# Calcium Buffer Injections Delay Cleavage in *Xenopus laevis* Blastomeres

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*Abstract.* Microinjection of calcium buffers into the two-cell *Xenopus laevis* embryo delays cell division in a dose-dependent manner. Four calcium buffers in the BAPTA series with different affinities for calcium were used to distinguish between a localized calcium gradient regulating cleavage and the global calcium concentration regulating this event. DibromoBAPTA ($K_d$ = 1.5 $\mu$M) was found to delay cleavage at the lowest intracellular concentration (1.3 mM) of the four buffers tested. The effectiveness of the calcium buffers was dependent upon the buffer dissociation constant but not in a linear fashion. The concentration of buffer required to delay cleavage increased as the buffer's dissociation constant shifted above or below that of the optimum buffer, dibromoBAPTA. This relationship between a calcium buffer's effectiveness at delaying cleavage and its calcium affinity provides support for the hypothesis that a calcium concentration gradient is required for normal cell cycle progression (Speksnijder, J. E., A. L. Miller, M. H. Weisenseel, T.-H. Chen, and L. F. Jaffe. 1989. *Proc. Natl. Acad. Sci. USA.* 86:6607–6611). DibromoBAPTA was also injected with two different amounts of coinjected calcium to test the possibility that the free calcium concentration of the buffer solution is the important parameter for delaying cleavage. However, we found that changes in buffer concentration have a much stronger effect than changes in the free calcium concentration. This observation supports the hypothesis that BAPTA-type buffers exert their effect by shuttling calcium from regions of high concentration to those of lower concentration, reducing any calcium concentration gradients present in the *Xenopus* embryo.

A transient increase in cytoplasmic $[Ca^{2+}]_i$ during the events of mitosis and cell cleavage is considered to be an important regulatory step in both plant (11, 12, 36) and animal cells (15, 16, 22, 23, 25, 26, 28, 30). Elevation of free intracellular calcium ion concentration ($[Ca^{2+}]_i$) has been proposed to participate in the regulation of mitotic events. Imposed changes in $[Ca^{2+}]_i$ have provided evidence that $[Ca^{2+}]_i$ regulates the initiation of DNA synthesis, nuclear envelope breakdown and cell cleavage. The addition of growth factors or serum to cells in culture stimulates an increase in $[Ca^{2+}]_i$ that leads to the initiation of DNA synthesis and buffering these $[Ca^{2+}]_i$ increases with quin 2 or dimethyl BAPTA blocked subsequent DNA synthesis (6, 29, 32, 35). Imposed increases in $[Ca^{2+}]_i$ have been shown to induce nuclear envelope breakdown in both sea urchin eggs and mammalian 3T3 fibroblasts, while suppressing $[Ca^{2+}]_i$ changes with the calcium buffer, BAPTA, inhibited nuclear envelope breakdown in sea urchin eggs and Swiss 3T3 cells (15, 28). Elevation of $[Ca^{2+}]_i$ in squid eggs by treatment with the calcium ionophore, A23187 (1), and in *Ilyanassa* eggs by treatment with caffeine (5), has been shown to speed the onset of cleavage. Cell division was retarded in sea ur-

chin (33), medaka (8), and *Xenopus* (10, 19) embryos in which $[Ca^{2+}]_i$ changes were suppressed with the BAPTA family of buffers and earlier work with much higher concentrations of EGTA also resulted in some cleavage inhibition (2, 31).

Further evidence for a regulatory role for $[Ca^{2+}]_i$ in mitotic events comes from observations of endogenous $[Ca^{2+}]_i$ changes. Transient increases in $[Ca^{2+}]_i$ have been frequently associated with the initiation of DNA synthesis, nuclear envelope breakdown, anaphase onset, and cell division in sea urchin eggs (23, 28, 33), sand dollar eggs (26), and Swiss 3T3 cells (15, 32). Slow waves of elevated $[Ca^{2+}]_i$ associated with cell division has been reported in the cleaving medaka embryo (8) and oscillations in $[Ca^{2+}]_i$ have been measured with $Ca^{2+}$-selective microelectrodes in the cleaving *Xenopus* embryo which correspond in frequency to cell division (9). However, contradictory reports also exist, such as a decrease in $[Ca^{2+}]_i$ measured in the cleaving sea urchin and medaka embryos (34) or the absence of $[Ca^{2+}]_i$ changes in cleaving *Xenopus* embryos (24).

