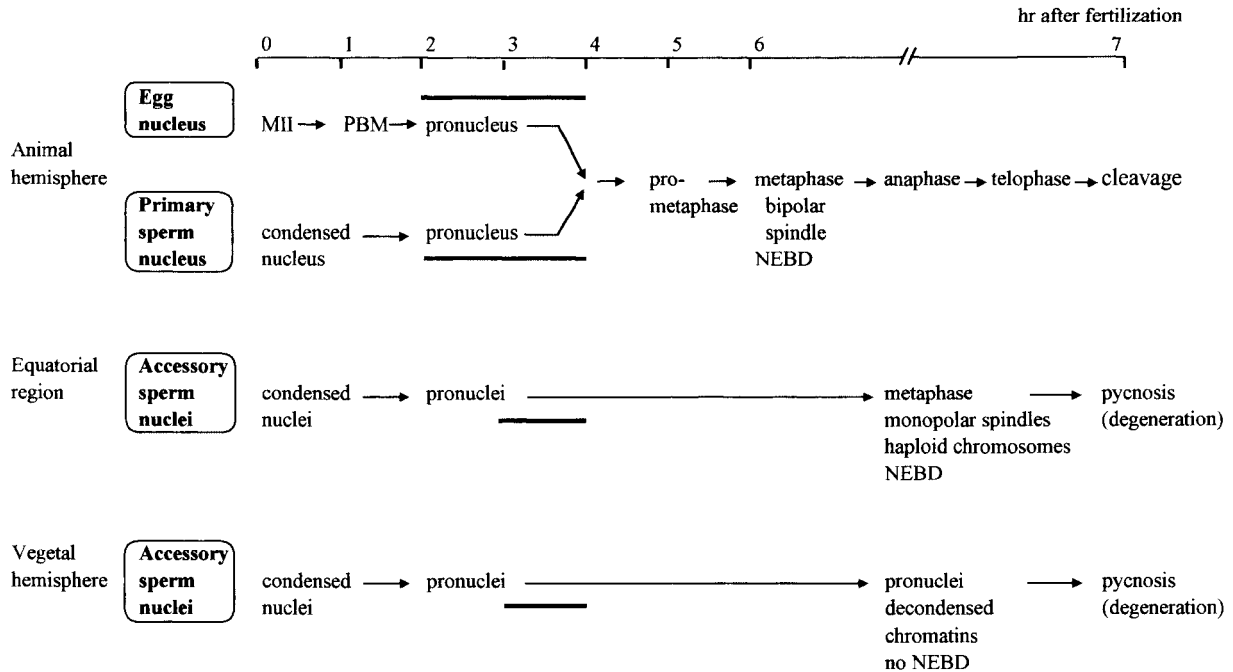
conditions, both sperm entry and egg activation are blocked (Cross and Elinson, 1980; Charbonneau *et al.*, 1983; Jaffe *et al.*, 1983a; Iwao 1989; Iwao *et al.*, 1994). In contrast, polyspermy occurs when the egg membrane potential remains below 0 mV under voltage-clamp (Cross and Elinson, 1980; Charbonneau *et al.*, 1983; Jaffe *et al.*, 1983a; Iwao and Jaffe, 1989; Iwao *et al.*, 1994) or in the presence of concentrated external halide ions (Grey *et al.*, 1982). The voltages that inhibit fertilization correspond well to those of fertilization potentials induced by sperm (Iwao, 2000). Thus, a positive fertilization potential functions as a fast, electrical block to polyspermy in voltage-sensitive species. In physiologically polyspermic urodeles, fertilization is not blocked by any positive potentials (Charbonneau *et al.*, 1983; Iwao and Jaffe, 1989). Cross-fertilization between the eggs of voltage-sensitive species and the sperm of voltage-insensitive species is not affected by positive potentials, resulting in polyspermy (Jaffe *et al.*, 1983a; Iwao and Jaffe, 1989), while a cross between the eggs of voltage-insensitive species and the sperm of voltage-sensitive species is sensitive to the voltage of the egg membrane (Iwao and Jaffe, 1989). These results indicate that the voltage-sensor for fertilization is localized on the membrane of the sperm, and not on the egg membrane. Potential candidates for the voltage sensor are the sperm protease and the xMDC16 protein, because egg activation by their molecules is voltage-dependent (Iwao *et al.*, 1994; Shilling *et al.*, 1998). While the fast block is transient, the eggs accomplish a complete polyspermy block by the formation of a fertilization envelope and by hydration of the jelly layers (Iwao, 2000).

