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Calcium Buffer Injections Inhibit Ooplasmic Segregation in Medaka Eggs

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Abstract. Injection of the weak (K_D = 1.5 μM) calcium buffer 5,5′-dibromo-BAPTA into fertilized medaka eggs inhibited the formation of the blastodisc at the animal pole, the movement of oil droplets toward the vegetal pole, and cytokinesis. These inhibitory actions were dependent upon the concentration of the buffer but were independent of free [Ca^{2+}] in the injectate. Because this buffer has previously been shown to substantially suppress zones of elevated calcium at the animal and vegetal poles of the medaka egg, the results of the present study suggest that these zones are necessary for normal segregation of the ooplasm and its inclusions in the medaka egg.

Introduction

Nearly 20 years ago, cytosolic calcium gradients were postulated to organize developmental localization in eggs of plants and animals (Jaffe *et al.*, 1974; for reviews, see Jaffe and Nuccitelli, 1977; and Jaffe, 1986). Proof of this theory should have three parts (Gilkey *et al.*, 1978): (1) evidence of the existence of cytosolic calcium gradients in systems undergoing localization; (2) evidence that artificial induction of a cytosolic calcium gradient will cause localization; and (3) evidence that dispersion of a cytosolic calcium gradient will inhibit localization. Evidence for the existence of cytosolic calcium gradients in eggs was at first indirect, based on studies suggesting that, in developing fucoid eggs, rhizoids grow in the direction of high [Ca^{2+}] (Robinson and Jaffe, 1975; Nuccitelli, 1978). Since then such gradients have been directly demonstrated in a number of developing systems in which zones of elevated cytosolic [Ca^{2+}] have been identified: near the tip of the rhizoid of fucoid embryos (Berger and Brownlee, 1993), near the tip of growing pollen tubes (Miller *et al.*, 1992b), near the vegetal pole of fertilized *Xenopus laevis* eggs (Miller *et al.*, 1991), and near the animal and vegetal poles of fertilized medaka eggs (Fluck *et al.*, 1992b). In medaka eggs, these zones are present throughout the period of ooplasmic segregation (Fluck *et al.*, 1992b).

Regarding the second criterion—that artificial induction of a cytosolic calcium gradient will organize developmental localization—there is only indirect evidence. This evidence includes the occurrence of localization in fucoid eggs (Robinson and Cone, 1980) and ascidian eggs (Jeffery, 1982; Bates and Jeffery, 1988) exposed to a gradient of the calcium ionophore A23187. Other indirect evidence comes from the medaka egg, in which additional axes of ooplasmic segregation can be induced by "strong pricking" of the egg (Sakai, 1964), an action that might create a zone of elevated cytosolic [Ca^{2+}] at the wound.

The present study addresses the third criterion—that dispersion of a cytosolic calcium gradient will inhibit localization. We have pursued this question in the large (diameter = 1.2 mm) and remarkably clear medaka fish egg. In this egg, which consists of a thin (about 25 μm thick) peripheral layer of cytoplasm bounded by a plasma membrane and separated from a large yolk vacuole by a yolk membrane, ooplasmic segregation consists of the roughly simultaneous streaming of the bulk of the ooplasm and its inclusions toward the animal pole, the movement of oil droplets toward the vegetal pole, and the saltatory movement of some inclusions toward the vegetal pole (Sakai, 1965; Iwamatsu, 1973; Abraham *et al.*, 1993a). During and after ooplasmic segregation, zones of elevated cytosolic [Ca^{2+}] are present at both the animal and vegetal poles of the egg (Fluck *et al.*, 1992b).

Injection of the calcium buffer 5,5′ dibromo-BAPTA (referred to hereafter as dibromo-BAPTA) substantially dissipates these polar zones of elevated [Ca^{2+}] (Fluck *et*

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Abbreviations: BCECF, 2,7-bis-(2-carboxyethyl)-5-(and 6-)-carboxyfluorescein.

al., 1992b). The buffer also blocks the development of fucoid eggs (Speksnijder *et al.*, 1989); suppresses high calcium zones in, and inhibits the growth of, pollen tubes (Miller *et al.*, 1992b); inhibits cell cycle contraction waves (Miller *et al.*, 1992a) and cytokinesis (Miller *et al.*, 1993; Snow and Nuccitelli, 1993) in *X. laevis* embryos; and inhibits *X. laevis* nuclear membrane vesicle fusion *in vitro* (Sullivan *et al.*, 1993). This relatively weak calcium buffer (K_D = 1.5 μM; Pethig *et al.*, 1989) is believed to act as a shuttle buffer, one that binds Ca^{2+} at the high end of a $[Ca^{2+}]$ gradient and releases it at the low end of the gradient. In other words, the buffer facilitates the diffusion of Ca^{2+} within the cell. In the present study, we demonstrate that injection of dibromo-BAPTA into fertilized medaka eggs inhibits ooplasmic segregation. A preliminary account of these findings has been published (Fluck *et al.*, 1992a).