The calcium gradient model proposed by Lionel Jaffe (27) could account for many of the discrepancies encountered during the investigations of calcium as a regulator of mitotic events. In particular, this could explain the lack of consistency in measured $[Ca^{2+}]_i$ changes. This model asserts that calcium increases only in localized areas of the cell, generating highly localized calcium gradients that might not

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be detected by some $Ca^{2+}$ imaging techniques. This model predicts that such highly localized calcium gradients could be reduced by introducing calcium buffers into the cytoplasm. The buffer is proposed to disrupt a calcium gradient by facilitating the diffusion of calcium away from regions of high concentration by binding calcium in regions of high concentration, diffusing away, and releasing calcium in regions of low calcium concentration. A calcium gradient could be disrupted by the shuttling of calcium without significantly changing the average $[Ca^{2+}]_i$. Although the basal $[Ca^{2+}]_i$ may be altered by the initial introduction of a calcium buffer into the cell, the basal concentration should be rapidly restored by the cell's normal calcium homeostasis mechanisms.

Previous investigators have envisioned that calcium buffers influenced cell functions by binding calcium present in the cytoplasm and thus lowering the $[Ca^{2+}]_i$ or suppressing global calcium transients. If calcium buffers act to disrupt cellular events by reducing global $[Ca^{2+}]_i$ then an increase in the buffer's calcium affinity would be predicted to increase the buffer's effectiveness. In contrast, if calcium buffers are disrupting mitotic events by dissipating calcium gradients, we would expect there to be a buffer with an optimum dissociation constant for dissipating the gradient. The prediction of an optimum dissociation constant arises because of the necessity of the buffer to both bind calcium at the region of high $[Ca^{2+}]_i$ and release calcium at the region of low $[Ca^{2+}]_i$. If a buffer exhibits a very high affinity for $Ca^{2+}$ and binds $Ca^{2+}$ strongly at physiological levels, it will not release $Ca^{2+}$ as it diffuses away from a high $Ca^{2+}$ region and will not continuously shuttle $Ca^{2+}$ from regions of high to low concentration to dissipate any concentration gradients. Conversely, a low affinity buffer may bind calcium at a region of high $[Ca^{2+}]_i$ but the calcium would dissociate before being transported very far away from the region of high concentration. The calcium gradient model predicts that an optimum dissociation constant for disrupting a gradient exists and that as the dissociation constant of a buffer differs from the optimum dissociation constant the buffer becomes less effective. The value of the optimum dissociation constant is predicted to be close to the average calcium concentration across the gradient (27).

To test the predictions made by the calcium gradient model several $Ca^{2+}$ buffers in the BAPTA family with a range of calcium affinities have been utilized to disrupt cellular events. This approach was first used to investigate the importance of $[Ca^{2+}]_i$ gradients in the early growth of embryos of the brown alga, *Pelvetia*, where they found that $Ca^{2+}$ buffers inhibited rhizoidal tip outgrowth, a process thought to be initiated by a zone of elevated $[Ca^{2+}]_i$ (27). As predicted by the calcium gradient model, a buffer (dibromoBAPTA) with an optimal calcium affinity for blocking fucoid egg development was identified and buffers exhibiting either lower or higher calcium affinities were less effective at blocking development. We have recently reported that microinjection of the calcium buffer, dibromoBAPTA, delays cleavage in the two-cell stage frog embryo (10). In this study, we have characterized the efficacy of four calcium buffers with a range of calcium affinities to further investigate the requirement for calcium concentration gradients in normal cleavage and the cell cycle.

## Materials and Methods

### Handling of Gametes and Embryos

Eggs were obtained by squeezing a female *Xenopus laevis* which had been induced to ovulate by a subcutaneous injection of 600–800 IU of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO) given 10–16 h earlier. Testes were dissected from *X. laevis* which had been anesthetized by submersion in ice water and killed by decapitation and pithing. Approximately one-eighth of a testis was macerated in 0.6 ml of F1 medium (in mM: 41.25 NaCl, 1.75 KCl, 0.5 $Na_2HPO_4$, 0.06 $MgCl_2$, 0.25 $CaCl_2$, 5.0 Hepes, pH adjusted to 7.8 with NaOH). The macerated testis solution was applied to eggs bathed in F1. At 5–15 min postfertilization, eggs were dejellied by agitation in 2% L-cysteine. Eggs were then washed five times with F1 and incubated in fresh F1 at 22–25°C.

