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Original Article

The redistribution of Ca^{2+} stores with inositol 1,4,5-trisphosphate receptor to the cleavage furrow in a microtubule-dependent manner

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ABSTRACT We reported that microinjection of Ca^{2+} store-enriched microsome fractions from cultured CHO cells and mouse cerebella to dividing newt eggs induced extra-cleavage furrows via inositol 1,4,5-trisphosphate-induced Ca^{2+} release (Mitsuyama *et al.,* 1999). Our observation strongly suggested that Ca^{2+} stores with inositol 1,4,5-trisphosphate receptor (IP_3R) induce and position a cleavage furrow, as Ca^{2+}-releasing machinery, and that such is itself a putative cleavage stimulus. For confirmation, we immunocytochemically examined mitotic CHO cells using antibodies against Ca^{2+} store-related proteins. We found that polar dominant Ca^{2+} stores with IP_3R during metaphase were re-distributed to the future cleavage cortex just preceding the onset of furrowing, and that this redistributing IP_3R was present on microtubule bundles. When a microsome fraction from sacro/endoplasmic reticulum Ca^{2+}-ATPase (SERCA)-GFP stably expressing CHO cells was microinjected into dividing newt eggs and observed by confocal microscopy, the microinjected Ca^{2+} stores with IP_3R moved linearly toward the next cleavage furrow and this movement was blocked by nocodazole, a microtubule-depolarizing agent, but not by cytochalasin B, an F-actin-depolarizing agent. These observations strongly suggest that Ca^{2+} stores with IP_3R are transferred and accumulate to the cleavage furrow by microtubule-based motility, as a cleavage stimulus.

KEY WORDS: *cytokinesis, cleavage furrow, Ca^{2+} transient, Ca^{2+} store, IP_3 receptor*

Introduction

During cytokinesis the spatio-temporal pattern of Ca^{2+} transients was evident (Fluck *et al.,* 1991; Chang and Meng, 1995; Muto *et al.,* 1996; Webb *et al.,* 1997; Creton *et al.,* 1998) and localized Ca^{2+} transients were particularly apparent at the cleavage furrow. Ca^{2+} transients were seen to occur at the site where the cleavage furrow would form in zebra fish embryos (Chang and Meng, 1995; Webb *et al.,* 1997; Creton *et al.,* 1998). However elimination of external Ca^{2+} as well as the addition of the Ca^{2+} channel blocker, La^{3+}, nifedipine, does not block Ca^{2+} transients at the cleavage furrow (Fluck *et al.,* 1991; Chang and Meng, 1995; Muto *et al.,* 1996; Webb *et al.,* 1997), hence Ca^{2+} influx may not have an important role.

There is a report that the second messenger signal, IP_3 was elevated together with Ca^{2+} transients during cytokinesis (Ciapa *et al.,* 1994). An antagonist of the IP_3R, such as heparin blocked Ca^{2+} transients during cytokinesis (Ciapa *et al.,* 1994; Chang and Meng, 1995; Muto *et al.,* 1996). The blocker of phosphoinositide turnover (anti-phosphatidyl inositol 4,5-bisphosphate antibody and lithium) inhibited the cell cycle (Han *et al.,* 1992; Becchetti and Whitaker, 1997). These reports suggest that the IP_3 induced Ca^{2+} release (IICR) (Berridge, 1993) from internal Ca^{2+} stores plays an important role in cytokinesis.

The signal to determine cleavage position was termed "cleavage stimulus" by Rappaport (Rappaport, 1961). Our observation (Mitsuyama *et al.,* 1999) strongly suggested that Ca^{2+} stores with IP_3R induce and position a cleavage furrow, as Ca^{2+}-releasing machinery, and is in itself a putative cleavage stimulus. To determine if these Ca^{2+} stores with IP_3R in cultured CHO cells redistribute to the cleavage furrow, we immunocytochemically studied mitotic cultured CHO cells, using antibodies against Ca^{2+} store-related proteins, and a microsome fraction from SERCA-

Abbreviations used in this paper: ER, endoplasmic reticulum; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; IICR, IP_3 induced Ca^{2+} release; mAb, monoclonal antibody; pAb, polyclonal antibody; SERCA-GFP, sacro / endoplasmic reticulum Ca^{2+}-ATPase-green fluorescent protein.

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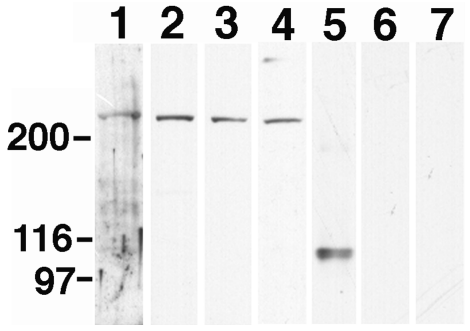

Fig. 1. Identification of IP_3R, SERCA and ryanodine receptor by immunoblot analysis of CHO cells. *Lanes correspond to immunoreaction of nitrocellulose blots probed with* **(1)** *rabbit polyclonal anti-IP_3R antibody;* **(2)** *mouse mAb to type 1-IP_3R(KM1112);* **(3)** *mouse mAb to type 2-IP_3R (KM1083);* **(4)** *mouse mAb to type 3-IP_3R (KM1082);* **(5)** *mouse mAb to SERCA 2 (II D8);* **(6)** *rabbit polyclonal antibody to the ryanodine receptor (C2) and* **(7)** *mouse mAb to ryanodine receptor. The positions of the M_r standards are given on the left.*

GFP stably expressed CHO cells was microinjected into 2-cell stage newt eggs, then observed under a confocal microscope. The role of Ca^{2+} stores with the IP_3R as a possible source of local Ca^{2+} transients and a possible "cleavage stimulus" signal in cytokinesis is discussed.