Materials and Methods

Biological material

Methods for removing gonads from breeding medaka (Yamamoto, 1967; Kirchen and West, 1976; Fluck, 1978; Abraham *et al.*, 1993a), preparing eggs for experimental use (Abraham *et al.*, 1993a), and fertilizing eggs *in vitro* (Yamamoto, 1967; Abraham *et al.*, 1993a) have been described previously. Gonads, gametes, and zygotes were placed in the same buffered saline solution used in other studies of the fertilized medaka egg (Fluck *et al.*, 1991, 1992b; Abraham *et al.*, 1993a): 111 mM NaCl; 5.37 mM KCl; 1.0 mM $CaCl_2$, 0.6 mM $MgSO_4$; 5 mM HEPES, pH 7.3.

Preparation of micropipettes

Glass micropipettes (0.8-mm diameter, Drummond Scientific Co., Broomall, Pennsylvania, Series I; or filamented, 1-mm diameter, Narashige U.S.A., Inc., Greenvale, New York, Series II and III) were made with a Narashige PN-3 microelectrode puller. The tips of the micropipettes were beveled at an angle of 20°C with a Narashige EG-4 microgrinder; this process was monitored with an audio system (Miller *et al.*, 1993). Tip diameter was approximately 5 μm at the widest part of the bevel.

Buffer solutions injected

Solutions of 50 mM (eggs in Series I–III) or 100 mM (some eggs in Series I) dibromo-BAPTA (Molecular Probes, Eugene, Oregon; tetrapotassium salt), $CaCl_2$, and 5 mM HEPES (Sigma Chemical Co., St. Louis, Missouri, titrated to pH 7.2 with KOH) were prepared in which $[Ca^{2+}]_{free}$ was set at 0.2 μM (Series I), 0.14 μM (Series II), or 0.3 μM (Series III). These values of $[Ca^{2+}]_{free}$ are within the range reported for the ooplasm of the fertilized medaka egg (Schantz, 1985).

Three types of controls were used. First, each batch of eggs included at least one egg into which we injected no fluids. A second type of control egg received 1.0–1.6 nl of 5 mM HEPES, pH 7.2, containing 50 or 100 mM K_2SO_4 or 125 mM KCl. A third type of control egg received 1.4 nl of a solution of 5,5′-dimethyl-BAPTA, a BAPTA-type buffer with a higher affinity for calcium (K_D = 0.15 μM; 50 mM 5,5′-dimethyl-BAPTA, tetrapotassium salt; 5 mM HEPES, pH 7.2; and sufficient $CaCl_2$ to set $[Ca^{2+}]_{free}$ at 0.2 μM).

Determining the amount of buffer to inject

According to facilitated diffusion theory, a final concentration of about 2 mM dibromo-BAPTA in the ooplasm should be sufficient to dissipate zones of elevated $[Ca^{2+}]$ in the micromolar range (Speksnijder *et al.*, 1989). Moreover, 2.7 mM dibromo-BAPTA has been shown to dissipate zones of elevated $[Ca^{2+}]$ at the poles of the fertilized medaka egg (Fluck *et al.*, 1992b). We thus microinjected enough 50 mM or 100 mM dibromo-BAPTA to achieve final buffer concentrations in the ooplasm of 0.5–7.0 mM. We estimated that the accessible ooplasmic volume was 27.6 nl (Fluck *et al.*, 1992b) and thus injected 0.26–1.96 nl of buffer. A number of investigators have shown that injectate volumes in this range have no detectable effect on the development of the medaka egg (Ridgway *et al.*, 1977; Gilkey, 1983; Yoshimoto *et al.*, 1985, 1986; Fluck *et al.*, 1991, 1992b); thus a purely mechanical effect of the injectate on development of the egg seems unlikely.

Microinjection

We used a low-pressure method (Hiramoto, 1962; Kiehart, 1982; Fluck *et al.*, 1991, 1992b) in Series I and a high-pressure method (Narashige IM-200 microinjection system, Fluck *et al.*, 1992b) in Series II and III. In both methods, we front-loaded the micropipettes. The pipettes were calibrated by injecting a small volume of the solution into vegetable oil and measuring the diameter of the spherical droplets.

Batches of two to six eggs were fertilized simultaneously in buffered saline solution, transferred to closely fitting holes drilled in a transparent plastic holder, and injected sequentially with fluid soon after fertilization (range, 5–30 min after fertilization; 12.5 ± 6.5 min, X ± SD, N = 104 eggs). Every batch included at least one control egg into which no fluid was injected.

