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Calcium buffer injections inhibit cytokinesis in *Xenopus* eggs

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SUMMARY

A slow cortical wave of high calcium accompanies the elongation of cleavage furrows in medaka fish eggs as well as in *Xenopus* eggs. We explored the role of such waves by injecting calcium buffers into *Xenopus* eggs at various times before and during first and second cleavage. Injection earlier than about 15 minutes before first cleavage normally starts delays it for hours. Injection between about 15 minutes and a few minutes before cleavage normally starts allows a (short) furrow to form on time but usually yields an eccentric one. This forms away from the injection side, often as far off-center as the egg's equator, and then regresses. Injection soon after it starts quickly arrests elongation of the furrow and eventually induces its regression; while injection a bit later likewise soon arrests elongation but allows delocalized furrow deepening to continue. The dependence of these inhibitory actions upon the dissociation constants and final cytosolic concentrations of the injected buffers indicates that they act as shuttle buffers to suppress needed zones of high calcium in the micromolar range. We conclude that the high calcium that is found within these furrows is needed to induce them, to extend them and even to maintain them.

Moreover, while short, eccentric furrows often form as far off center as the equator, they somehow always form along a meridian through the animal pole. This seems difficult to explain by the orthodox, diastral model. Rather, it suggests that the cleavage furrows in *Xenopus* - and perhaps in animal cells quite generally - are directly induced by a diastema or telophase disc rather than by the asters.

Key words: calcium waves, cytokinesis, shuttle buffers, telophase disc

INTRODUCTION

The uncertain relationships of free calcium patterns to cytokinesis have been critically reviewed by Hepler (1989), considered by several investigators in a recent symposium volume (Conrad and Schroeder, 1990) and most recently reviewed by Satterwhite and Pollard (1992). As the latter have pointed out, the visualization of high free calcium within the advancing cleavage furrows of medaka fish eggs (see Fluck et al., 1991) has finally provided visual evidence of a direct role for calcium in cytokinesis. Moreover, retrospective evidence that such calcium waves act back to favor furrowing lies in older observations that applications of a calcium ionophore speed the cleavage of squid eggs and that applications of caffeine can speed the cleavage of snail eggs (Arnold, 1975; Conrad et al., 1987). However, attempts to inhibit furrowing with injected calcium buffers have given ambiguous results: both Baker and Warner (1972) and more recently Vincent et al. (1987, p. 149) have reported the effects of injecting *Xenopus* eggs with EGTA. They both found that it takes a final cytosolic concentration of what we now estimate to have been about 30 millimolar unbound EGTA to half-inhibit cleavage - a concentration so high that it might even be considered as evidence *against* a needed high-calcium zone.

On the other hand, a recent study of the effects of calcium buffer injections on fucoid eggs indicates that EGTA injections are so ineffective only because EGTA - with a K_D, or calcium dissociation constant, of 0.01 μM within *Xenopus* eggs - is too strong a buffer. Living cells turn over cytosolic calcium so rapidly that even quite high concentrations of such a strong buffer are soon largely saturated with calcium flowing into the cytosol from inner stores or from the medium. Thereafter, EGTA would be expected to sit in the cytosol - bound, inert and impotent to affect the cell's physiology (Speksnijder et al., 1989).

Injections of weaker buffers, on the other hand, permanently block the division of fucoid eggs at final cytosolic concentrations as low as 1 mM. They do so by suppressing the development of localized regions of high calcium, which are somehow needed for cytokinesis. When such a high-calcium region begins to emerge, buffer molecules pick up calcium there and diffuse to a low-calcium region where the bound calcium is then released. They can then repeatedly shuttle back and forth from high-to-low calcium regions and in this way facilitate the diffusion of calcium down a concentration gradient and thus suppress the emergence of a high-calcium region. In brief, they act as what we will call *shuttle buffers*.

This process is most efficient when the calcium dissoci-

ation constant, or K_D, of the buffer approximates the needed level of raised local calcium. For with this match between its K_D and the needed calcium level, the buffer can efficiently pick up *and release* calcium. It is in this way that injections of dibromo BAPTA (which has a K_D of about 5 μM at the ionic strength of a marine egg) can in fact block cytokinesis in fucoid eggs at final cytosolic concentrations of only about 1 mM.

With these ideas in mind, we have explored the effects of injecting *Xenopus* eggs with dibromo BAPTA (1,2-bis(*o*-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid) as well as with other BAPTA-type buffers that have both lower and higher K_D values. In a preliminary study, we found that when dibromo BAPTA is injected long before first cleavage, that it detectably delays cleavage at final cytosolic concentrations of only 0.1 mM and delays it for about 3 hours at only 0.6 mM. Thus it delays cleavage at concentrations that are 1/300 to 1/50 of those reported for EGTA and comparable to those found effective in fucoid eggs (McLaughlin et al., 1991). Here we report the effects of such injections made after first as well as second furrowing has begun. We find that they soon suppress and eventually induce regression of both first and second furrowing in *Xenopus* eggs at buffer concentrations and buffer K_D values that are comparable to those that are found in fucoid eggs. Moreover, Snow and Nuccitelli (1993) report a remarkably similar dependence of the inhibition of later *Xenopus* cleavages upon injected buffer concentrations and K_D values. These new results also support a model of cytokinesis in which a local rise in free calcium is part of the cycle that lengthens growing cleavage furrows.

We also explored the effects of making such injections somewhat before first cleavage starts. We discovered that those made between about 5 and 20 minutes before cleavage starts usually induce an eccentric furrow to start with. Such furrows are shifted away from the injection side and well away from the usual site of furrow initiation at the animal pole. This indicates that furrow initiation as well as elongation requires a local rise in cortical calcium.

While such eccentric furrows often form as far as 700 μm away from the spindle, they nevertheless always do so along a meridian through the animal pole and do so with little delay. This observation has induced us to question the widespread idea that the centrosomal mictrotubules somehow directly position the cleavage furrows. Instead, we offer arguments for an intermediate between these tubules and the cortex: namely, a growing extension of the metaphase plate called the diastema or telophase disc. The metaphase plate, of course, is in turn positioned by the interaction of those centrosomal tubules that make up the spindle.

MATERIALS AND METHODS

Biological material

Eggs were stripped from the cloacas of female *Xenopus laevis* frogs (obtained from Carolina Biological Supply Co., Burlington, NC 27215) that had been injected the previous day with 500 i.u. of human chorionic gonadotropin (Sigma CG-10). Testes were dissected from a male frog the same day or up to 6 days earlier and stored at 5ºC for use as needed. Slices were macerated in 0.5 ml of an 'R33' or 33% Gerhart solution, which contained 33 mM NaCl, 0.67 mM KCl, 0.67 mM $CaCl_2{\cdot}2H_2O$ and 0.33 mM $MgCl_2{\cdot}6H_2O$, with addition of $NaHCO_3$ to pH7.4 (Gimlich and Gerhart, 1984). Sperm and eggs were mixed at room temperature (20°C) and allowed to stand for 10 minutes. The time of mixing was regarded as being the insemination time. Eggs were then dejellied by swirling them for 3-5 minutes in an R33 solution containing 200 mM cysteine (Sigma C-7755) at pH 8.0. The eggs were then transferred to a modified version of Hollinger and Corton's (1980) artificial pond water (our F1: 41.3 mM NaCl, 1.75 mM KCl, 0.5 mM Na $H_2PO_4{\cdot}H_2O$, 2.0 mM NaOH, 2.5 mM HEPES, 0.25 mM $CaCl_2{\cdot}2H_2O$, 0.06 mM $MgCl_2{\cdot}6H_2O$ adjusted to pH 7.8 with KOH) via washing through a series of R33/F1 solutions (80/20, 60/40, 40/60, 20/80) with two washes with 100% R33 at the beginning and two washes with 100% F1 at the end.