Results

CHO cells express all three types of IP_3R but not the ryanodine receptor

Western blot analysis was done, using anti-IP_3R pAb, mAb KM1112, mAb KM1083, mAb KM1082, ll D8, C2, and monoclonal anti-ryanodine receptor antibody (Fig. 1). All three types of IP_3R were expressed, but the ryanodine receptor was hardly detectable. Polyclonal anti-IP_3R antibody reacted with a single band of M_r 250kD (Fig. 1, lane 1). Mouse mAb to SERCA reacted with a single band of M_r 115kD (Fig. 1, lane 5).

Polar dominant Ca^{2+} stores with IP_3R during metaphase were re-distributed to the future cleavage cortex just preceding the onset of furrowing together with F-actin

To determine if these Ca^{2+} stores with IP_3R in cultured CHO cells re-distribute to the cleavage furrow, we examined subcellular localization of the IP_3R, SERCA, ER, F-actin, and DNA in whole CHO cells from metaphase (Fig. 2 A-D) until just before the onset of furrowing (Fig. 2 U-X). F-actin was predominantly present at the polar cortex but rarely at the equatorial cell cortex during metaphase (Fig. 2C). Before the onset of furrowing, F-actin gradually accumulated as an actin ring at the equatorial cell cortex (inner surface of presumptive furrow) (Fig. 2S), and much accumulation was evident at the presumptive cleavage furrow just before the onset of furrowing (Fig. 2W). This drastic change of F-actin distribution closely mirrored that of the IP_3R, SERCA, and ER: During metaphase, ER was present at the pole to polar cortex (Fig. 2B), and the distribution was similar to that of the IP_3R (Fig. 2A). Before the onset of furrowing, ER together with the IP_3R and SERCA re-distributed mainly at the equatorial cell cortex (Fig. 2 Q,R) together with F-actin (Fig. 2S), and much accumulation was evident at the presumptive cleavage furrow just before the onset of furrowing (Fig. 2 U,V) where reactivity for F-actin was predominant as actin rings (Fig. 2W). In early~mid-anaphase cells, immunoreactivities for IP_3R, SERCA, ER, and F-actin began to be re-distributed and did not accumulate at the equatorial cell cortex (Fig. 2 E-P). Negative controls stained with normal IgG instead of each primary antibody showed practically no immunoreactivity (data not shown). These observations indicate that Ca^{2+} stores with IP_3R are redistributed and accumulated to the future cleavage cortex just preceding the onset of furrowing together with F-actin.

IP_3R was present on microtubule bundles

To identify the involvement of microtubule-based motility as a motor source of this re-distribution of Ca^{2+} stores, double immunolabelling with anti-IP_3R pAb and anti-tubulin mAb in CHO cells was done. To confirm the co-localization of IP_3R with microtubules, double immunolabelling on a direct ultra-thin frozen section (0.2 μm in width) was done. IP_3R was present on the microtubule bundles in a late anaphase cell, in which it appeared that to a great extent IP_3R are dragged away from the site of a future cleavage (Fig. 3 A-D). In a telophase cell, IP_3R accumulated at the cleavage furrow and was present on microtubule bundles as seen under a confocal microscope (Fig. 3 E-H).

Ca^{2+} stores with IP_3R microinjected into the dividing newt egg moved linearly and accumulated to the next cleavage furrow

To confirm that the Ca^{2+} stores with IP_3R from CHO cells can move from the polar area to the equatorial cortex, a microsome fraction from SERCA-GFP stably expressing CHO cells was microinjected just beneath the cortex of dividing newt eggs when the normal first cleavage furrow was completed (at the 2-cell stage) and the signals from SERCA-GFP-labeled Ca^{2+} stores with IP_3R were observed under a confocal microscope. When the first cleavage furrow was completed, the capillary was inserted from one side (IP: inserted point in Fig. 4C) and was pushed through the egg and brought close to the egg surface on the opposite side to avoid insertion artifact, and then the microsome fraction was injected (IA: injected area in Fig. 4C). We tried to inject the microsome fraction only into the injected area, but the small amount of microinjection at the inserted point could not be avoided (IP in Fig. 4A). The weak fluorescent signals from the microinjected microsome fraction were seen around the injected area (IA in Fig. 4A), because the focus was adjusted to the inserted point. The signals from SERCA-GFP-labeled Ca^{2+} stores with IP_3R moved linearly with time toward the next (the 2nd) cleavage furrow, and accumulated at the 2nd cleavage furrow (Table 1 and Fig. 4B,

TABLE 1

MOVEMENT OF MICIROINJECTED SERCA-GFP-LABELED MICIROSOME FRACTIONS

Inhibitor	No. of eggs	Moved	Not moved	Normal 2nd furrow	
				Suppressed	Not suppressed
No drug	13	10	3	3	10
Nocodazole	19	0	19	19	0
Cytochalasin B	13	10	3	13	0

Movement of the GFP signal was detected under a confocal miciroscope.