Embryos were microinjected during the two-cell stage. Individual embryos were selected and placed in 5% ficoll dissolved in F1. Embryos were injected in the ficoll solution, which reduced turgor pressure, to minimize leakage during impalement. In each embryo, one blastomere was microinjected with 30 nl of a BAPTA-type buffer or control solution (distilled water or KCl) using a Drummond Digital Microdispensor (model 510, Drummond Scientific Co., Broomall, PA). The site of injection was approximately in the center of the animal hemisphere portion of the blastomere. Due to occasional clogging of the pipette, we found it necessary to calibrate before and after each injection. The volume dispensed by the pipette was assessed by expelling the injection solution into silicone oil and measuring the diameter of the resulting sphere. After injection, embryos were immediately removed from the ficoll solution, rinsed with F1, and incubated in fresh F1.

### Injection Techniques

Micropipettes used for injection were pulled on a vertical pipette puller from borosilicate glass capillary tubing initially 1.2 mm OD × 0.7 mm ID. After pulling, micropipettes were 6–7 mm in length from the shoulder to the tip. The tips were beveled at a 45° angle until an opening 20 $\mu$m in length was obtained. The settings used to pull micropipettes were chosen so that after beveling the shaft diameter was 20 $\mu$m at a distance of 100 $\mu$m from the tip.

The calculation of the actual intracellular concentration of the BAPTA-type buffer injected included an egg volume correction factor to include only cytoplasmic volume in the calculation. The total volume of a *Xenopus laevis* egg 1.2 mm in diameter is 900 nl. Nonorganellar cytoplasm comprises 49% of the total volume of the animal hemisphere and 30% of the vegetal hemisphere (13). If each hemisphere is assumed to comprise 50% of the egg, the mean cytoplasmic volume is 37% of the total volume (333 nl). At the two-cell stage, the volume of the fluid cytoplasm of one blastomere is one-half the fluid cytoplasm of the egg (167 nl).

Injection solutions were prepared by dissolving crystalline BAPTA analog and $CaCl_2$ in $H_2O$ (distilled, deionized), adjusting pH to 7.0 and storing at −20°C. MethylnitroBAPTA was the generous gift of Dr. Lionel Jaffe. All other BAPTA-type buffers used were purchased from Molecular Probes, Inc. (Eugene, OR). *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI). Buffer dissociation constants were taken from Pethig et al. (21).

### Scoring Embryos

Embryos microinjected with a calcium buffer following first cleavage were evaluated based on the following criteria. Approximately 30 min after the initiation of first cleavage, embryos in which second cleavage had occurred in the uninjected blastomere, but not in the injected blastomere were considered to exhibit unilateral delay of cleavage. Such embryos were composed of three blastomeres: one blastomere comprising half of the embryo and two blastomeres together comprising the other half. Embryos in which cleavage occurred in both injected and uninjected blastomeres were considered to have normal bilateral cleavage.

Embryos were deemed to have cleaved bilaterally at third cleavage when cell division occurred in all blastomeres existing after second cleavage. Delay of third cleavage was scored when the blastomeres of one-half of the embryo cleaved, while the blastomeres of the other half did not (each half being comprised of one of the original blastomeres of the two-cell stage embryo). Embryos that were delayed at second cleavage sometimes cleaved bilaterally at third cleavage. 91% of these cleavage furrows were in the meridional orientation which is the normal orientation for the second cleavage furrow.

The remaining 9% ($n = 60$) were equatorial, the orientation of a normal third cleavage furrow. Embryos which had cleaved bilaterally at second cleavage were also observed to be unilaterally delayed at third cleavage.