The method for injecting fluid into the thin peripheral layer of ooplasm of the medaka egg has been described elsewhere (Ridgway *et al.*, 1977; Gilkey, 1983; Fluck *et al.*, 1991) and will only be summarized here. The micropipette, held in a micromanipulator, was advanced far enough to penetrate the chorion (shell) and plasma membrane but not the yolk membrane, which roughly con-

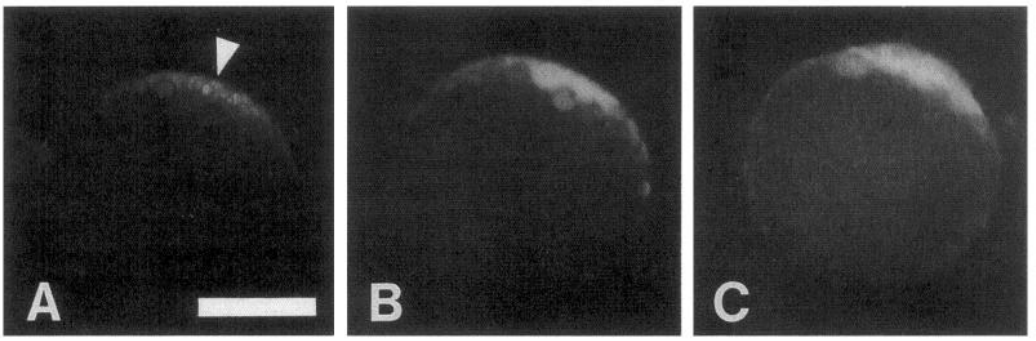

Figure 1. Diffusion of fluorescein around the medaka egg. The approximate position of the injection site is marked with an arrowhead. (A) By 5 min after injection of the dye, it had spread more than 50° arc in all directions around the egg. (B) By 18 min, it was present about 90° arc from the injection site. (C) At 74 min, it had spread to the antipode of the injection site. Scale bar, 250 μm.

formed to the shape of the micropipette. When fluid was injected into the ooplasm, it distended the yolk membrane near the tip of the micropipette, thus visually confirming that fluid had been injected into the ooplasm and not the yolk vacuole. As the pipette was slowly withdrawn, the yolk membrane returned to its normal position, causing the injectate to be spread quickly over a large area of the ooplasm. Fluid was injected either equatorially (within 30° arc of the equator) or near the vegetal pole (within 30° arc of the vegetal pole).

Dibromo-BAPTA was injected into the yolk vacuole of four eggs. Such eggs were penetrated with a micropipette in the usual manner, except that the tip of the pipette also penetrated the yolk membrane. The injectate thus did not distend the yolk membrane but did transiently change the optical properties of the yolk near the tip of the pipette, confirming that fluid had been injected.

We injected dibromo-BAPTA into eggs in three series of experiments: I (winter 1990, in which we successfully injected buffer into the ooplasm of 45 eggs from 12 females), II (winter 1992, 27 eggs from 4 females), and III (summer 1992, 20 eggs from 4 females). In addition, we injected solutions of K_2SO_4 or KCl into 15 eggs; injected dimethyl-BAPTA into 10 eggs from two females; injected dibromo-BAPTA into the yolk vacuole of 4 eggs; and monitored the development of 44 uninjected control eggs.

Observation of the effects of buffer injection

Ooplasmic movements in at least one egg in each batch were recorded by time-lapse video microscopy (Abraham *et al.*, 1993a), while other eggs were observed at regular intervals with a stereomicroscope. Room temperature was 18–19°C for Series I and II and 23–24°C for Series III; at these temperatures, the first cleavage begins, respectively, 130–140 min and 85–95 min after fertilization. In order to compare data from experiments at different temperatures, we have reported our results not only as "minutes after fertilization" but also as t_n, or normalized time, where $t_n = 1.0$ represents the time at which cytokinesis begins.

Injection of fluorescein into eggs

To estimate both the size of the area over which an injectate spreads immediately after injection and the time required for fluorescein to diffuse around the egg, we injected 1.5–2.0 nl of 50 μ*M* fluorescein (sodium salt, dissolved in 100 m*M* K_2SO_4, 5 m*M* HEPES, pH 7.2; Sigma Chemical Co.) into eggs. Using epi-illumination and a SIT camera (Dage/MTI, model 66DX), we examined these eggs within 5 min after injection and at regular intervals thereafter for up to 3 h.

Results

Movement of fluorescein around the egg

Within 5 min after injection, fluorescein was present in ooplasm within 50° arc (or 530 μm, assuming an egg diameter of 1190 μm) in all directions from the injection site (Fig. 1); this is equivalent to the molecule having spread initially into an area equal to 13% of the total surface of the egg and thus into a volume equal to 13% of the total volume of the ooplasm. The molecule spread as far as 90° arc in less than 15 min and reached the antipode of the injection site in about 75 min.