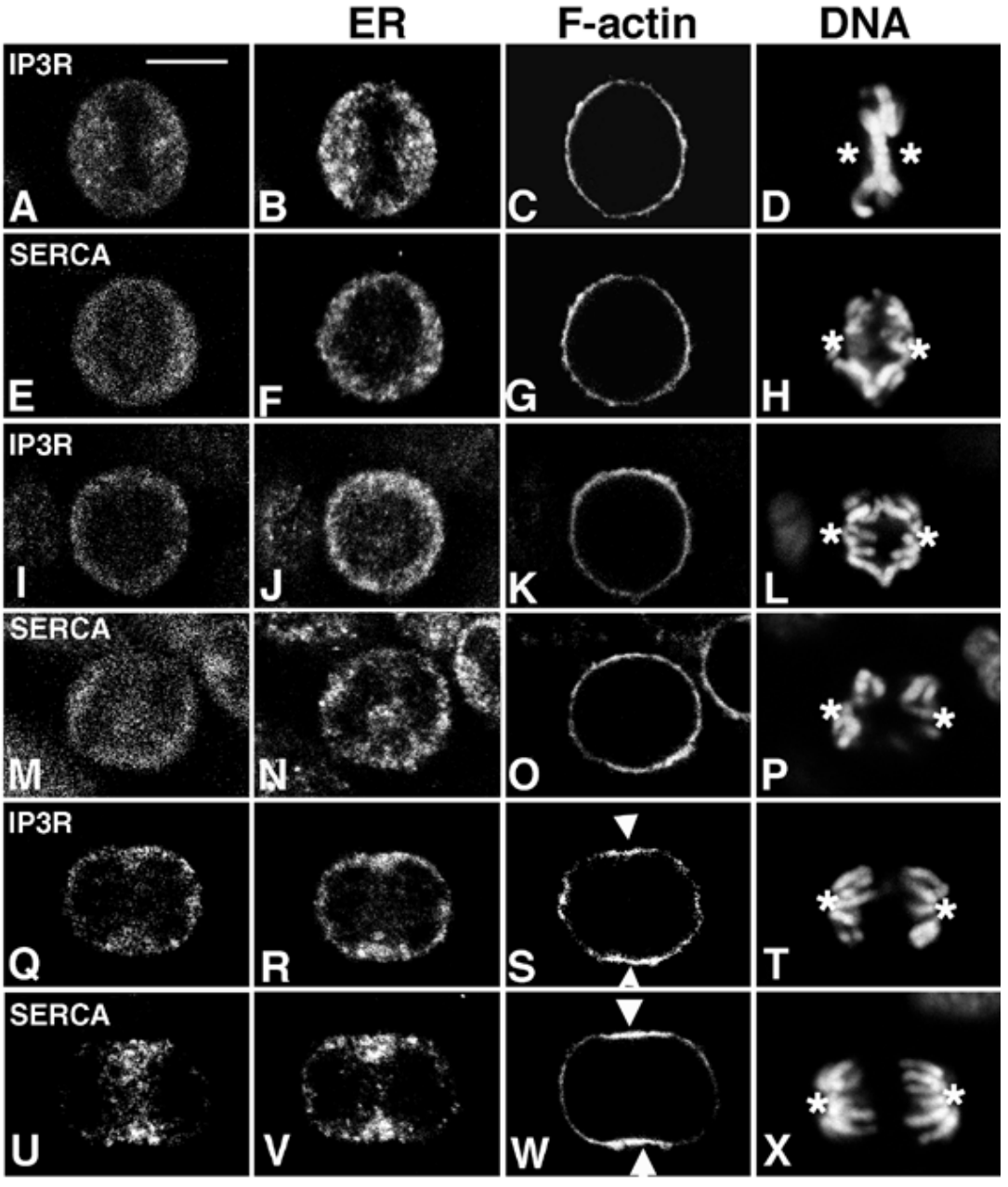

Fig. 2. Dynamic redistribution of IP_3R, SERCA, ER and F-actin to the equatorial cell cortex from metaphase to just before the onset of furrowing. *CHO cells attached to coverslips were fixed, immunostained and observed under a confocal microscope. The same cells from metaphase (A-D) to just before the onset of furrowing (U-X) were simultaneously stained for IP_3R (IP3R)* **(A,I,Q)** *or SERCA* **(E,M,U)**, *ER* **(B,F,J,N,R,V)**, *F-actin* **(C,G,K,O,S,W)**, *and DNA* **(D,H,L,P,T,X)**. *IP_3R was detected by anti-type 3-IP_3R mAb (KM1082). ER and IP_3R were present at the pole to polar cortex together with F-actin during metaphase (A-D). In early~mid-anaphase cells, IP_3R, SERCA, ER and F-actin began to re-distribute and did not accumulate at the equatorial cell cortex (E-P). Before the onset of furrowing, ER, IP_3R and SERCA together with F-actin gradually accumulated at the equatorial cell cortex (inner surface of presumptive furrow) (Q-T) and finally much accumulation was evident at the presumptive cleavage furrow just before the onset of furrowing (U-X). Arrowheads in S and W indicate actin rings which also indicate the site of presumptive furrows. Asterisks indicate the sites of spindle poles. Bar, 10 μm.*

arrow). Microinjected rhodamine-conjugated normal mouse IgG as control did not move (data not shown).