*Oryzias* (bony fish, Osteichthyes) eggs do not elicit a positive fertilization potential and their fertilization is voltage-insensitive (Nuccitelli, 1980), indicating lack of a fast electrical block. Limitation of sperm entry through a narrow micropyle on the egg envelope (chorion) is necessary to ensure monospermy in bony fishes (Kobayashi and Yamamoto, 1981). In contrast, monospermic fertilization of the lamprey (jawless fishes, Agantha) in fresh water is ensured by a fast electrical block (Kobayashi and Yamamoto, 1994; Kobayashi *et al.*, 1994). Lamprey eggs elicit a large positive fertilization potential mediated by the opening of Cl<sup>-</sup> channels which mainly localize in the animal pole region (Kobayashi *et al.*, 1994). The positive potential blocks sperm-oocyte fusion, but not egg activation. These results suggest that the membrane receptor model can be applied to lamprey fertilization and that molecules with different voltage-sensitivities are involved in sperm-egg membrane fusion and the signaling pathway for egg activation. Fertilized mammalian eggs elicit repetitive hyperpolarizations (negative-going potentials) mediated by the opening of K<sup>+</sup> channels in response to the Ca<sup>2+</sup> oscillation (Miyazaki and Igusa, 1981; 1982). A fast electrical block does not operate in mammalian eggs (Jaffe *et al.*, 1983b), where monospermy is generally accomplished by a zona reaction mediated by cortical granule exocytosis (Wassermann, 1999).

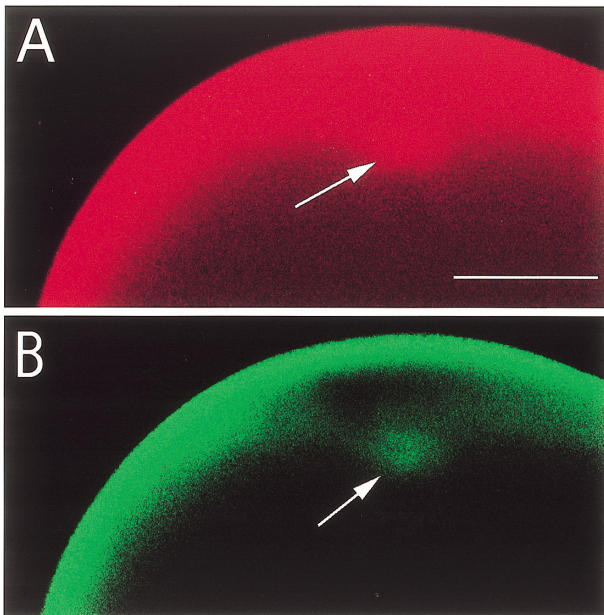
### Behavior of sperm nuclei and centrosomes in physiologically polyspermic urodele eggs