Immediate localized effects of dibromo-BAPTA

An immediate reaction of the eggs to injection of dibromo-BAPTA was an apparent expansion of the yolk membrane near the injection site, which caused the yolk vacuole to bulge into the ooplasm (Fig. 2A). This bulge usually subsided within 10 min in eggs receiving low concentrations of buffer (final cytosolic concentration < 4 m*M*). But in some of these eggs and in most eggs receiving higher concentrations of buffer, the bulge persisted and the yolk membrane over the bulge lysed (Table

I), releasing yolk into the ooplasm or, more often, into the perivitelline space. To determine whether [dibromo-BAPTA] and $[Ca^{2+}]_{free}$ significantly affected egg lysis, the probability of egg lysis was analyzed using a generalized additive model with $[Ca^{2+}]_{free}$ as a factor variable and a slightly nonlinear, nonparametric relationship, with 1.5 degrees of freedom (intermediate between a linear and a quadratic relationship) between final buffer concentration and the probability of lysis (function gam of the S-PLUS statistical package, S-PLUS Reference Manual, Version 3.0, Statistical Services, Inc., Seattle, Washington). An analysis of variance indicated that *P* values for the effect of $[Ca^{2+}]_{free}$ and [dibromo-BAPTA] on lysis were 0.012 and 3.4×10^{-7}, respectively.

Another immediate response to the injection of dibromo-BAPTA (final cytosolic concentration ≥ 1 m*M*), which made a convenient marker of the injection site, was the movement of nearby oil droplets away from the injection site and toward the top of the egg (Figs. 1B, C); the droplets appeared simply to float to the top of the egg. This movement began immediately upon injection of the buffer and was essentially over within 15 min. For comparison, the normal movement of oil droplets toward the vegetal pole during ooplasmic segregation continues for more than 140 min at 18–19°C and more than 95 min at 23–24°C (Sakai, 1965; Abraham *et al.*, 1993a). These immediate localized responses were not due to the mechanical effects of injection because we saw no such responses in control eggs receiving either KCl or K_2SO_4. Moreover, they were less prominent in eggs into which we injected dimethyl-BAPTA. Instead, these early responses were likely a response to the initially high concentration of dibromo-BAPTA near the injection site. Assuming that (1) injected buffer initially spread as far as injected fluorescein and (2) the accessible volume of the ooplasm is 27.6 nl (Fluck *et al.*, 1992b), we can estimate the initial concentration of buffer in the ooplasm soon after injection. For example, in eggs receiving enough 50 m*M* dibromo-BAPTA to achieve an estimated final concentration of 2.7 m*M* throughout the ooplasm, the initial concentration within 50° arc of the injection site would be about 21 m*M*.

Effects of dibromo-BAPTA on ooplasmic segregation

Dibromo-BAPTA inhibited ooplasmic segregation and the subsequent development of the embryos in a concentration-dependent manner (Table I and Fig. 2B–G), and these effects were independent of $[Ca^{2+}]_{free}$ in the injectate. Except for causing the early movement of oil droplets (described above) and a slight delay in the onset of cytokinesis (see below), low concentrations of dibromo-BAPTA (<2.0 m*M*) had no substantial effect on either the formation of the blastodisc or the movement of oil droplets toward the vegetal pole (Fig. 2H). However, buffer concentrations ≥ 2.6 m*M* inhibited the movement of oil droplets toward the vegetal pole and slowed the growth of the blastodisc (Table I; Fig. 2C, E–G). In addition, small aggregates of ooplasm often formed outside the blastodisc proper and on the side of the egg into which buffer had been injected (Fig. 2E, G). The specific effects of the buffer on oil droplet movement varied with the site of injection. Injection near the equator strongly inhibited oil droplet movement throughout the contralateral half of the embryo and caused the retention of oil droplets within the blastodisc at the animal pole (Fig. 2C, E, G). Injection near the vegetal pole resulted in the accumulation of oil droplets near the equator of the egg (Fig. 2F), the result of droplets in the vegetal hemisphere having floated to the top of the egg and those in the animal hemisphere having moved normally toward the equator.

Effects of dibromo-BAPTA on cytokinesis and embryonic axis formation

Dibromo-BAPTA delayed or blocked cytokinesis in a concentration-dependent manner. For example, when one group of eggs—from the same female and injected with differing amounts of buffer within a few minutes of each other—were examined 170 min after fertilization (18–19°C), the eggs had developed to the following stages: uninjected control, had completed first cleavage; 0.64 m*M* dibromo-BAPTA, was similar to control; 1.1 m*M* dibromo-BAPTA, was forming two unequal blastomeres; 2.0 m*M* dibromo-BAPTA, had not begun cytokinesis. The last egg in this group formed two incomplete furrows by 4.5 h after fertilization but did not go on to form an embryonic axis, whereas the two eggs that received lower amounts of buffer did form an embryonic axis. The inhibition of cytokinesis was more pronounced in eggs receiving buffer concentrations ≥ 2.6 m*M*. For example, of 24 eggs (Series I and II) receiving 2.6–3.6 m*M* dibromo-BAPTA, 9 did not divide and the other 12 divided from 40 min to more than 3 h after the controls did; whereas of eggs receiving >4 m*M* buffer, none underwent cytokinesis (Table I; Fig. 2E–G). Embryos receiving ≥2.6 m*M* dibromo-BAPTA did not form an embryonic axis.