Linear movement of Ca^{2+} stores with IP_3R was blocked by the microtubule-depolarizing agent, nocodazole but not by the F-actin-depolarizing agent, cytochalasin B

To ascertain that this linear movement of Ca^{2+} stores with IP_3R toward the 2nd cleavage furrow is mediated by microtubules, we tested effects of nocodazole and cytochalasin B (Table 1 and Figs. 5,6). Co-injection of nocodazole (18.75 μM in injecting solution) with a microsome fraction blocked the formation of the 2nd cleavage furrow in an injected cell (Table 1 and Fig. 5 C,D), where the movement of Ca^{2+} stores with IP_3R was also blocked (Table 1 and Fig. 5 A,B). Co-injection of cytochalasin B (0.22 μg/ml in the injecting solution) with a microsome fraction also blocked formation of the cleavage furrow in an injected cell (Table 1 and Fig. 6 C,D), yet movement of the Ca^{2+} stores with IP_3R was not blocked (Table 1 and Fig. 6 A,B) and accumulation of the Ca^{2+} stores with IP_3R at the presumptive cleavage furrow was observed (Fig. 6B, arrow). These observatons suggest that movement of Ca^{2+} stores with IP_3R is mediated by microtubules.

Discussion

Transportation of Ca^{2+} Stores with IP_3R in a microtubule-dependent manner

In general, two main mechanisms, microtubule-based motility (Vale *et al.,* 1985; Scholey *et al.,* 1985; Hirokawa, 1996) and actin-

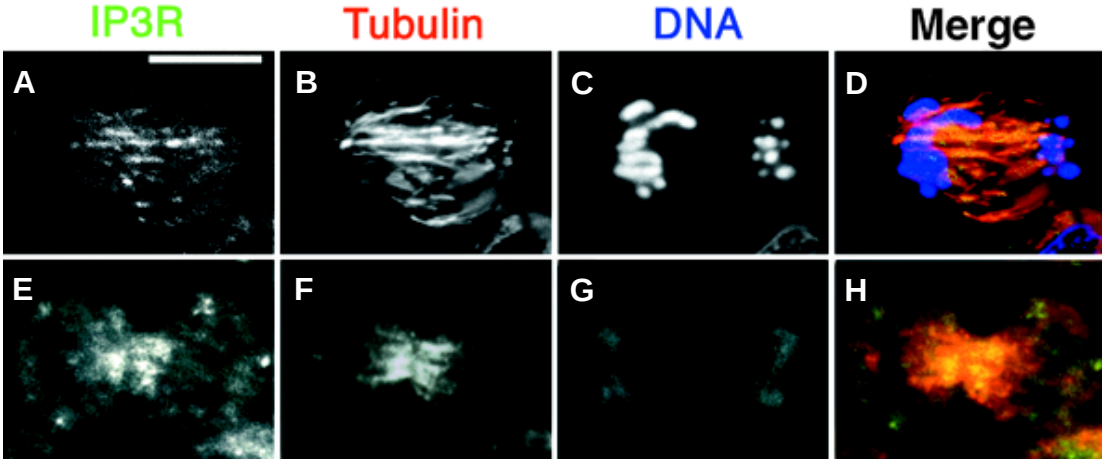

Fig. 3. IP_3R was localized along microtubules. *An ultrathin frozen section of CHO cells during late anaphase* **(A-D)** *and a whole CHO cell during telophase* **(E-H)** *were simultaneously stained for the IP_3R (IP3R), microtubules (Tubulin), and DNA, and observed under a conventional fluorescent microscope and a confocal microscope, respectively. Simultaneous localization of the three signals is shown as pseudocolored images (Merge). IP_3R was present on the microtubule bundles in a late anaphase cell, in which it appears that to a great extent the IP_3R are dragged away from the site of future cleavage (A-D). In a telophase cell, IP_3R accumulated at the cleavage furrow and was present on microtubule bundles, as seen under a confocal microscope (E-H). Bar, 10 μm.*

based motility (Mitchison and Cramer, 1996) are mechanisms involved in vesicle transport.

We found that redistributing Ca^{2+} stores with IP_3R were present along microtubules and the microinjected Ca^{2+} stores with IP_3R moved linearly toward the next cleavage furrow in a microtubule-dependent manner. These observations indicate that the movement is mediated by microtubules and microtubule-based motility and not by actin and actin-based motility. These results are consistent with reports that microtubules are required for cytokinesis (Jesuthasan, 1998; Danilchik *et al.,* 1998; Larkin and Danilchik, 1999).

It has been reported that the telophase disc may be important to position the cleavage furrow (Margolis and Andreassen, 1993). The image in Fig. 3F may perhaps be an expanding telophase disc. The images in Fig. 3 E-H may be those from a section which is optically cut and observed as indicated by an asterisk in Fig. 7C. The telophase disc may be the bilateral peripheral astral microtubules which encountered at the equatorial cortex.