Physiological polyspermy is seen in some invertebrates and in several species of vertebrates, including fishes, urodele amphibians, reptiles, and birds (Austin, 1965). The mechanism of a polyspermy block in physiologically polyspermic eggs is well understood in urodeles (Fankhauser, 1948; Elinson, 1986; Iwao, 2000). Several sperm (2–20 sperm/egg) enter the egg at normal fertilization of *Cynops* (Iwao *et al.*, 1985; 1993). While most sperm enter animal hemispheres or equatorial regions, some enter at the vegetal hemispheres (Iwao *et al.*, 1993). The number of fertilizing sperm is limited by the hydration of the jelly layers (McLaughlin and Humphries, 1978; Matsuda and Onitake, 1984). All incorporated sperm undergo nuclear decondensation and form sperm pronuclei with functional centrosomes (Fig. 4). Each sperm pronucleus is associated with each sperm aster, but the asters in animal hemispheres are larger than those in vegetal hemispheres (Iwao *et al.*, 1997). The size of the asters is dependent upon the state of the egg cytoplasm (Iwao *et al.*, 1997), and probably upon the amount of  $\gamma$ -tubulin in the centrosomes which is responsible for microtubule polymerization (Iwao Y, unpublished data). A single sperm pronucleus, the “principal sperm nucleus”, forms a zygote nucleus with an egg pronucleus in the animal hemisphere. Although the exact mechanism for selection of the principal sperm nucleus remains unclear, the sperm nucleus nearest the egg nucleus appears capable of contacting the egg nucleus. All sperm and egg nuclei enter the S phase, but both the onset and the completion of DNA synthesis are earlier in the zygote nucleus (the egg and the principal sperm nuclei) than they are in other accessory sperm nuclei (Iwao *et al.*, 1993). When the zygote nucleus enters the pro-metaphase, its single centrosome divides and forms a bipolar spindle with a diploid set of condensed chromosomes (Fig. 4). When the zygote nucleus enters the anaphase, centrosomes in the accessory sperm nuclei do not separate, and the nuclear membranes of the sperm nuclei in the vegetal hemispheres remain distinct and enclose the decondensed chromatins. Accessory sperm nuclei in the equatorial region sometimes form mono-polar spindles with a haploid set of chromosomes. After the first cleavage, all accessory sperm nuclei undergo degeneration. Their chromatins undergo pycnosis and the materials of their centrosomes are dispersed in the egg cytoplasm. Thus, only one sperm nucleus with a centrosome (a centriole) participates in the embryonic development of urodele eggs (Fig. 4).

The degeneration of accessory sperm nuclei is closely related to their failure to enter the M-phase (Iwao, 2000). M-phase promoting factor (MPF), consisting of cdc2 kinase (cdk1) and cyclin B (Lohka *et al.*, 1988; Gautier *et al.*, 1990), is a key component involved in entering the M-phase in many animal cells (Masui, 1992). Injection of an MPF-rich cytoplasmic fraction prevents some accessory sperm nuclei from degenerating in animal hemispheres or equatorial regions (Iwao and Elinson, 1990). The rescued accessory sperm nuclei form extra bipolar spindles with haploid sets of chromosomes, and then



**Fig. 4.** Fate of sperm nuclei in a physiologically polyspermic *Cynops* egg. MII, the second meiotic metaphase; NEBD, nuclear membrane breakdown; PBM, the second polar body emission; Bars, S phase.



**Fig. 5.** Localization of cyclin B (A) and  $\alpha$ -tubulin (B) in a *Cynops* egg 4 hr after fertilization, showing cyclin B in the cortex and around the zygote nucleus (arrows) in association with microtubules. Bar, 0.2 mm.

induce multipolar cleavage. In addition to the increased MPF activity, there are greater amounts of both cdc2 kinase and cyclin B in animal hemispheres compared with vegetal hemispheres (Sakamoto *et al.*, 1998). Cyclin B is mainly distributed in the egg cortex (Sakamoto *et al.*, 1998), and a large amount of cyclin B is associated with the zygote nucleus (Fig.

5A). Microtubule structures, such as sperm asters and cortical microtubules (Iwao *et al.*, 1997), probably regulate the localization of cyclins in egg cytoplasm (Fig. 5B). Since DNA synthesis and centrosome separation in *Xenopus* eggs is dependent upon cdc2/cdk2 kinases (Chevalier *et al.*, 1995) and cdk2/cyclins (Hinchcliffe *et al.*, 1999), respectively, earlier entry of the zygote nucleus into both the S-phase and the M-phase, as well as centrosome separation, is probably due to the abundant cdc2 or cdk2 around the zygote nucleus. Furthermore, earlier entry into the anaphase in the zygote nucleus is likely stimulated by anaphase-promoting factor (APF) (Peters *et al.*, 1996), which inactivates MPF by destruction of cyclin B through ubiquitin-dependent proteolysis (Aizawa *et al.*, 1996; Tokumoto *et al.*, 1997). Earlier disappearance of cyclin B around the zygote nucleus is seen around the anaphase of the first cleavage in *Cynops* eggs (Iwao Y, unpublished data). These results indicate that the accessory sperm nuclei are exposed to APF without sufficient exposure of MPF, which causes degeneration of accessory sperm nuclei. However, the molecular mechanism of degradation of accessory sperm chromatins and centrosomes remains unknown.