## Statistical Methods

The SEM was calculated separately for each data point using the total number of embryos scored and an estimate of the sample standard deviation. The sample standard deviation was calculated using the following formula for grouped data (18):

$$s^2 = \frac{\Sigma f \cdot m^2 - (\Sigma f \cdot m)^2/n}{n - 1} \tag{1}$$

where $f$ is the number of embryos scored in an individual experiment, $m$ is the fraction of embryos which were delayed and $n$ is the total number of embryos scored from all experiments performed. The standard deviation was calculated using a formula for grouped data because these data are dichotomous; each embryo either cleaved or was delayed.

Because these data are dichotomous and not normally distributed we also used a logistic model to calculate 95% confidence intervals. The SAS statistical program was used to perform a probit analysis in which a log transformation of the dose was performed and confidence limits (fiducial limits) were obtained (14). The logistic model used passed a goodness of fit test for some data sets and not others. The confidence intervals are presented for those data sets in which the logistic model fit the data as assessed by the goodness of fit test ($P > 0.05$).

## Results

We have investigated the involvement of intracellular calcium gradients in the early cleavage divisions of the frog embryo by microinjecting various calcium buffers into one blastomere of the two-cell stage embryo. We found that these buffers delayed cleavage in the injected blastomere in a concentration-dependent manner that varied with the calcium affinity of the buffer (Fig. 1).

### DibromoBAPTA, $K_D = 1.5\ \mu M$

The percentage of dibromoBAPTA-injected embryos which exhibit unilateral delay of cleavage depends on the intracellular concentration of dibromoBAPTA within the injected blastomere (Fig. 2 A). The occurrence of cleavage delay increases sharply as the intracellular concentration of dibromoBAPTA rises. At second cleavage, an intracellular concentration of 0.26 mM dibromoBAPTA results in a delay of cleavage in 50% ($DC_{50}$) of the injected embryos and a concentration of 1.3 mM results in 100% occurrence of delay of cleavage ($DC_{100}$). DibromoBAPTA delays cleavage at lower concentrations than the other BAPTA-type buffers injected.

### MethylnitroBAPTA, $K_D = 22\ \mu M$

The percentage of embryos exhibiting a delay of cleavage at a given concentration of methylnitroBAPTA was reduced in comparison with the percent delayed when dibromoBAPTA was injected (Fig. 3). MethylnitroBAPTA is a calcium buffer that has a lower affinity for calcium than dibromoBAPTA ($K_D = 22\ \mu M$ compared with 1.5 $\mu M$ for dibromoBAPTA). The slope of the methylnitroBAPTA curve was also reduced in comparison to the dibromoBAPTA curve. The $DC_{50}$ for methylnitroBAPTA occurs at an intracellular concentration of 1 mM and the $DC_{100}$ occurs at 3.9 mM. In the lower concentration range of between 0.05 and 0.25 mM methylnitroBAPTA the percent of embryos exhibiting a delay of
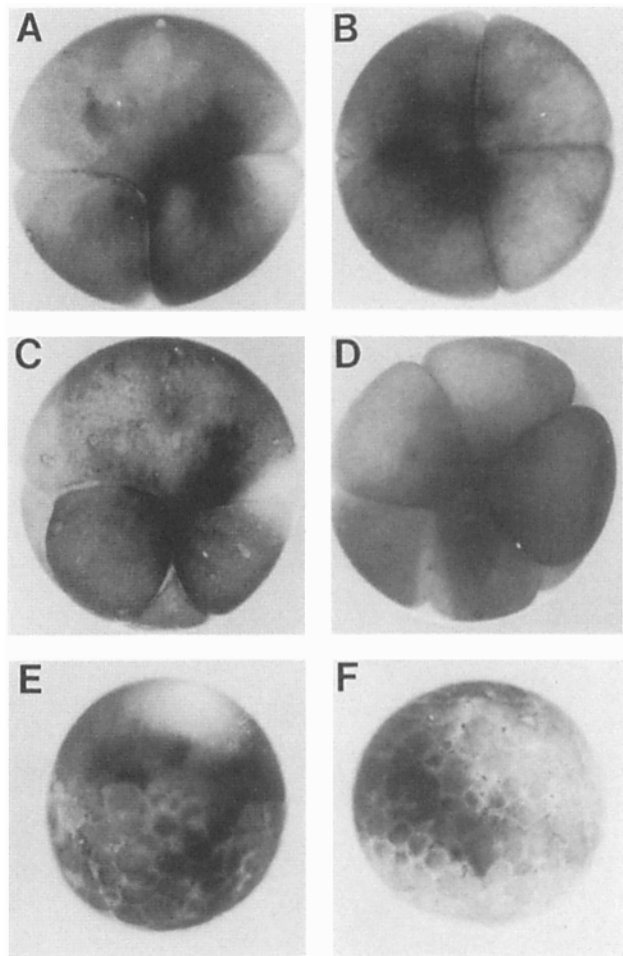