At late anaphase there are still microtubules running from pole to pole, along which IP_3R (ER) could possibly relocate back to the middle of the spindle. It would appear from Fig. 3A-B that some IP_3R remain in the middle of the spindle (at least at late anaphase). Perhaps these (i.e., the ER that contains them) may act as a kind of nucleation center for the subsequent redistribution of ER as the potential “cleavage signal”.

Microtubule motors (kinesin family and cytoplasmic dynein) are responsible for the movement of membranous organelle on microtubules. (Vale *et al.,* 1985; Scholey *et al.,* 1985; Hirokawa, 1996). Microinjection of a microsome fraction was done during the metaphase-early anaphase in a 2-cell stage newt egg. The polarity of microtubules in a mitotic apparatus during these stages is constant. Plus ends of almost all microtubules in a mitotic apparatus are directed toward the equator (Polar side is the minus end) (Euteneuer *et al.,* 1982). Therefore, Ca^{2+} stores with IP_3R which are transferred toward the equatorial cortex on microtubules must be topologically powered by a plus-ended microtubule

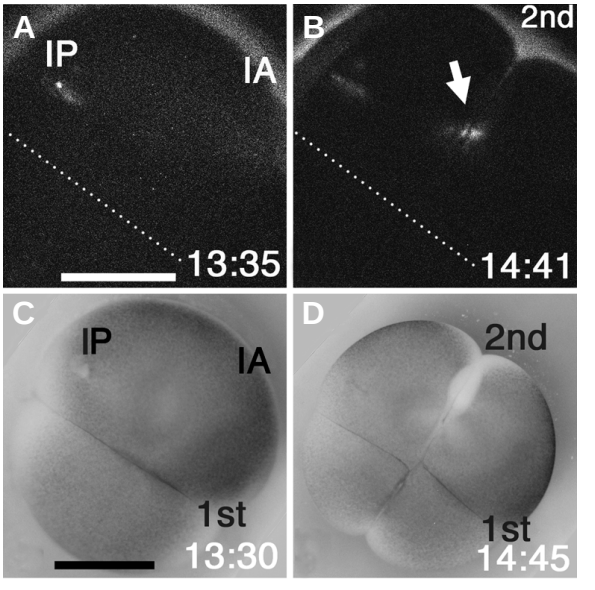

Fig. 4. Ca^{2+} stores with IP_3R microinjected into the dividing newt egg move linearly and accumulate to the next cleavage furrow. *A SERCA-GFP-labeled microsome fraction from SERCA-GFP stably expressing CHO cells was microinjected into a cell of a 2-cell stage newt egg, and observed under a confocal microscope. Representative profiles were obtained using a confocal microscope* **(A-B)** *and a light microscope* **(C-D)**. *When the first cleavage furrow was completed, the capillary was inserted from one side (IP, inserted point in C) and was pushed through the egg and brought close to the egg surface on the opposite side to avoid insertion artifact; then the microsome fraction was injected (IA, actual injected area in C). We tried to inject the microsome fraction only in the injected area, but we could not avoid a small amount of microinjection at the inserted point (IP in A). The weak fluorescent signals from the microinjected microsome fraction were seen around the injected area (IA in A), because the focus was adjusted to the inserted point. 20-40 minutes after the injection, the second normal cleavage furrow was initiated. SERCA-GFP-labeled Ca^{2+} stores with IP_3R moved linearly toward the next (2nd) cleavage furrow from both IP and IA with time, and accumulated at the second cleavage furrow (B, arrow). The time the confocal image was taken is shown at the bottom of the respective panels in hours and minutes. Broken lines represent positions of the first cleavage furrow. IP, inserted point; IA, actual injected area. Scale bar, 1 mm.*

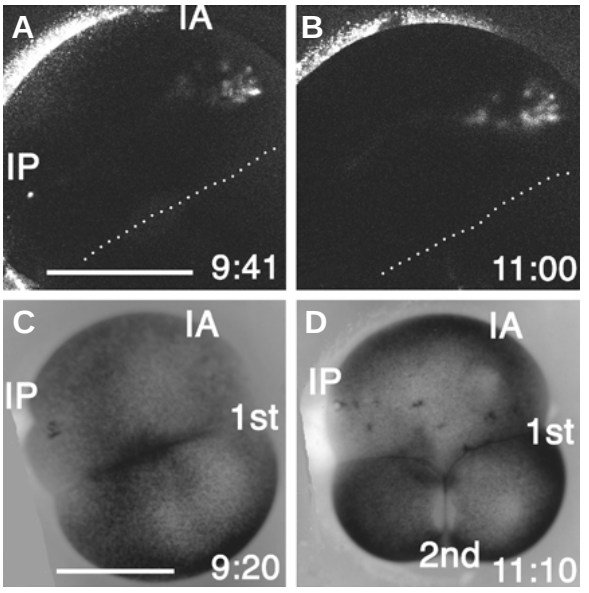

Fig. 5. The linear movement of Ca^{2+} stores with IP_3R towards the next cleavage furrow was blocked by co-injection of nocodazole, a microtubule-depolarizing agent. *A SERCA-GFP-labeled microsome fraction was microinjected into a cell of a 2-cell stage newt egg together with nocodazole (18.75 μM in injecting solution), and observed under a confocal microscope. The microinjected cell did not divide* **(C,D)** *and microinjected Ca^{2+} stores with IP_3R did not move* **(A,B)**. *Broken lines represent positions of the first cleavage furrow. IP, inserted point; IA, actual injected area. Scale bar, 1 mm.*

motor, the kinesin family. Calculating from the distance and the time of the locomotion of Ca^{2+} stores, the velocity of Ca^{2+} stores is about 0.3-0.5 μm/second. This velocity is consistent with the velocity of the reported kinesin families.