Controls

Eggs injected with KCl (125 m*M*, 6 embryos) or K_2SO_4 (50 m*M*, 6 embryos; 100 m*M*, 3 embryos) near either the equator (12 embryos) or the vegetal pole (3 embryos) developed normally. There was neither an immediate response to the injectate nor an apparent effect on ooplasmic segregation, cell division, or formation of the embryonic axis. Moreover, injecting dibromo-BAPTA into the yolk vacuole had no apparent effect on the embryos.

In contrast to the pronounced effects of ≥2.6 m*M* dibromo-BAPTA on segregation, the effects of 2.6 m*M* dimethyl-BAPTA were slight or apparently absent. As

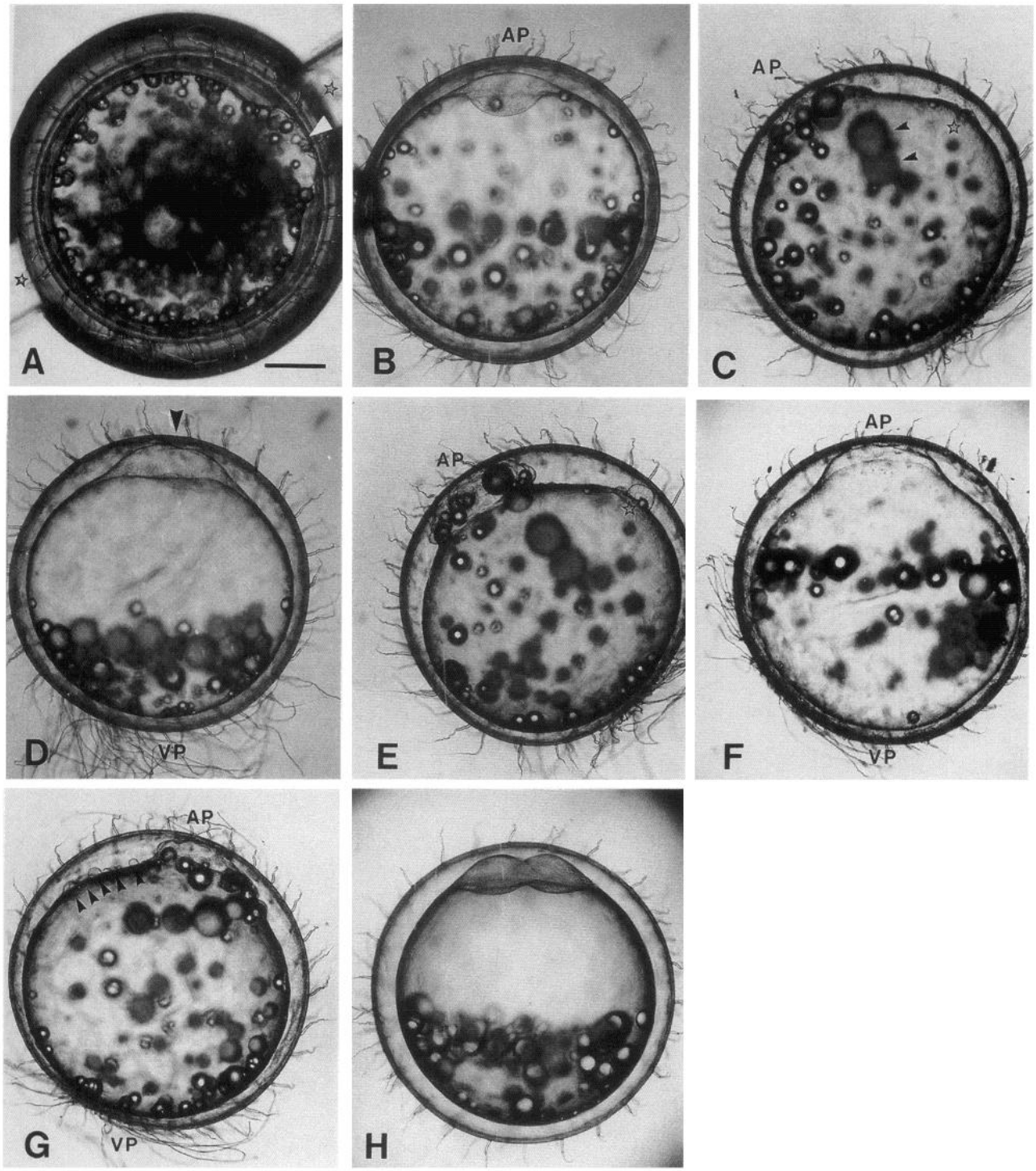

Figure 2. Effect of dibromo-BAPTA on segregation and cytokinesis in the medaka egg. Abbreviations: AP, animal pole; VP, vegetal pole. (A) Immediate local response to injection of 2.6 m*M* dibromo-BAPTA (free $[Ca^{2+}] = 0.2\ \mu M$). This photograph was taken 7 min after injecting enough dibromo-BAPTA to achieve a final concentration of 2.6 m*M* in the ooplasm. The egg is still in the injection chamber, the channels of which can be seen at the lower left and upper right (*). Note the bulge (white arrowhead) in the egg near the injection site. The dark mass near the center of the image is an accumulation of oil droplets on the top of the egg, that is, toward the viewer. The bulge eventually subsided, but after 177 min, when an uninjected control egg was at the four-cell stage, this egg had not yet divided. Scale bar, 250 μM. (B) Segregation in an