Buffer solutions injected

Four BAPTA-type buffers were used in this study: 5,5′-dimethyl BAPTA, 5,5′-dibromo BAPTA, 5-mononitro BAPTA and BAPTA itself. These buffers have K_D values in the range of 0.15 μM to 40 μM (Table 1). Buffers were made up in ultra-pure water with 5 mM HEPES set at pH 7.0. The K^+ level in the injectate was kept at 200 mM wherever possible. As the BAPTA-type buffers used were tetrapotassium salts, and in most cases the buffer injectate concentration was 50 mM, this was a simple matter. When lower buffer injectate concentrations were used, KCl was added to bring the injectate [K^+] up to 200 mM. The same (7 to 8 nl) volume was always injected so the desired final cytosolic concentration of each buffer was achieved by altering the concentration of buffer within the injectate. However, in order to reach inhibitory concentrations of dimethyl BAPTA while keeping the injection volume the same, an injectate concentration of 100 mM buffer had to be used, which unavoidably doubled its K^+ level.

In most experiments (see Results) we added enough $CaCl_2$ to each buffer to set its free Ca^{2+} at 0.4 μM, which is known to be the resting level in *Xenopus* zygotes (Rink et al., 1980; Busa and Nuccitelli, 1985). The buffers used, along with their K_D values and Ca/B ratios, are given in Table 1. For the purpose of comparison with the results of Vincent et al. (1987), we also list the K_D of EGTA. In order to determine what concentration of buffer to inject in order to achieve a desired final cytosolic buffer concentration, the volume of each *Xenopus* egg was assumed to be 900 nl from an egg diameter of ≈1200 μm, 30% of the egg was assumed to be cytosol (Kline, 1988) and 70% of the cytosol assumed to be available water (de Laat et al., 1974).

Microinjection of buffers

The micropipettes used for microinjection were pulled from 1.0 mm diameter borosilicate glass capillaries with filling fibers (Medical Systems Corp PLI-CFS, Greenvale, NY 11548) using a

Table l. Calcium buffer values

Buffer	K_D (μM)*	Ca/B
EGTA	0.01	≈0
5,5′-dimethyl BAPTA	0.15	0.73
BAPTA	0.21	0.66
5,5′-dibromo BAPTA	**1.50**	**0.21**
5-mononitro BAPTA	40.00	0.01

*From Pethig et al. (1989) for BAPTA-type buffers in 100 mM KCl and thus at approximately the ionic strength of a fresh water egg like that of *Xenopus.* From the graphs of Campbell (1983, pp. 79-80) for EGTA in a medium of pH 7.7, which is taken to be the pH of cleaving *Xenopus* eggs from the data of Webb and Nuccitelli (1982). Moreover, Rink et al. (1980) also give 0.01 μM as the K_D of EGTA within a *Xenopus* zygote.

Narishige PN-3 horizontal puller (Narishige Scientific Instruments, Tokyo, Japan). These were then given a single 25° bevel using a Narishige EG-4 beveller. The EG-4 was modified by connecting the pipette holder to a simple audio amplifier/headphone arrangement via a phonograph stylus. This arrangement provided an accurate means of determining when the unbevelled pipette tip made contact with the grinding surface of the beveller. These procedures resulted in micropipettes of a consistent tip shape and size (3-5 μm in diameter at the widest part of the bevel), which did minimal damage to eggs throughout experimentation. Micropipettes were back-filled with buffer solution using a microliter syringe (Hamilton 701-N, Hamilton Co., Reno, NV 89520).

In the case of injections made well before the appearance of the furrow, no attempt was made to relate the injection site to any other physical feature of the egg (i.e. gray crescent, sperm entry point etc.) However, when eggs were injected after the appearance of the first furrow, the injections were either at right angles to or along the future plane of furrowing. In all cases, eggs were immersed in a modified F1 solution during injection. This sometimes contained 5% Ficoll (Ficoll 70, Pharmacia Fine Chemicals), which helped to prevent leakage from the egg during injection. About 5 minutes after injection, an egg was returned to normal F1 for observation.

The 900 nl eggs were injected with 7 to 8 nl of fluid using a Medical Systems Corp PLI-100 high-pressure injection system (Medical Systems Corp, Greenvale, NY 11548) in combination with a Leitz micromanipulator and Zeiss SV-11 stereoscope. For each experiment micropipettes were calibrated by injecting buffer under Wesson vegetable oil and measuring droplet diameters with an eye-piece reticule. Injection pressure was kept at a constant 15 lbf/in^2 (1 $lbf/in^2 \approx 6.9$ kPa) and the duration of the pressure burst varied from about 1 to 2 seconds so as to deliver the desired volume. Pipettes were checked before, during and after each series of injections. This eliminated confusing results caused by pipette clogging. Prior to injection, up to 60 eggs were placed in the lid of a 35 mm diameter Petri dish (Falcon 1008) and 20 or so were positioned in a row against a back-stop cut from a standard 1.2 mm thick microscope slide. Pipettes were inserted axially into eggs at an angle of 15° from the horizontal. The point of entry was just above the equator, in the pigmented animal hemisphere. The tip of the pipette was inserted to a depth that was approximately half-way between the surface and center of the egg. From these facts, the injection point was estimated to lie approximately within the equatorial plane and about half-way between each egg's surface and its center. This was later confirmed by observations of fixed sections (see Fig. 8, below).

Determining the injectates' spread

The time course of the injectates' spread was estimated from experiments in which eggs were injected in a similar way with a 3,000 M_r, fluorescein-labelled dextran (Molecular Probes' D-3305; 1% in distilled water), fixed at various times after injection and eventually embedded and sectioned. We resorted to fixing and sectioning because patterns of internal fluorescence cannot be clearly seen in living, normally pigmented *Xenopus* eggs. Eggs were prepared for sectioning by a methanol freeze-substitution procedure modified from that of Fraser and Bronner-Fraser (1991). We used this procedure, together with a fluorescein-labelled dextran marker, because it was known to preserve the position of this marker within the cytosol.

In order to determine the plane of fluorescein-dextran injection and hence the desired planes of later sectioning, each egg was first firmly attached near one end of its own (5 mm × 9 mm) tab of Whatman no.41 filter paper, injection was given from this end and subsequent fixation, embedding and sectioning were all done with the egg still firmly attached to its tab. Firm attachments were attained by forming a shallow depression in each wet tab with a round-tipped 1 mm diameter glass rod, then coating each depression with 'Cel Tak' (a biocompatible, adhesive protein obtained from a marine mussel and sold by Biopolymers Inc., Farmington, CT) by pipetting in 0.5 μl of a Cel Tak solution, air drying it, dipping the tab in 100% ethanol and again air drying it. Then at least 10 minutes before injection, these tabs were rewet, and the *Xenopus* eggs gently pressed into their sticky cups, vegetal pole down. Right after injecting each egg with fluorescein-dextran, its tab was drained by placing it tab side down on a piece of dry filter paper. This tab was then dipped into cold, liquid isopentane (at −160°C via liquid nitrogen) for ten seconds, transferred to cold, liquid 100% methanol (at −80°C via dry ice), then kept at −80°C for 3 days in a freezer, shifted to −20°C for another 3 days and finally to 4°C for 1 to 7 days, changing the methanol with each of the latter two shifts in temperature. The eggs were embedded in Epon before making 20 μm sections parallel to the injection plane. Sections were then immersed in Permount (Fisher SP-15) and covered with a slip for examination and photography.