Since ER is dissociated into small fragments by entering mitosis (Koch and Booth, 1988), our results strongly suggest that ER fragments, especially Ca^{2+} stores with IP_3R are transported along microtubules to the equatorial cell cortex by a microtubule-based organelle transporting system, especially by kinesin families, resulting in accumulation of Ca^{2+} stores with IP_3R to the equatorial cell cortex (Fig. 7).

Ca^{2+} stores with IP_3R as the Ca^{2+} source

Two pools, internal Ca^{2+} stores or an extracellular Ca^{2+} pool, can be considered as the source of the Ca^{2+} transients along the cleavage furrow, but many authors suggested that internal Ca^{2+} stores are good candidates, as described in our introduction.

In general, there are two kinds of Ca^{2+} channels on the Ca^{2+} stores, IP_3R and the ryanodine receptor, but it has been suggested that the Ca^{2+} transient along the cleavage furrow which occurs via a IP_3R-mediated manner, as described in the introduction. While it is not known if the ryanodine receptor is present in newt eggs, the ryanodine receptor was not detected in *Xenopus* embryos (Parys *et al.,* 1992), a species close to the newt.

Reported local Ca^{2+} transients along the cleavage furrows were precisely localized to the cleavage furrows (Fluck *et al.,* 1991; Chang and Meng, 1995; Muto *et al.,* 1996; Webb *et al.,* 1997; Creton *et al.,* 1998). We reported that Ca^{2+} stores with IP_3R in microsome fractions from mouse cerebella and CHO cells can induce extra-cleavage furrow via IICR (Mitsuyama *et al.,* 1999). We showed that polar dominant Ca^{2+} stores with IP_3R during metaphase were redistributed and accumulated to the presumptive cleavage furrow until just before the onset of furrowing along microtubules, and that microinjected Ca^{2+} stores with IP_3R move linearly and accumulate to the next cleavage furrow in a microtubule-dependent manner. Therefore, it appears that endogenous Ca^{2+} stores with IP_3R are transported and accumulate at the equatorial cell cortex as the result of microtubule-based motility then function as its Ca^{2+}-source via IICR, at least in formation of the cleavage furrow.

In Fig. 6D, there was clearly a surface feature that corresponded to the pattern of fluorescence shown in 6B. This means that pigmentation of the surface that corresponded to the pattern of accumulation of Ca^{2+} stores with IP_3R at the presumptive cleavage furrow occurred. The pigmentation may be due to the IP_3 induced Ca^{2+} release from the accumulated Ca^{2+} stores with IP_3R at the presumptive cleavage furrow as Ca^{2+} releasing machinery and a furrow positioning signal.

Ca^{2+} stores with IP_3R as the cleavage furrow inducing signal

The signal to determine cleavage position was termed "cleavage stimulus" by Rappaport (Rappaport, 1961). There are reports to show characteristics of the "cleavage stimulus". 1) It could be transferred to the equatorial cell cortex from the polar area (Spek, 1918; Harvey, 1934; Dan, 1943; Swann and Mitchison, 1953; Rappaport, 1961; Eckley *et al.,* 1997), 2) it was seen to be mediated by astral microtubules (Wilson, 1928; Rappaport, 1961; Dan, 1943; Hiramoto, 1971; Eckley *et al.,* 1997), 3) it was

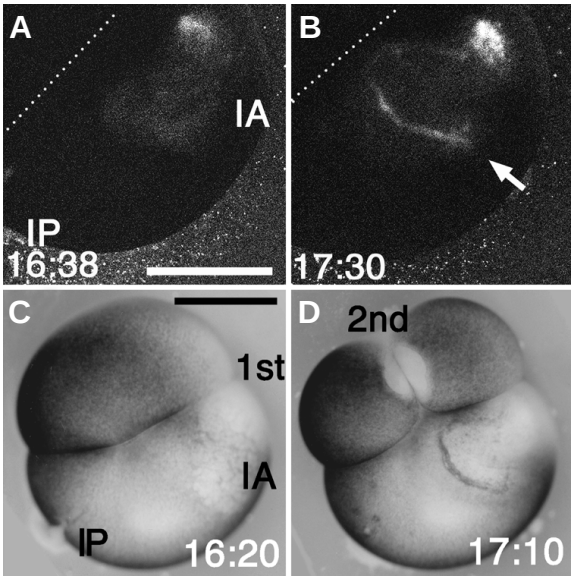

Fig. 6. The movement of Ca^{2+} stores with IP_3R towards the next cleavage furrow was not blocked by co-injection of cytochalasin B, an F-actin-depolarizing agent. *A SERCA-GFP-labeled microsome fraction was microinjected into a cell of a 2-cell stage newt egg together with cytochalasin B (0.22 μg/ml in the injecting solution), and observed under a confocal microscope. A microinjected cell did not divide* **(C,D)**, *but microinjected Ca^{2+} stores with IP_3R accumulated at the presumptive cleavage furrow* **(A,B)**, *arrow. Broken lines represent positions of the first cleavage furrow. IP, inserted point; IA, actual injected area. Scale bar, 1 mm.*