Table I

Inhibition of ooplasmic segregation and cytokinesis in the medaka egg by the injection of 5,5'-dibromo-BAPTA

[Free Ca^{2+}] μM[a]	[Dibromo-BAPTA] mM[b]	N	Number of embryos			
			Lysed[c]	Segregated normally	Cleaved normally	Formed embryonic axis
0.14	0.5–2.0	6	0	6	6[d]	6
0.14	2.6–3.6	11	1	0	0[e]	0
0.14	4.0–7.0	10	4	0	0	0
0.20	0.5–2.0	5	0	5	5[d]	4
0.20	2.6–3.6	21	7	0	0[e]	0
0.20	4.0–7.0	19	17	0	0	0
0.30	0.5–2.0	5	0	5	5[d]	5
0.30	2.6–3.6	12	6	0	0[e]	0
0.30	4.0–7.0	3	3	0	0	0

[a] Series I, 0.20 μM; Series II, 0.14 μM; Series III, 0.30 μM.
[b] Estimated final ooplasmic concentration. See text.
[c] The yolk membrane of these embryos lysed within 10 min after injection of the buffer.
[d] The cleavage appeared normal and occurred within 15 min after cleavage began in control eggs.
[e] If an egg did not divide within 40 min after cleavage began in control eggs, we classified it as "not dividing."

already noted, injection of this buffer produced a transient, small bulge at the injection site and caused a few oil droplets near the injection site to float to the top of the egg. However, ooplasmic segregation—both the formation of the blastodisc and the movement of oil droplets toward the vegetal pole—proceeded in a manner nearly indistinguishable from that in uninjected control eggs or eggs into which we injected KCl or K_2SO_4 (Fig. 2B, D). Of the 10 eggs receiving 2.6 mM dimethyl-BAPTA, all 10 cleaved, 7 of them dividing within 10 min (at 21°C) after the uninjected control eggs divided (Fig. 2D). Nine of these embryos went on

egg receiving 2.6 mM 5,5'-dimethyl-BAPTA, t_n = 0.65. The buffer was injected near the equator. The blastodisc that has begun to form at the animal pole is indistinguishable in its shape and size from the blastodisc in uninjected control eggs (Abraham *et al.*, 1993a) and eggs into which we injected KCl or K_2SO_4. All oil droplets but one have moved out of the blastodisc and toward the vegetal pole; the animal hemisphere is relatively devoid of oil droplets, which have moved toward the vegetal pole and formed a prominent ring just below the equator of the egg. (C) Effect of 2.7 mM dibromo-BAPTA (free [Ca^{2+}] = 0.14 μM) on oil droplet movement and formation of the blastodisc, t_n = 0.62. This egg is about the same age as the one shown in (B); dibromo-BAPTA was injected near the equator. Note the large number of oil droplets in the blastodisc at the animal pole of the egg, the large number of oil droplets in the animal hemisphere of the half of the egg opposite the injection site (which is marked by a star), and the absence of a ring of oil droplets near the equator of the egg. Oil droplets that were originally near the injection site have floated to the top of the egg and coalesced into two large oil droplets (arrowheads); this early effect of the buffer provided a convenient marker of the injection site. (D) Segregation and cytokinesis in an egg receiving 2.6 mM 5,5'-dimethyl-BAPTA, t_n = 1.11. This egg is nearly the same age as those shown in (E) and (F). Note the nascent cleavage furrow in the blastodisc (arrowhead) and the apparently normal accumulation of oil droplets near the vegetal pole. This egg was indistinguishable from uninjected control eggs except that it divided about 10 min (at 21°C) later than control eggs. (E) Inhibition of ooplasmic segregation and cytokinesis by 2.7 mM dibromo-BAPTA (free [Ca^{2+}] = 0.14 μM) on segregation, t_n = 1.07. This is the same egg shown in (C). Note the large number of oil droplets near the animal pole, the inhibition of the movement of oil droplets toward the vegetal pole, and the small accumulation of ooplasm near the injection site (*). (F) Inhibition of ooplasmic segregation and cytokinesis by 2.7 mM dibromo-BAPTA (free [Ca^{2+}] = 0.30 μM) on segregation, t_n = 1.16. Dibromo-BAPTA was injected near the vegetal pole of this egg. Note the small blastodisc at the animal pole and the ring of oil droplets near the equator. This egg did not cleave. (G) Accumulations of ooplasm outside the blastodisc, t_n = 1.05. Dibromo-BAPTA (2.7 mM, free [Ca^{2+}] = 0.14 μM, injected near the equator). Note the blebs of ooplasm (arrowheads) on the side of the egg on which the buffer was injected, the presence of oil droplets in the blastodisc, and the inhibition of the movement of oil droplets toward the vegetal pole. (H) Delay of cytokinesis caused by 1 mM dibromo-BAPTA (free [Ca^{2+}] = 0.30 μM), injected near the vegetal pole. The first cleavage has just begun in this egg, which was photographed at t_n = 1.31, a time when sibling control eggs had completed this cleavage. Note the normal appearance of the blastodisc and that all oil droplets have moved close to the vegetal pole.