Observing cytokinesis and relating it to the time of injection

Eggs were observed with a Zeiss SV-11 stereoscope equipped for photomicroscopy. Black and white 35 mm photographs (using Kodak Tri-X pan 400 ASA film) were taken using a Zeiss MC 100 camera attachment and control unit. Time-lapse video records were also taken using a Dage/MTI CCD-72 camera and control unit combined with a JVC SR-9000U recorder. Appropriate lighting was provided by fiber optics (Dolan-Jenner, Fiber-Lite Series 180).

In the experiments where eggs were injected after the appearance of the furrow at the surface, relating the time of injection to that of cytokinesis was simply a matter of noting the physical appearance of the furrow at the egg surface at the time of injection. However, in studies on the effects of injecting buffers before furrowing began - while we were necessarily guided during injection by the time after insemination - we ultimately related the time of injection to the time when half of the uninjected control eggs (from the same insemination batch) showed the first clear signs of furrowing and were thus in the single stripe stage. This allowed us to compare the effects of injecting buffers into different batches of eggs, from different frogs.

We also noted considerable variation among egg batches in the time after insemination when half the eggs initiated cleavage. Most of this could clearly be attributed to small changes in the temperature on the laboratory bench in the injection room where the eggs developed (see Fig. 1), while up to 5-10 minutes of it might

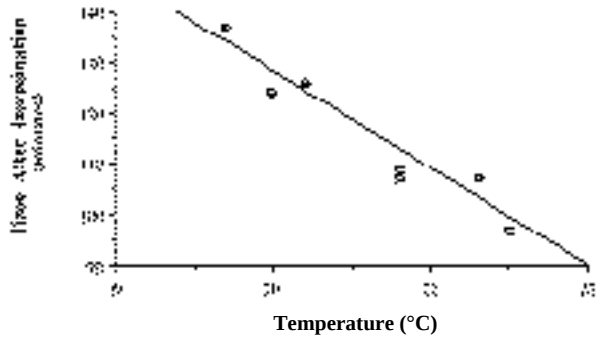

Fig. 1. 50% cleavage time as a function of temperature in *Xenopus*. Each symbol represents a separate experiment with eggs from a separate frog. The slope indicates a Q_{10} of about twofold per 10deg. C.

have resulted from variable delays between insemination and fertilization (R. Nuccitelli, unpublished).

RESULTS

Injections during first cleavage

Cleaving eggs were injected with various buffers at the single stripe, double stripe and just completed groove stages of Singal and Sanders (1974). The single stripe stage marks the very beginning of externally visible cleavage and is seen as a fine black line (formed by a row of pigment granules) that is about a third of an egg diameter or half a millimeter long. One to two minutes after this line forms, it can be seen to split into two parallel lines, which mark the double stripe stage shown in Fig. 5A (below). The latter lasts for a few minutes until a broader groove is seen to form. The point at which this elongating groove first reaches the vegetal pole, which is about half an hour later, marks the just completed groove stage.

Responses to the most effective buffer (dibromo BAPTA) at its just effective final concentration of 1.5 mM (and mononitro BAPTA at its just effective 4 mM)

In all 17 eggs that were injected with dibromo BAPTA at the *single stripe stage*, the furrows stopped growing and regressed, to leave nothing but some curious pigment patterns. When the injection was nearly equidistant from a furrow's two growing ends, both stopped lengthening and deepening within 5 to 10 minutes as shown in Fig. 2A and then regressed as shown in Fig. 2B. When the injection was directly ahead of one end, this closer end soon stopped growing. However, in this case, the further one continued to grow for 30 to 60 minutes before it too finally stopped and regressed as shown in Fig. 2C and D.

In all 21 eggs that were injected with dibromo BAPTA in the *double streak* stage, the furrows likewise stopped growing and then regressed to leave nothing but odd pigment patterns. Their elongation soon stopped abruptly, but in this case they continued to deepen in an abnormal, characteristic way. This deepening was not restricted to the narrow plane that cuts a control egg in two, but rather forms a wide shallow trough with white, unpigmented sides and floor. Two characteristic, 'pigmented cones' form on either side of this trough and fine, deeply pigmented, finger-like structures often protrude from the cones' inner surfaces (Fig. 3A and B). These 'fingers' resemble the stretch-induced folds that appear at the animal pole when a control furrow begins to deepen. Such folds may normally contribute to the new membrane that forms between the daughter cells. However, the lack of localized deepening in buffer-injected eggs may result in their continued appearance late in the disrupted cytokinetic cycle. Altogether, while buffer injected at the single stripe stage completely blocked furrow deepening, it seemed to delocalize rather

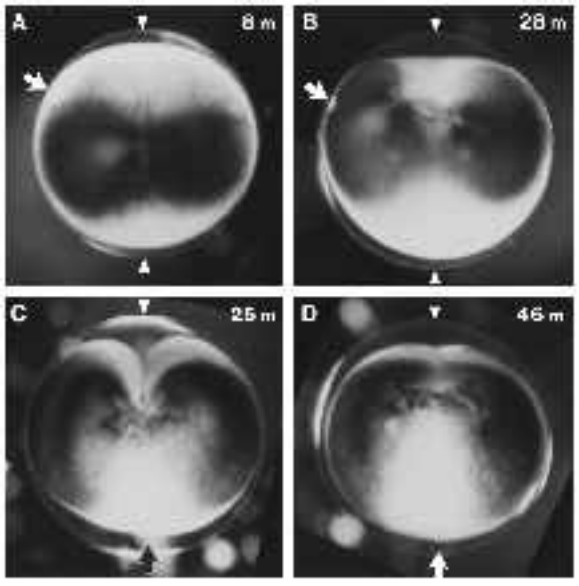

Fig. 2. Eggs injected at the single stripe stage with dibromo BAPTA to reach a final cytosolic concentration of 1.5 mM. These illustrate the arrest and regression of furrowing at the times shown after injection (m, minutes). (A) and (B) show an egg injected roughly at right angles to the cleavage plane; (C) and (D) along this plane. The arrows indicate the injection hole and the arrowheads the plane of the first furrow. The residual flattening seen in (B) is a common feature, which is not understood. This and later micrographs of live eggs are all shown from the animal pole. The scale can be obtained from the eggs' 1200 μm diameter.

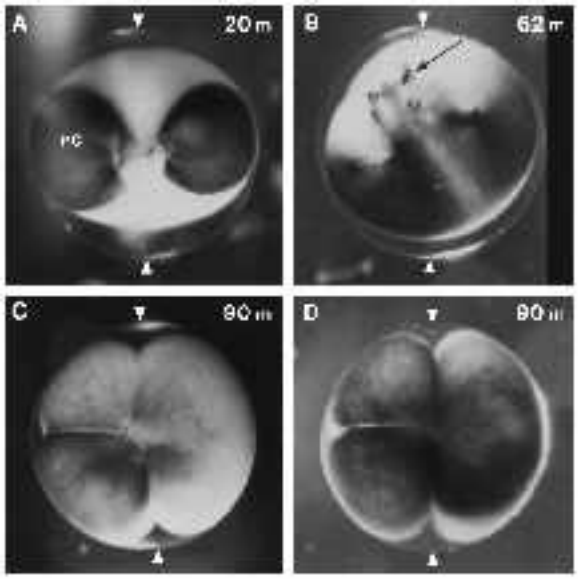

Fig. 3. Eggs injected at the double stripe stage. The eggs in (A) and (B) were injected with dibromo BAPTA to reach a final cytosolic concentration of 1.5 mM; (C) and (D) with the stronger and weaker buffers BAPTA and mononitro BAPTA, respectively, both reach a final cytosolic concentration of 1.5 mM. The right-hand cells in (C) and (D) cleaved soon after the the photographs were taken. As before, the injection holes are shown by large white arrows and the first cleavage plane by small ones. PC in A shows one of the 'pigmented cones' that develop on either side of a delocalized furrow. The presence of unpigmented membrane in the furrow floor and walls is also clear. The black arrow in (B) shows the fine, pigmented projections that are often left behind when a furrow regresses. The times are postinjection (m, minutes).

than inhibit the contractile process in double stripe-injected eggs.