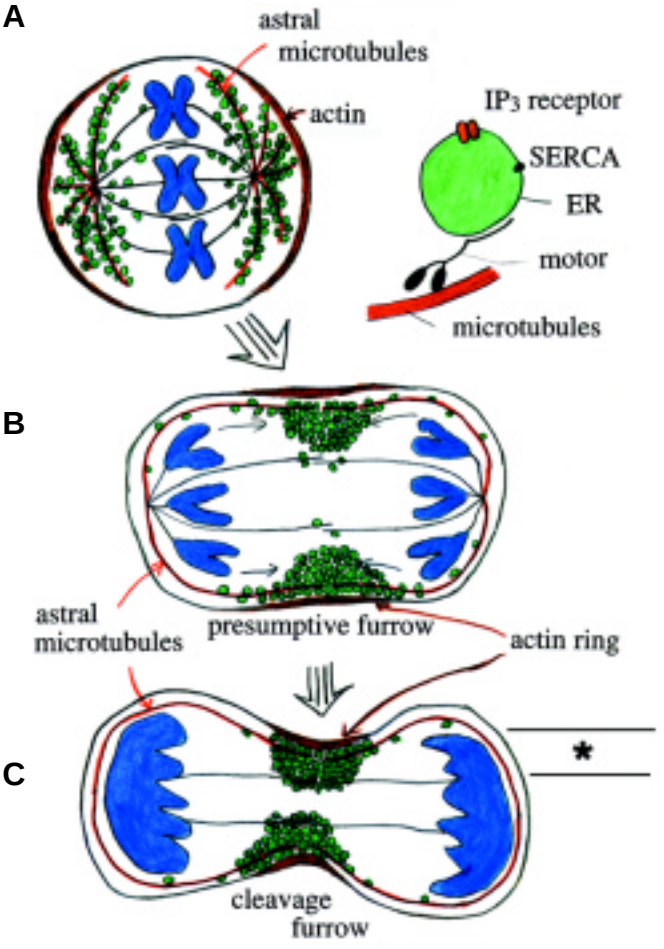

Fig. 7. We hypothesize that the Ca^{2+} stores with IP_3R are transferred along astral microtubules by microtubule motors, resulting in redistribution of Ca^{2+} stores with IP_3R to the presumptive cleavage furrow, as Ca^{2+} releasing machinery and as a "cleavage stimulus" which induce and position the cleavage furrow. *The local Ca^{2+} increase from these Ca^{2+} stores by IP_3 induced Ca^{2+} release (IICR) may bring about contraction of the actomyosin ring by activating Ca^{2+}-calmodulin dependent myosin light chain kinase.* **(A)** *Metaphase.* **(B)** *Just before the onset of furrowing (late anaphase).* **(C)** *Telophase. The images in Fig. 3 E-H could be those from a section which is optically cut and observed as indicated by an asterisk in C.*

transferred before the onset of furrowing (Harvey, 1934; Beams and Evans, 1940; Swann and Mitchison,1953; Rappaport and Ebstein, 1965), 4) it induces the furrowing (Rappaport and Ebstein, 1965; Tilney and Marsland, 1969; Sawai,1972 and 1983), 5) it is not diffusible (Mabuchi, 1986; Sawai,1972 and 1983), and 6) it is present in the cytoplasm just beneath the furrow and is transplantable (Sawai, 1972, 1983).

We reported that microinjection of Ca^{2+} store-enriched microsome fractions to dividing newt eggs induced extra-cleavage furrows via inositol 1,4,5-trisphosphate-induced Ca^{2+} release (F. Mitsuyama *et al.,* 1999). This means that Ca^{2+} stores with IP_3R have the above 4th characteristic of a cleavage stimulus.

In the present work, we showed that polar dominant Ca^{2+} stores with IP_3R during metaphase were re-distributed to the equatorial cortex until just before the onset of furrowing. Thus, Ca^{2+} stores with IP_3R also have the above 1st and 3rd characteristics of a cleavage stimulus.

We also showed that Ca^{2+} stores with IP_3R was relocated to the cleavage furrow along microtubules and microinjected Ca^{2+} stores with IP_3R move linearly and accumulate to the next cleavage furrow in a microtubule-dependent manner during anaphase-cytokinesis. Therefore Ca^{2+} stores with IP_3R have the above 2nd characteristic of cleavage stimulus.

Ca^{2+} ion is diffusible, but Ca^{2+} ion in the Ca^{2+} stores is not diffusible by the sequestering with ER membranes. As the ER is present in the cytoplasm by anchoring to the microtubules mediated with microtubule motors, the Ca^{2+} store is not diffusible. This satisfies the 5th characteristic of a cleavage stimulus.

We also showed that Ca^{2+} stores with IP_3R are present just beneath the cleavage furrow. Transplantation of the cytoplasm just beneath the furrow would induce the extra-cleavage furrow mediated by the transplanted Ca^{2+} stores with IP_3R in the cytoplasm, as a cleavage stimulus.

These results indicate that Ca^{2+} stores with IP_3R are not only the first to be observed to induce a local cleavage furrow among the candidates for the cleavage stimulus, but also satisfy all other characteristics of a cleavage stimulus. Therefore, Ca^{2+} stores with IP_3R may well be "cleavage stimulus" itself to induce and position cleavage furrows.