to form an embryonic axis and appeared to develop normally.

Discussion

The final ooplasmic concentration of dibromo-BAPTA required to block ooplasmic segregation and cell division in the medaka embryo (*ca.* 2.6 m*M*) was comparable to that required to inhibit cell division in fucoid eggs (Speksnijder *et al.*, 1989), inhibit cell cycle contraction waves and cytokinesis in *X. laevis* eggs (Miller *et al.*, 1992a; Miller *et al.*, 1993; Snow and Nuccitelli, 1993), and dissipate cytosolic calcium gradients near the animal and vegetal poles of the medaka egg (Fluck *et al.*, 1992b). According to facilitated diffusion theory, the primary effect of such buffers is to dissipate cytosolic Ca^{2+} gradients by facilitating the diffusion of Ca^{2+} away from zones of elevated cytosolic $[Ca^{2+}]$. Thus, when high calcium regions begin to emerge within a cell, as they do at the animal and vegetal poles of the fertilized medaka egg, buffer molecules pick up Ca^{2+} there and diffuse to low calcium regions, where the bound Ca^{2+} is then released. The results of the present study suggest that these polar zones are necessary for the normal segregation of ooplasm and its inclusions in this egg. However, it is theoretically possible that the inhibition of segregation is caused by either an increase in $[Ca^{2+}]$ at sites away from the poles or the dissipation of the cytosolic calcium gradients.

Facilitated diffusion theory predicts that the Ca^{2+} buffer will be relatively ineffective as a shuttle buffer if its affinity for Ca^{2+} is too high. Such a buffer, for example 5,5'-dimethyl-BAPTA (K_D = 0.15 μM), will become saturated with Ca^{2+} at the high end of the Ca^{2+} gradient but will not release Ca^{2+} when it diffuses away from this region. The results of three studies, which have reported dimethyl-BAPTA to be from 2.7- to 18-fold less effective than dibromo-BAPTA (Speksnijder *et al.*, 1989; Snow and Nuccitelli, 1993; Sullivan *et al.*, 1993), are consistent with this prediction. The results of the present study, that dimethyl-BAPTA had far less effect on ooplasmic segregation and cytokinesis in the medaka than did dibromo-BAPTA, are consistent with these three previous studies and with this prediction of the theory.

Facilitated diffusion theory also predicts that the effect of a shuttle buffer should be independent of $[Ca^{2+}]$ in the injectate (Speksnijder *et al.*, 1989). In other words, the effect of the buffer is not to clamp cytosolic $[Ca^{2+}]$ at any particular value, but rather to facilitate calcium diffusion and thus dissipate calcium gradients in the cytoplasm. Our data are also consistent with this aspect of theory, at least as far as effects on ooplasmic segregation and cytokinesis are concerned. However, we did find that immediate localized responses to the injectate—herniation and subsequent lysis, the movement of oil droplets to the top of the egg—were a function of $[Ca^{2+}]$ in the injectate over the range 0.14–0.30 μM, with the probability of lysis increasing with $[Ca^{2+}]$. Our choice of $[Ca^{2+}]$ in the injectate was based primarily on a study of the medaka egg in which cytosolic $[Ca^{2+}]$ was measured with a calcium microelectrode (Schantz, 1985; see his Table 1). The occurrence of lysis in our study suggests that in the future we should set $[Ca^{2+}]$ in the injectate lower, perhaps to 50 nM or 100 nM, to reduce the probability of embryo lysis.