Nine eggs were injected with dibromo BAPTA at the *just completed groove stage*. Although the leading edges of the furrow have reached the vegetal pole at this stage, the deepening of the furrow is by no means complete and one would expect that at the time of injection there would be a degree of cytoplasmic continuity between the two cells especially in the vegetal hemisphere. Following buffer injection, the deepening of the furrow through shortening of the contractile band continues to completion, separating the two daughter cells. The apposition or 'zipping together' of the two daughter cells is delayed by the buffer, but not irreversibly inhibited. Subsequent cell divisions are inhibited in the daughter cell formed on the injected side but the opposite daughter divides quite normally.

Eggs injected with *mononitro to reach 4 mM at both stripe stages* gave similar responses to dibromo-injected ones. In all 25 cases, the progression of the furrow across the egg was arrested. Once arrested, a furrow was never seen to recover and continue its progression around the egg circumference. A difference, however, was recorded with regard to the effects on deepening of the furrow when eggs were injected at the double stripe stage. Like the furrows of eggs injected at the single stripe stage, these did not deepen.

Responses of eggs injected with dibromo BAPTA to reach 0.6 mM

Eggs injected during both stripe stages showed normal, undelayed first divisions. Furrow progression was not arrested, furrows deepened normally, and the resulting daughter cells zipped together like uninjected controls. Marginal effects of the buffer were seen in less than half of the daughter cells that arose from the injected side of eggs, while daughter cells from the uninjected sides of eggs showed no effects of the buffer. The former showed a few minutes inhibition of second cleavage, compared to daughter cells from the uninjected side of eggs (as well as uninjected control eggs). Nevertheless, such eggs went on to develop normally.

Responses of eggs injected with mononitro BAPTA and BAPTA to reach 1.5 mM

Both the stronger buffer (BAPTA, K_D=0.21) and the weaker one (mononitro BAPTA, K_D=40) have no effect on the first cell division when injected to a final cytosolic concentration of 1.5 mM at either stripe stage. Progression and deepening of the first furrow were unaffected and the two daughter cells zipped up like uninjected controls. BAPTA had a marginal effect on the second division of about half of the daughter cells arising from the injected side of eggs, delaying division for up to about 10 minutes as compared to the opposite daughter cells (Fig. 3C) as well as uninjected controls. Mononitro BAPTA occasionally had a similar marginal effect on the second division of daughter cells arising from the injected side of the egg (Fig. 3D). Both the mononitro BAPTA- and BAPTA-injected eggs went on to develop normally.

Injections before cleavage has begun

Eggs injected before the first precleavage transition

In the first study, summarized in Table 2, 404 eggs were successfully injected with various buffers at various times between 0.2 and 0.6 of the first cell cycle and we focussed upon the resultant delay in furrowing rather than its location. These delays depend upon the final cytosolic concentration and K_D in a manner that again indicates that the injectates act as shuttle buffers to suppress normal local rises in free calcium in the micromolar range. When

Table 2. Inhibition of the *Xenopus* egg's first cleavage by injecting BAPTA-type calcium buffers between 0.2 and 0.6 of the time to cleavage

BAPTA buffer concentration* (mM)	*n* injected	Average first cleavage delay (minutes)	Death (hours)	K_D (μM) in 100 mM KCl
5,5′-Dibromo				1.5
2.0-6.0	40	180	≈ 6	
0.8-3.3	69	100	≤ 24	
0.2-1.4	40	25	None	
0.1-0.5	120	7	None	
0.01-0.2	50	No effect	None	
5,5′-Dimethyl				0.15
7.9-26.7	10	No cleavage	≈ 1	
4.1-13.8	9	No cleavage	≈ 4	
2.1-7.1	8	133	≈ 3.5	
0.6-2.0	10	No effect	None	
5-Mononitro				40.0
4.8-19.9	19	No cleavage	≈ 6	
3.9-13.7	13	237	≤ 20	
2.1-7.1	7	13	≤ 12	
0.4-1.2	9	No effect	None	

Eggs were co-injected with 0.1 mole of Ca^{2+} per mole of buffer for dibromo and mononitro, and 0.7 mole of Ca^{2+} per mole of buffer for dimethyl BAPTA; Done at 15°C.

*The lower value in each range is the final cytosolic concentration, assuming that about 21% of the egg's 900 nl volume is accessible to the injected buffer, in turn calculated by taking 70% of the egg volume as inaccessible yolk platelets and other organelles (Kline, 1988) and about 70% of the cytosol as accessible water (de Laat et al., 1974). The higher value in each range takes into account the likelihood that at the time of action, the buffer had diffused out to a sphere about 800 μm in diameter instead of filling the whole 1200 μm diameter egg.

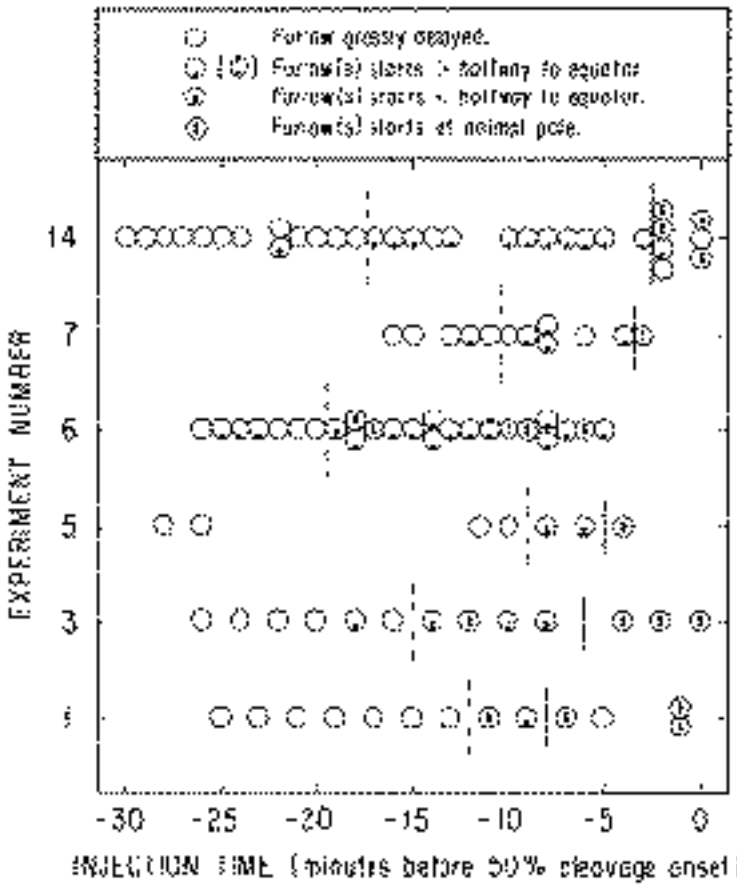

Fig. 4. The loci of furrowing in eggs injected with dibromo BAPTA (to reach 1.5 mM) at various times before cleavage starts. Each circle represents a successful injection. The broken lines show the first precleavage transitions, i.e. the times before which few eggs cleave; the continuous lines show the second precleavage transitions, i.e. the times after which most form furrows at the animal pole. Between these times, most eggs formed more or less eccentric furrows as indicated by the symbols in the key above.

dibromo BAPTA - a buffer with the optimal or near optimal K_D of 1.5 μM - is injected to reach a final cytosolic concentration of about 1 mM or more, cleavage is delayed for hours. This delay is so great that when cleavage eventually occurs we take it to indicate loss of buffer from the cytosol rather than true adaptation. One reason for this supposition is that BAPTA-type buffers with fluorescent side groups - the fura-type dyes used to report the presence of free calcium - are well known to leave the cytosol of a variety of cells in comparable times (Read et al., 1992).