There are few reports of the nuclear behavior in other physiologically polyspermic animals that are comparable with urodele amphibians. In the domestic fowl, some accessory sperm form bipolar spindles at the M-phase for the first cleavage, but they never cause the extra cleavage furrow (Perry, 1987). The accessory sperm nuclei that disperse slightly towards the margin of a germinal disc degenerate after one round of mitosis (Perry, 1987; Waddington *et al.*, 1998). There may be some difference in the ability to induce cleavage in the egg cortex. In the polyspermic invertebrate ctenophore *Bore ovata*,



one sperm nuclei is selected to form the zygote nucleus after an egg nucleus approaches different sperm nuclei along the microtubules in egg cytoplasm (Carré and Sardet, 1984; Rouvière *et al.*, 1994). Although each sperm pronucleus is associated with a sperm aster, they do not migrate (Houliston *et al.*, 1993). The egg nucleus which enters into the center of the sperm aster forms the zygote nucleus (Carré *et al.*, 1991). The mechanisms for suppression of accessory sperm in these species remain to be investigated.

### Concluding remarks

Two different models for egg activation may apply in amphibian fertilization: the membrane receptor model for the anuran *Xenopus* and the sperm factor model for the urodele *Cynops*. Although the molecular mechanisms of egg activation are not fully understood in amphibians, the observed voltage-sensitive and voltage-insensitive fertilization seems to correspond well to the membrane receptor model and the sperm factor model, respectively. Since it is estimated that the second sperm probably reaches the egg membrane within several seconds of the arrival of the first sperm (Iwao, 2000), the signal transmission through the membrane receptor seems to be suitable for faster egg activation (faster generation of a positive fertilization potential) to prevent polyspermy in monospermic species. From the phylogenetic perspective, voltage-insensitive fertilization was probably acquired concomitant with the emergence of physiological polyspermy in urodeles. Recent molecular studies indicate that the anuran group may have branched relatively early from the urodele/caecilian (limbless amphibians) group, perhaps during the beginning of the Mesozoic period (240 million years ago), while the urodele and caecilians groups probably branched relatively late, in the late Mesozoic period (160–190 million years ago) (Feller and Hedges, 1998). In this connection, monospermy with the fast electrical block, but without the cortical granule-mediated block, in urodeles belonging to the genus *Hynobius* (Iwao, 1989; 2000), apparently shows an intermediate mode between monospermic anurans, with both fast and cortical granule-mediated blocks, and physiologically polyspermic urodeles, which lack both blocks.

In this context, the mode of fertilization in the ancestor of amphibians may provide an important view of the relationship between the type of polyspermy block and the mode of egg activation. Amphibians are believed to share a common ancestor with the bony fishes of subclass Sarcopterygii (lobe-finned fishes), which contains Crossopterygii (coelacanth, *Latimeria*) and Dipnoi (lung fishes) (Meyer and Dolven, 1992). The molecular analysis in extant animals suggests that lung-fishes comprise the closest sister group of tetrapods (amphibians) (Zardoya and Meyer, 1997). *Latimeria* is ovoviviparous (Smith *et al.*, 1975), but the mode of fertilization in sarcopterygian fishes remains unknown. As is the case in monospermic amphibians, lampreys exhibit voltage-sensitive fertilization and their eggs generate a positive fertilization potential mediated by  $\text{Cl}^-$  channels. A  $\text{Cl}^-$ -dependent fertilization potential may be necessary for monospermic fertilization in

vertebrates that live in fresh water. Since fish sperm contain cleavage-initiation activities (Iwamatsu and Ohta, 1974), molecular mechanisms of egg activation in voltage-insensitive monospermic bony fishes and polyspermic cartilaginous fishes merit further investigation. Mammalian eggs exhibit voltage-insensitive fertilization and appear to be activated by the sperm factor. However, the mechanisms of activation of polyspermic yolky eggs of birds and reptiles remain to be investigated.

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