*Figure 1.* Delay of cleavage induced by the microinjection of a BAPTA-family buffer into one blastomere of a two-cell stage embryo. (A) Delay of second cleavage induced by the microinjection of difluoroBAPTA (intracellular concentration 1.3 mM). (B) A control embryo exhibiting normal second cleavage. (C) Delay of third cleavage induced by the microinjection of methylnitroBAPTA (intracellular concentration of 0.65 mM). (D) A control embryo exhibiting normal third cleavage. (E) Delay of cleavage at 4 h post-fertilization induced by the microinjection of dibromoBAPTA (intracellular concentration 1.3 mM; 11:1 ratio of dibromoBAPTA:calcium). (F) A control embryo exhibiting normal development at 4 h postfertilization.

cleavage does not increase sharply as was seen with the lower concentrations of dibromoBAPTA but remains near 20% delay of cleavage throughout this concentration range. These data fit a logistic model well and the 95% confidence intervals are plotted in Fig. 3, B and C.

### DifluoroBAPTA, $K_D = 0.25\ \mu M$

Blastomeres injected with difluoroBAPTA exhibit a delay of cleavage only 40% of the time at the $DC_{100}$ concentration for dibromoBAPTA (1.3 mM) (Fig. 2 B) and the inhibitory response was biphasic. When injected with concentrations ranging from 0.2 through 2.5 mM difluoroBAPTA, about half of the embryos exhibit delayed cleavage and at higher
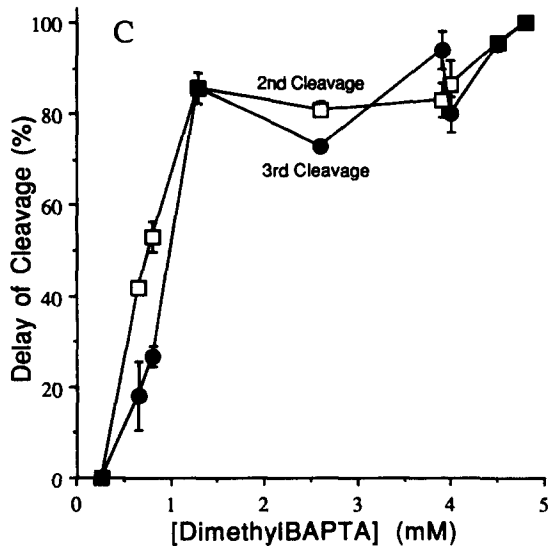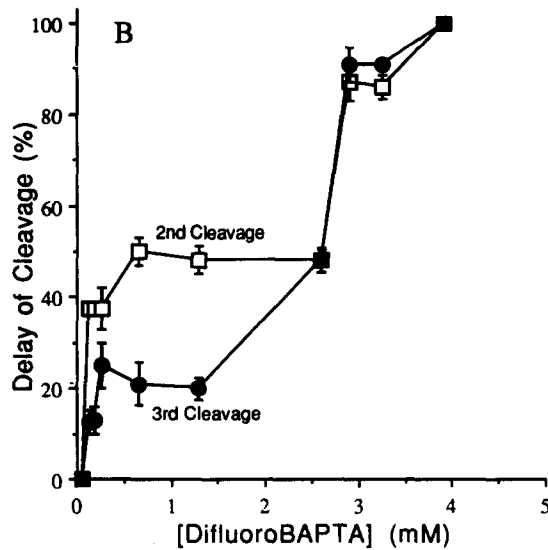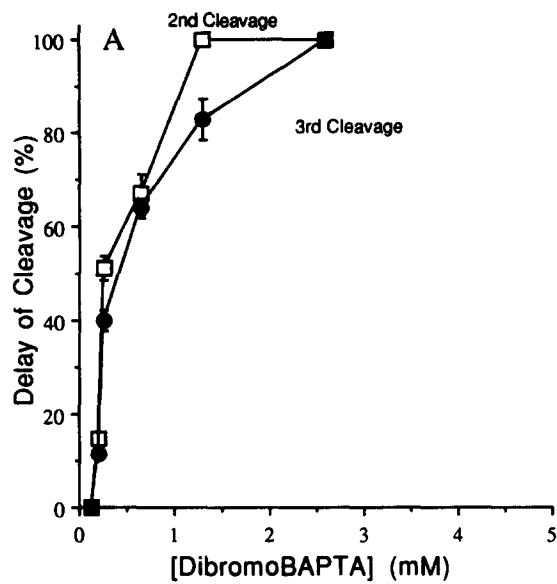
concentrations nearly all embryos are inhibited. The greatest difference between the percentage of embryos delayed at second cleavage and the percentage delayed at third cleavage for all buffers injected is observed in this plateau region of the difluoroBAPTA curve. The $DC_{100}$ for difluoroBAPTA occurred at 3.9 mM, which was identical to that observed for methylnitroBAPTA. This biphasic inhibitory response suggests that there might be two target mechanisms with which the difluoroBAPTA is interacting.