In the second study, summarized in Fig. 4, 102 eggs were successfully injected to reach 1.5 mM dibromo BAPTA at times between about 0.7 and 1.0 of the first cell cycle. In these eggs, we focussed upon the locus of initial furrowing rather than the delay before it. If none was seen within about half an hour after cleavage normally started in that batch, we refer to such eggs as 'furrow grossly delayed' as they were sometimes seen to undergo very delayed cleavage.

There is a distinct transition from this grossly delayed cleavage to abortive cleavage, which occurs at about 10 to 20 minutes before half the eggs start cleaving. As a fraction of the time (from insemination) to 50% cleavage, this first transition occurred at 0.88 ± 0.02 (s.d.m.) of the first cell cycle. We term this distinct change in the response to buffer injection the first precleavage transition.

Eggs injected after the second precleavage transition

Eggs injected after what we will term the second precleavage transition generally form an abortive furrow at the animal pole. This second transition occurs at about 3 to 6 minutes before half the eggs start cleaving and thus at about 0.96 of the first cell cycle. This response is similar or identical to those elicited by injections made at the single stripe stage just after furrowing begins.

Eggs injected between the first and second precleavage transitions

However, eggs that are injected between these two transitions, respond in an unexpected and remarkable way. They generally form an abortive furrow, which is shifted away from the normal site of furrow initiation at the animal pole (Fig. 5A). In extreme cases, such eccentric furrows may even be centered on an egg's equator (Fig. 5B-D). In a few cases, such eggs actually form *two* eccentric furrows on opposite equatorial sides of the egg (Fig. 5E-F). In all cases where the relationship could be determined, eccentric furrows were seen to have formed away from the injection side (Fig. 5B-D). In general, the degree of shift from the animal pole was related to how soon after the first precleavage transition the buffer was injected. Furrows that were shifted all the way to the equator tended to result from injections made soon after the first transition, while later injections tended to induce smaller shifts.

We also observed the angle between each eccentric furrow and the meridian (or great circle) that traversed this furrows' center. In all 47 cases where this angle could be determined, the (eccentric) furrow lay along such a merid-

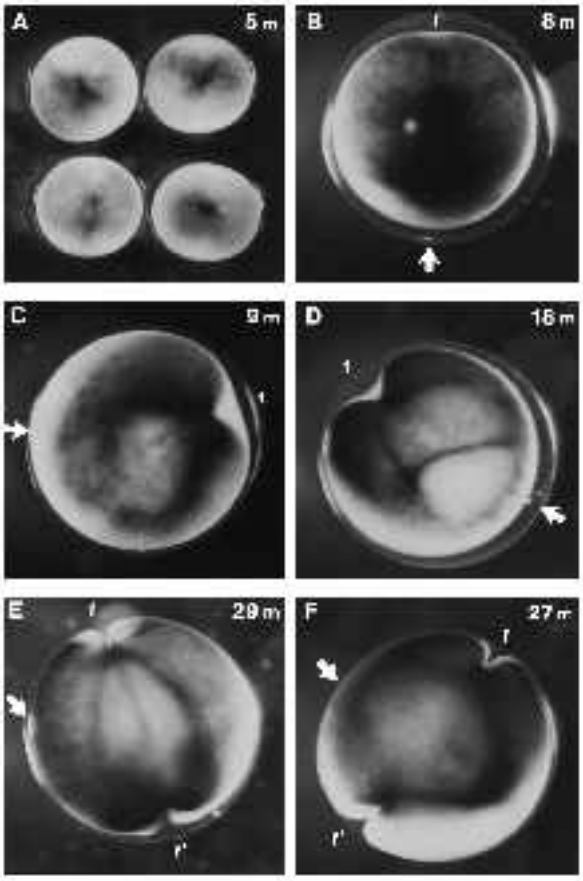

Fig. 5. The shift of furrowing towards the equator by buffer injections between about 15 and 5 minutes before cleavage starts. (A) shows four uninjected control eggs at the double stripe stage. These normal first furrows are seen to start at the animal pole. (B-D) show eggs in which a single equatorial furrow (f) has formed. Such equatorial furrows always start away from the injection site (shown by an arrow) and always lie on a meridian or great circle that goes through an egg's poles. (E) and (F) show two eggs that formed two equatorial furrows, f and f'. Note that these pairs lie along the same meridian. The times are postinjection m, minutes.

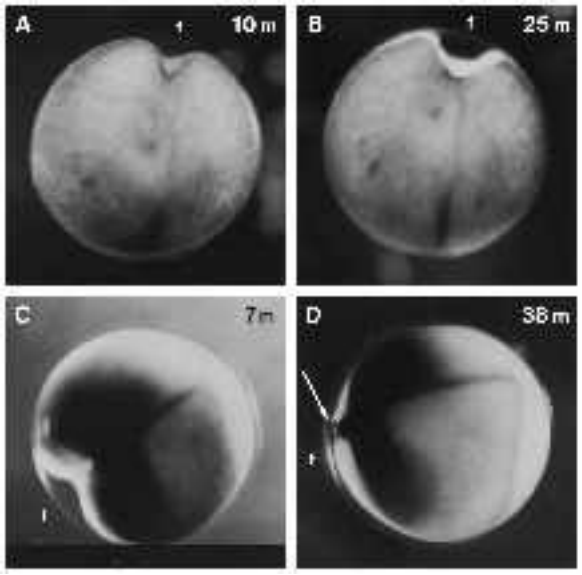

Fig. 6. The fates of equatorial furrows (f). (A) plus (B), and (C) plus (D), show these in two representative eggs at two different times after injection. Such furrows do not extend much beyond their initial lengths and scarcely deepen. Eventually, they regress so as to return the egg to a nearly spherical shape as shown in (D). Occasionally, black fingers (shown by an arrow in D) remain as vestiges of the former furrow.

ian. In those cases where two eccentric furrows formed, both lay along the same meridian.

Moreover, we found little or no delay between the times when eccentric furrows began and the times when half of the uninjected control eggs began to furrow. The control values should be reliable, since they were obtained by scanning groups of eggs from time to time and interpolating the time of 50% cleavage. However, most observations of injected eggs were clearly done long after furrowing began but when we restricted our consideration to 14 cases in which there was no evidence for such gross observational delay, the delays between first seeing an eccentric furrow and the control time in that batch averaged 4±4 minutes, where ±4 minutes was the standard deviation of the group and ±1 minute that of the mean. Even this 4 minute value may well be attributed to the time that a normal, polar furrow would have taken to reach the average site of eccentric furrowing.