Two cytoskeletal systems—one involving microfilaments and another involving microtubules—are involved in ooplasmic segregation in the medaka egg. Formation of the blastodisc at the animal pole of the medaka and other teleost embryos is inhibited by cytochalasins (Katow, 1983; Ivanenkov *et al.*, 1987; Webb and Fluck, 1993) and DNase I (Ivanenkov *et al.*, 1987) and thus presumably involves the action of microfilaments, whereas the movement of oil droplets and the saltatory movement of other inclusions toward the animal and vegetal poles of the medaka egg are both inhibited by microtubule poisons—colchicine, colcemid, nocodazole—and thus presumably involve microtubules (Abraham *et al.*, 1993a; Webb and Fluck, 1993). Moreover, a microtubule-organizing center (MTOC) is present in the animal pole region of the medaka egg by t_n = 0.24, and the convergent array of microtubules near this center is disrupted by the injection of dibromo-BAPTA (Abraham *et al.*, 1993b). The assembly/disassembly and the function of both microfilaments and microtubules are controlled by a number of calcium-binding regulatory proteins (Weisenberg, 1972; Schliwa *et al.*, 1981; Keith *et al.*, 1983; Mooseker, 1985; Stossel *et al.*, 1985; Bray, 1992, pp. 147–148, 342). Facilitated diffusion theory predicts that calcium buffer concentrations similar to those found effective in the present study act by dissipating zones of cytosolic $[Ca^{2+}]_{free}$ in the micromolar range (Speksnijder *et al.*, 1989). An example of a protein that is responsive to changes in $[Ca^{2+}]_{free}$ in this range is calmodulin (Cheung, 1980), a protein that regulates the activity of proteins that interact with both microfilaments (Wolenski *et al.*, 1993) and microtubules (Ishikawa *et al.*, 1992).

Oil droplet movement in the animal hemisphere of the egg was more strongly inhibited when buffer was injected near the equator *versus* near the vegetal pole. One interpretation of these results is that structures/events in the animal pole region, for example the MTOC, are essential for oil droplet movement and that these are disrupted by dibromo-BAPTA. Given the circumference of the medaka egg (about 3700 μm), buffer injected near the equator would reach the animal pole of the egg sooner than buffer injected near the vegetal pole. To estimate the time required for dibromo-BAPTA to diffuse around the medaka egg, we used published values for the diffusion coefficients of molecules similar to dibromo-BAPTA (fura-2 in skeletal muscle, 3.6×10^{-7} cm^2 s^{-1}, Baylor and Hollingworth, 1988) and fluorescein (BCECF in fibroblasts, 2

$\times 10^{-6}$ cm^2 s^{-1}, Kao *et al.*, 1993). Assuming that the cytoplasm of the medaka egg is more similar to the cytoplasm of a fibroblast than to that of a skeletal muscle, we corrected the diffusion constant of fura-2 upward to 5.9 $\times 10^{-7}$ cm^2 s^{-1} (Kushmerick and Podolsky, 1969; Kao *et al.*, 1993) and then estimated the times required for molecules with these diffusion coefficients to diffuse around the egg (Einstein, 1956). The predicted values for BCECF were consistent with the rate of movement of fluorescein around the egg (Fig. 1), suggesting that our assumptions are valid. We estimate that the times required for dibromo-BAPTA to diffuse 90° and 180° arc from the injection site would thus be about 35 min and >3 h, respectively. In other words, buffer injected near the equator of an egg would reach the animal pole while segregation of the oil droplets was ongoing, but buffer injected near the vegetal pole would not.

The medaka egg appears to be a particularly good system in which to investigate the importance of cytosolic calcium gradients in ooplasmic segregation and the cytoplasmic localization of morphogenetic determinants in teleost embryos. If a fate map exists during early cleavage of the medaka embryo as it does in *Brachydanio rerio* embryo (Strehlow and Gilbert, 1993), this would suggest that particular cytoplasmic determinants might be found along particular meridians of the egg. If these determinants streamed into specific regions of the blastodisc during ooplasmic segregation, they would eventually segregate into particular blastomeres during cleavage. The geometry of the medaka egg makes it possible to pursue this problem by selectively interfering with segregation along particular meridians. Such interference could perhaps be achieved by the localized photolysis of caged calcium or caged calcium buffers that have been injected into the ooplasm (McCray and Trentham, 1989; Ashley *et al.*, 1991a, b).

The delay and inhibition of cytokinesis in eggs receiving dibromo-BAPTA, though suggestive and consistent with the results of other studies (Speksnijder *et al.*, 1989; Snow and Nuccitelli, 1993; Miller *et al.*, 1993), are difficult to interpret. Because the time of injection was not proximal enough to specific events accompanying cytokinesis—formation of the contractile band, formation of the furrow, zipping of the furrow, *etc.* (Fluck *et al.*, 1991)—the effect on cytokinesis could be indirect, caused by the inhibition of earlier events that are accompanied by a calcium pulse and which are themselves inhibited by BAPTA-type buffers. These events include nuclear envelope breakdown (Steinhardt and Alderton, 1988; Tombes *et al.*, 1992; Browne *et al.*, 1992) and the metaphase/anaphase transition (reviewed in Hepler, 1989). To determine whether dibromo-BAPTA specifically affects cytokinesis in the medaka egg, buffer injections must be timed much closer to cytokinesis. Such an approach has been successful in *X. laevis* (Snow and Nuccitelli, 1993; Miller *et al.*, 1993), and preliminary experiments (Abraham and Fluck, unpublished) suggest that it will be successful in the medaka as well.

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