Materials and Methods

Antibodies

Polyclonal anti-IP_3R antibody was developed, as described (Nakade *et al.,* 1994). MAbs to type 1 (KM1112), type 2 (KM1083) and type 3 (KM1082)-IP_3R were raised, as described (Sugiyama *et al.,* 1994). Rabbit pAb against ER was a kind gift from Dr. Daniel Louvard in Institute Curie (Louvard *et al.,* 1982). Rabbit polyclonal antibody to the ryanodine receptor (C2) was raised, as described (Kuwajima *et al.*, 1992). Mouse monoclonal anti-SERCA2 antibody (ll D8, Affinity Bioreagents Inc., Golden, CO), mouse monoclonal anti-alpha-tubulin antibody (Oncogene Research Products, Cambridge, MA), mouse monoclonal anti-ryanodine receptor antibody (Calbiochem-Novabiochem Corp. San Diego, CA), normal rabbit IgG (Zymed, San Francisco, CA), and normal mouse IgG (Sigma, St. Louis, MO) were used. Anti-rabbit IgG-HRP (Amersham), anti-mouse IgG-HRP (Amersham), anti-rabbit IgG-FITC (Vector, Burlingame, CA), anti-mouse IgG-TRITC (Boehringer Mannheim, Indianapolis, IN), anti-rabbit IgG-Texas Red (Amersham), anti-mouse IgG-FITC (Vector), and anti-rabbit IgG-Cy5 (Amersham) were used.

Western blotting

The method of Western blotting of membrane proteins prepared from CHO cells was, as described (Monkawa *et al.,* 1995). C2 pAb at 10 μg/ml and mouse monoclonal anti-ryanodine receptor antibody at 10 μg/ml were used as primary antibodies. Concentrations of other primary antibodies used were the same as described below. HRP conjugated secondary antibodies were used at a 1: 1,000 dilution.

Immunofluorescent microscopy

Frozen sections were made as described (Mitsuyama and Kanno, 1993). Immunofluorescent cytochemistry was done essentially as described (Sato *et al.,* 1985). Polyclonal anti-IP_3R antibody at 10 μg/ml, KM1112 at 10 μg/ml, KM1083 at 10 μg/ml, KM1082 at 10 μg/ml, anti-ER antibody at 1:100 dilution, ll D8 at 1:100 dilution, mouse anti-tubulin antibody at 1:100 dilution, normal rabbit IgG at 10 μg/ml, normal mouse IgG at 10 μg/ml, anti-rabbit IgG -FITC at 1:100 dilution, anti-mouse IgG-TRITC at 1:10 dilution, anti-rabbit IgG-Texas Red at 1:35 dilution, anti-

mouse IgG-FITC at 1:100 dilution, and anti-rabbit IgG-Cy5 at 1:150 dilution were used for immunofluorescence. F-actin was detected by phalloidin-TRITC (Sigma) at 0.5 μg/ml. Immunostained whole cells were observed under confocal laser microscopes (LSM 410, Zeiss, Oberkochen, Germany) adapted to an inverted microscope (Axiovert 135 TV, Zeiss) (objective lens: C-Apochromat 40x/1.2 NA, Zeiss) and a Bio-Rad MRC1024 confocal microscope (Bio-Rad Laboratories, Hercules, CA) adapted to a microscope (Optiphot-2, Nikon, Tokyo, Japan) (objective lens: Plan Apo 60x/1.4 NA, Nikon). Immunostained frozen sections were observed under a conventional fluorescent microscope (Axioplan, Zeiss,) (objective lens: Plan-Neofluar 100x/1.30 NA). Images viewed under a fluorescent microscope were photographed using Neopan 400 black and white films (Fuji, Tokyo, Japan) and converted into digital files using Polascan 35 Ultra (Japan Polaroid Inc., Tokyo, Japan). Staining image by each antibody on the same cell was pseudocolored and superimposed using Adobe Photoshop 4.0 J (Adobe Systems Inc., San Jose, CA).

Preparation of newt eggs and microsome fractions for microinjection

Eggs of the newt, *Cynops pyrrhogaster*, were used as described (Sawai, 1972, 1983, Mitsuyama *et al.,* 1999). SERCA-GFP-labeled microsome fractions from CHO cells were prepared by centrifugation and microinjected SERCA-GFP-labeled Ca^{2+} stores were observed, as described (Mitsuyama *et al.,* 1999). As a control, tetramethylrhodamine (FluoReporter Tetramethylrhodamine Protein Labelling Kit, Molecular Probes Inc., Eugene OR) was conjugated to normal mouse IgG (Sigma).

Blocking agents

Pilot experiments were done to determine the dose required to disrupt formation of the cleavage furrows in the injected cell. Blocking agents were used as co-injection with a microsome fraction. Nocodazole (Sigma) was used at 18.75 μM in the injecting solution. Cytochalasin B (Sigma) was used at 0.22 μg/ml in the injecting solution. Ca^{2+}-depleted cytosolic-like medium with 1% dimethyl sulfoxide (Sigma) was used as the control. Parallel observations were made, using a light microscope to determine the degree of inhibition of formation of the cleavage furrow.

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