Fig. 6 illustrates the fate of these abnormal eccentric furrows. Such furrows never extend beyond their initial lengths. They deepen slightly and unpigmented membrane appears within the grooves. Such furrows do not zip-up like those of control eggs, but the grooves regress, restoring the egg's spherical shape. Occasionally, vestigial black pigmented protrusions are left, marking the site of original induction (Fig. 6D). Buffer injections during this period were first done with dibromo BAPTA to reach 1.5 mM. This concentration was again chosen on the assumption that the buffer injections would block cytokinesis by preventing the emergence of a localized zone of elevated calcium in the micromolar range.

We also carried out six additional experiments, illustrated in Fig. 7, in order to check the assumption that buffer injections during this period act by inhibiting the emergence of a high-calcium zone in the micromolar range. To be more specific we spot-checked whether the dependence of furrow eccentricity and furrow inhibition on buffer concentration and K_D was similar to that first seen in fucoid eggs. In two experiments, eggs were injected at various times with dibromo BAPTA to reach 0.5 mM rather than 1.5 mM. These injections generated no detectable sign of cleavage off-set or arrest during the first cell division, but occasionally caused a temporary inhibition of second division in the daughter cell that arose from the injected side of the egg (Fig. 7A). In two other experiments, eggs were injected to reach a final 1.5 mM cytosolic concentration of the stronger buffer BAPTA (K_D = 0.21 μM). In most cases there was no detectable effect of the buffer on the first or any subsequent divisions (Fig. 7B). However, in a few cases an eccentric first furrow was induced, which eventually regressed. These eggs were not permanently arrested but

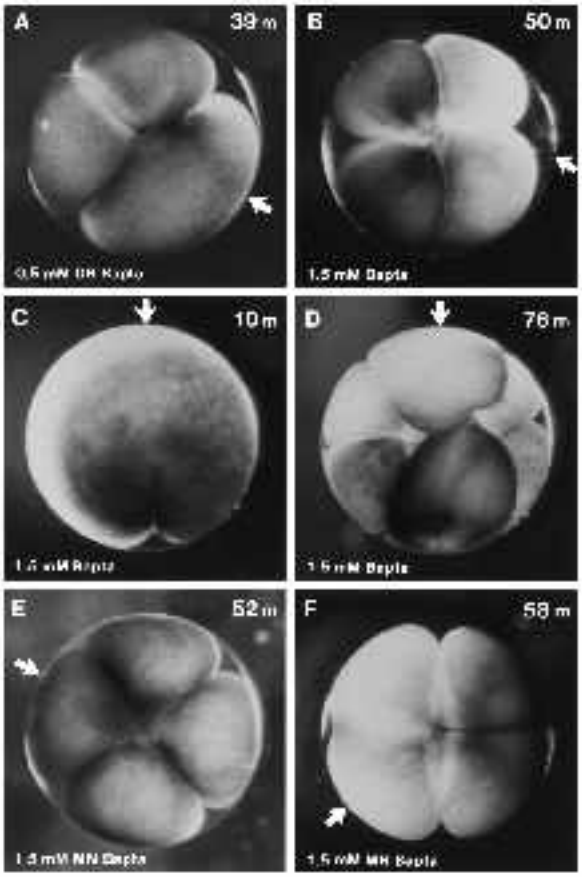

Fig. 7. The marginal effects of injecting buffers with suboptimal combinations of K_D and final cytosolic concentration at the stage (about 5 to 15 minutes before cleavage) when eccentric furrows can be induced. The injection sites are marked by arrows. In (A) dibromo BAPTA was injected to reach 0.5 mM instead of the fully effective 1.5 mM. This had no effect upon the initial site or subsequent growth of the first furrow. However, it did delay second cleavage on the injection side by about 5 minutes. (B) plus (C) and (D) illustrate the varied effects of injecting BAPTA (which has a suboptimal K_D of 0.21 μM) to reach a final cytosolic concentration of 1.5 mM. In (B) no effect was seen; while in (C) and (D) an equatorial furrow formed and regressed but later cleavages soon resumed and persisted. (E) and (F) show the varied effects of injecting mononitro BAPTA (which has a suboptimal K_D of 40 μM) to reach 1.5 mM. In (E) no effect was seen; but in (F) we saw a temporary, 5 minute inhibition of the second cleavage on the injected side.

proceeded to undergo subsequent divisions, which resulted in an abnormal pattern of development compared to controls (Fig. 7C-D).

In two further experiments eggs were injected with the weaker buffer mononitro BAPTA (K_D= 40 μM) to reach a final cytosolic concentration of 1.5 mM. Again, in most cases these injections yielded no detectable off-set or inhibition of the first or subsequent cleavages (Fig. 7E). Once again, in a few cases, there was a temporary inhibition of cleavage in the daughter cell, which arose from the injected side of the egg (Fig. 7F).

We should add one more curious point. Alerted to the possibility of such eccentric and abortive furrowing, we also checked our uninjected control eggs for them. They generally proved to be rare or absent in such controls. However, we did find one batch of control eggs from a particular frog (in our experiment no.16) in which a third of the first furrows had this eccentric character. We do not yet know if this anomaly had a genetic basis.

Injection of buffer during the second cell cycle

In another series of experiments, one of the first two blastomeres was injected with dibromo BAPTA to reach 1.5 to 3.0 mM soon after the appearance of its furrow. The results obtained were very similar to the effects of buffer on the first cell division. If the buffer was injected at the single stripe stage, the progression of the furrow in the injected blastomere was rapidly arrested, then seen to regress without any extensive deepening of the furrow. If, however, injection was made at the double stripe stage of the second division, the furrow was again arrested and, like an injection during this stage of the first cell cycle, it deepened in a characteristically delocalized way, with pigmented cones and unpigmented inner walls and floor. It was clear that the second furrow of the uninjected blastomere continues its progression across the cell surface eventually cutting it in two. Cytokinetic activity in the injected blastomere is clearly inhibited for several cell cycles, while that in the cells arising from the initially uninjected blastomere begin to be affected as buffer crosses to them from the arrested portion of the zygote.

Injections to determine the injectate's spread

Fig. 8 shows representative sections through the injection planes of eggs that were injected with a fluorescein-labelled 3,000 M_r dextran solution and then freeze-fixed 20 seconds, 3 minutes or 10 minutes later. These sections were obviously flattened. Indeed, the midsections of 8 of 9 eggs had an average height to diameter ratio of 0.54 ± 0.04. However, a ninth one had a ratio of 0.72 and was visibly less adherent to the paper. So we deduce that the flattening occurred as the recently injected, live eggs spread out on their sticky paper supports rather than during fixation.

In any case, each such 20 second midsection showed a big hole in its cytoplasm, which must indicate the injection site. Some of these injection holes were roughly spherical; others were elongated along what can be assumed to have been the axis of insertion of the injection pipette. Moreover, their volumes about equalled that of the 8 nl (250 μm diameter when spherical) injectate. So it can be inferred that the injectates initially forced back the cytoplasm and only later spread through it. By three minutes, the holes are gone and the dextran has spread well beyond its 20 second boundaries and by 10 minutes it has spread considerably further.

By making measurements on these midsections (as well as on serial lateral sections) we learned that after the initial, pressure-driven spread, the dextran moves out in about the manner expected of a molecule diffusing out at a few hundred $\mu m^2/s$. Since this is known to be about the diffusion constant of other molecules of this 3,000 M_r size when spreading through water (Tanford, 1961), we tentatively assume that the injected buffers spread out in a similar way within the eggs except that they diffused out at the some-

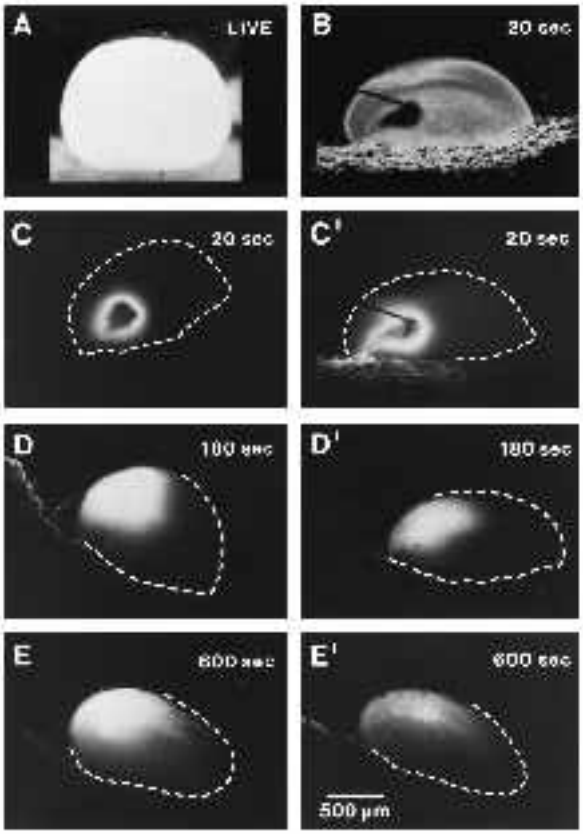

Fig. 8. The spread of injected, fluorescein-labeled, 3,000 M_r dextran through *Xenopus* eggs. These injections were done in the same way as we injected various BAPTA-type calcium buffers. (A) shows a bright-field image of a living egg taken at about the same stage as when the dextran injections were given. Subsequent panels show images of sections made approximately in the plane of the injection pipette of eggs that were freeze-fixed at various times after injection. (B) shows a bright-field image of a section from an egg fixed at 20 seconds after injection. Note the large injection cavity and the paper that supported the egg. Its flattening apparently occurred while the living egg was spreading out on this sticky paper. The crack in this section may have formed along a line of weakness produced by the injection pipette. (C-E) and (C′-E′) show representative fluorescent images of sections made from two eggs at 20, 180 and 600 seconds after fixation. Note the disappearance of the injection cavities by 180 seconds and the slow spread of fluorescent dextran during the first 10 minutes after injection.

what faster rate characteristic of 500 M_r molecules as opposed to 3,000 M_r ones in water. So we take the buffers' diffusion constants within an egg's cytoplasm to be that of the 500 M_r raffinose molecule in water or about 360 $\mu m^2/s$ (Höber, 1945). In Fig. 9, we show a rough model of the spread of the injected buffers that is made on this assumption.

DISCUSSION

For reasons that are discussed below, we believe that the injected calcium buffers acted as shuttle buffers to continually suppress zones of high calcium in the micromolar range (rather than as sponges that immediately soak up calcium). Moreover, we assume that the main targets of such suppression were the calcium waves that accompany furrowing. However, the exact cellular mechanisms involved must have varied with the stage at which the injections are made.

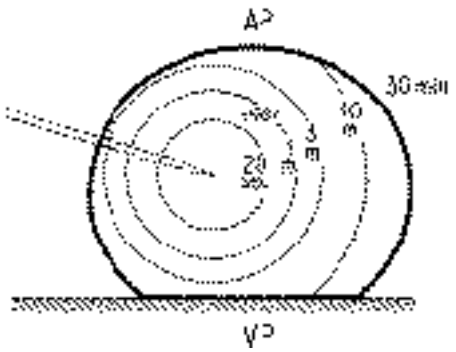

Fig. 9. An approximate model of the spread of BAPTA-type buffers injected at about 10 minutes before the first cleavage. The egg's profile is taken from Fig. 8A. From observations of the injection cavities in sections fixed 20 seconds after injection we took the injection center to be within the equatorial plane and 36% of the way across the egg. The radius of spread at 20 seconds is taken from that of the fluorescent dextran boli seen then. The subsequent increases in radius, r are calculated from the equation, $r = Dt$, where t is time after injection and D is taken to be 360 $\mu m^2/s$ as discussed in the text.

Injections made after cleavage starts

We know that the lengthening furrows in cleaving medaka and *Xenopus* eggs contain high free calcium in the micromolar range. In other words, furrowing in these giant cells is accompanied by a calcium wave (Fluck et al., 1991; Miller et al., 1991, and unpublished). Presumably, therefore, injections made at the single stripe stage arrest furrow extension by suppressing such a wave. It is true that 5 to 10 minutes elapsed between buffer injections made at the single stripe stage and the stoppage of furrowing. This delay might suggest a less direct mechanism than wave suppression. However, the evidence (summarized in Fig. 9) indicates that the time taken by the injectate to spread to the furrow accounts for most or almost all of this delay. This argument is strengthened when one realizes that the spreading times shown in Fig. 9 are based upon the questionable assumption that the injected, BAPTA-type buffers diffused as freely through a *Xenopus* egg as a small fluorescein-labelled dextran did; namely, as freely as molecules of these sizes diffuse through water. However, the BAPTA-type buffer, fura-2 is known to diffuse through muscle cells at only a third to a tenth of this rate, presumably because it is somewhat adherent to a muscle cell's cytoskeleton (Timmerman and Ashley, 1986; Baylor and Hollingworth, 1988). To the extent that our buffers were impeded by such adherence, their spread through a *Xenopus* egg would have been even slower than is shown in Fig. 9.

Such arrest supports the model of cytokinesis proposed by Fluck et al. (1991) - one in which a local increase in free calcium is induced by contraction of the contractile arc

and acts back to induce elongation of this arc. Moreover, in this model, arc contraction releases calcium by tugging on stretch-sensitive channels within the plasma membrane or the endoplasmic reticulum (ER). Since *Xenopus* furrows are known to elongate at normal rates in media kept at extremely low calcium levels with EGTA (Baker and Warner, 1972), we suggest that the putative stretch-sensitive channels in *Xenopus* might lie - in good part at least - within its cortical ER rather than its plasma membrane.

However, buffer injections made at the double stripe stages of cleavage - while they also arrest furrow elongation - are followed by the development of large areas of new, unpigmented membrane (Fig. 3A). Since the formation of such new membrane accompanies normal furrow deepening, it would seem that buffer injections do *not* block those later stages of furrow formation that generate the deeper parts of the new daughter cells' membranes. This finding fits evidence showing that there is a second phase of furrow deepening in amphibian eggs, which has a very different morphology than the first one (Bluemink, 1970; Singal and Sanders, 1974) and may require local calcium levels that are below rather than above the resting level. Thus a newt egg furrow that is isolated after the contractile ring has shrunk much of the way to extinction, continues to shorten but does so fastest at 10 to 100 nM calcium levels (Mabuchi et al., 1988) - levels that are well below those generally found in developing eggs. In particular, it is well below the 400 nM resting level found in *Xenopus* zygotes (Busa and Nuccitelli, 1985). This finding even suggests the possibility - put forward for sea urchin eggs by Yoshimoto and Hiramoto (1985) - that a second phase of amphibian egg furrowing may require a local *reduction* rather than a local rise in free calcium. Dibromo BAPTA, with a K_D of 1.5 μM would not be expected to effectively suppress local reductions in local calcium to levels well below 0.4 μM.

Injections done before cleavage starts

When buffer is injected between about 15 minutes and a few minutes before cleavage normally occurs, the injected egg usually forms an eccentric but abortive furrow that is shifted away from the injection site and toward the opposite equator. Our interpretation of such eccentric furrowing is that the injected buffer reaches the nearby but not the farther part of the cortex in time to suppress the induction of a needed zone of high calcium. In short, high cortical calcium is needed for furrows to start as well as to grow.

This interpretation may suffice to explain the suppression of furrowing near the injection side; however, three other accessory mediators of ectopic furrowing should be considered. The first are the asters. This possibility is doubtful because our shuttle buffers would be expected to preserve rather than dissolve microtubules, since they should block rises in calcium that should dissolve microtubules. Compelling evidence showing that increases in cytosolic calcium within the physiological range generally tend to dissolve microtubules can be found in the work of Kiehart (1981) on sea urchin eggs, of Izant (1983) on PtK_1 cells and of Zhang et al. (1992) on *Tradescantia* stamen hairs. It might be imagined that the shuttle buffers used block local *decreases* in calcium below the resting level, which naturally increase microtubule densities; however, as noted elsewhere, at the concentrations and K_D values used, the injected buffers would not effectively oppose such reductions.

A second, more plausible target, is the main precleavage surface contraction wave in *Xenopus* eggs, a 0.9 μm/s wave that may help prepare the surface for furrowing. According to Hara et al. (1977) it begins at the animal pole at about 19 minutes before and reaches the equator at about the time that cleavage starts at 20 to 21°C. We have some unpublished evidence that buffer injections can inhibit such waves. A third, highly speculative mediator is the growing diastema, a poorly characterized structure that is briefly discussed below.

When buffer is injected earlier than 15 to 25 minutes before cleavage normally starts, furrowing is completely suppressed for hours. An apparently sufficient interpretation of such global suppression is that the injected buffer reaches the whole animal cortex in 15 to 25 minutes, where it then everywhere suppresses the induction of a cortical zone of high calcium. However, we would also point out that buffer injections made that long before cleavage normally starts are likely also to suppress cleavage indirectly by suppressing mitosis and thence the spindle-to-cortex message that induces furrowing. In particular they may act to block a calcium pulse needed for nuclear envelope breakdown, since there is good evidence for such a pulse during the first cell cycle in sea urchin eggs (Steinhardt and Alderton, 1988; Browne et al., 1992) as well as in mouse zygotes (Tombes et al., 1992).

Furrow initiation may be mediated by a diastema

Eccentric furrows always lie along a meridian through the animal pole. In those cases where two eccentric furrows formed, both lay along the same meridian. It is as if the signal that positions a furrow moves from the mitotic apparatus to the surface in the form of a growing disc or plate. We would therefore suggest that this signal is carried by an extension of the metaphase plate called the *diastema* rather than the asters. Classical cytological evidence for the diastema's reality can be found in the papers of Selman (1982) and of Sawai and Yomota (1990) as well as in plate 13 of Hausen and Riebesell (1991). Moreover, we suggest that the so-called 'telophase disc', of Andreassen et al. (1991), is an immunocytochemical image of the diastema in a HeLa cell. A diastemal intermediate in amphibian eggs is also supported by Zotin's (1964) old report that heavy water treatment fragments the diastema of axolotl eggs and that its 'dispersed remnants....seemed to...[generate]...additional diastems which induce many furrows on the egg surface'. However, a full discussion of this issue is beyond the scope of this paper.

Comparison with earlier buffer injection studies: injected chelators act as shuttle buffers

Finally, we take our new findings - together with the complementary ones of Snow and Nuccitelli (1993) on second cleavage inhibition - as good evidence that injected chelators can easily act as shuttle buffers. We have not attempted as thorough and exact a study of the dependence of cleavage inhibition in *Xenopus* on buffer concentration and on K_D as Speksnijder et al. (1989) did for fucoid eggs. Nev-

ertheless, it is clear that our results for *Xenopus* eggs are remarkably similar to those reported for fucoid eggs. In both cases dibromo BAPTA is the most (or among the most) effective cleavage inhibitor among the buffers tested. In both cases, the dependence of inhibition upon final buffer concentration is really very sharp with most of the range of inhibitory responses occurring over a concentration range of twofold or less. In both cases, the final cytosolic concentration of dibromo BAPTA at which inhibition was largely complete was about 1 to 2 mM. In both cases, the weaker buffer, mononitro BAPTA, which has a K_D about 25-fold higher, and the *stronger* ones, BAPTA and dimethyl BAPTA, with K_D values 5- to 10-fold lower, were less effective than dibromo BAPTA. In both cases, the critical inhibitory concentrations of these suboptimal buffers seemed to be only a few-fold higher than that for dibromo BAPTA. Furthermore, in both cases, the inhibitory effects of injecting these calcium buffers was largely independent of the concentration of coinjected calcium.

Not only do the responses to the four BAPTA-type buffers used by us (as well as the four partly different ones used by Snow and Nuccitelli, 1993) fit facilitated diffusion theory, but even the effects of EGTA injections briefly reported by Baker and Warner (1972) as well as by Vincent et al. (1987) seem to be largely explicable by this analysis. The former injected one of the first two blastomeres of *Xenopus* with various Ca^{2+}/EGTA mixtures and found that what we now estimate to have been a final concentration of about 50 mM EGTA (of which 20 mM was preneutralized with Ca^{2+}) gave about half normally cleaving cells and half that were blocked or slowed; while the latter injected uncleaved eggs with what we now estimate to have been a final concentration of 30 mM EGTA and found that 'one-half of the eggs injected.....divided at first cleavage, though with a 20-min delay'. These old results seem to indicate a degree of inhibition comparable to that produced by a final concentration of a few tenths millimolar dibromo BAPTA (see Table 2) and thus of the order of a hundred times smaller than the needed concentration of EGTA. Application of the facilitated diffusion equation [7] of Speksnijder et al. (1989) indicates that a buffer with a K_D matched to the emerging high Ca^{2+} level will exceed the effectiveness of a hyperstrong one like EGTA by a factor about equal to the ratios of their K_D values. Our Table 1 shows this ratio to be about 150-fold and thus comparable to the observed effectiveness ratio. It seems remarkable that *Xenopus* eggs could recover from the enormous cytosolic concentration of 30 mM unneutralized EGTA by releasing or letting in enough calcium to neutralize it and then be inhibited by the residual shuttle activity of the EGTA; but that is what the numbers suggest happened, at least in part.

We think that our new data, together with that of Snow and Nuccitelli (1993), strengthen the shuttle buffer model both because frog and seaweed eggs are such different cells and because of the relative rapidity of some of the frog egg responses - some of which were evident within 5 to 10 minutes, as opposed to days as in fucoid eggs. Furthermore, nuclear envelope assembly in *Xenopus* egg extracts is also inhibited by various BAPTA-type buffers with a dependence upon K_D and buffer concentration that is similar to that seen for cleavage inhibition in *Xenopus* and in *Pelvetia* eggs (K. Sullivan et al., unpublished). Alternative mechanisms of calcium buffer effects - such as those mediated by direct effects on macromolecules as opposed to ions, those mediated by Zn^{2+} gradients rather than Ca^{2+} ones, or those mediated by impurities in the buffers - all seem very difficult indeed to square with the accumulating data.

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