### DimethylBAPTA, $K_D = 0.15 \ \mu M$

DimethylBAPTA exhibits the greatest affinity for calcium of the buffers tested and produced a delay of cleavage in 85% of the injected embryos at the $DC_{100}$ concentration of dibromoBAPTA (Fig. 2 C). The percentage of cleavage-delayed embryos increased sharply from 0% at 0.26 mM to 85% at 1.3 mM dimethylBAPTA. The $DC_{50}$ for dimethylBAPTA occurs at 0.8 mM. The curve plateaus near 85% delay of cleavage between 1.3 and 4.0 mM dimethylBAPTA. Dimethyl-BAPTA required the highest concentration (4.8 mM) to produce a delay of cleavage in all injected embryos.

### Subsequent Development

The number of BAPTA-injected embryos surviving to the tadpole stage is very low even at the buffer concentrations producing no delay of cleavage (Table I). Because the KCl-injected embryos regularly develop into tadpoles, the low rate of tadpole formation and high death rate of BAPTA-injected embryos is probably due to the cytotoxic effects of the buffer rather than from the trauma of injection. The percentage of injected embryos in which cleavage was still delayed at 4–6 h postfertilization (7th–10th cleavage cycle) is low for the $DC_{50}$ concentrations. BAPTA-family buffer concentrations producing 100% delay of second cleavage continue to block cleavage at 4–6 h postfertilization. By this time, 50% of the embryos receiving $DC_{100}$ concentrations are still blocked or have cleaved only once and the other 50% have turned white and lysed.

### Comparing the Efficacy of Calcium Buffers

The efficacy of the various calcium buffers injected versus

*Figure 2.* The concentration-dependent delay of second and third cleavage after microinjection of various members of the BAPTA $Ca^{2+}$ buffer family into one blastomere of two-cell stage *Xenopus* embryos. (*A*) Intracellular concentrations of dibromoBAPTA ranged from 0.13 to 2.6 mM and the dibromoBAPTA:calcium ratio of the injectate was 1:1. The number of embryos scored for each concentration ranged from 11 to 78. Error bars indicate SEM calculated as described in the Materials and Methods. If no error bar is shown, the error is smaller than the size of the symbol. Open squares denote the delay of second cleavage whereas closed circles denote the delay of third cleavage. (*B*) DifluoroBAPTA-injected two-cell stage *Xenopus* blastomeres. Intracellular concentrations of difluoroBAPTA ranged from 0.05 to 3.9 mM and the difluoroBAPTA:calcium ratio of the injectate was 1:1. The number of embryos scored for each concentration ranged from 12 to 30. (*C*) Dimethyl-BAPTA-injected, two-cell stage *Xenopus* blastomeres. Intracellular concentrations of dimethylBAPTA ranged from 0.26 to 4.8 mM and the dimethylBAPTA:calcium ratio of the injectate was 1:1. The number of embryos scored for each concentration ranged from 12 to 32.
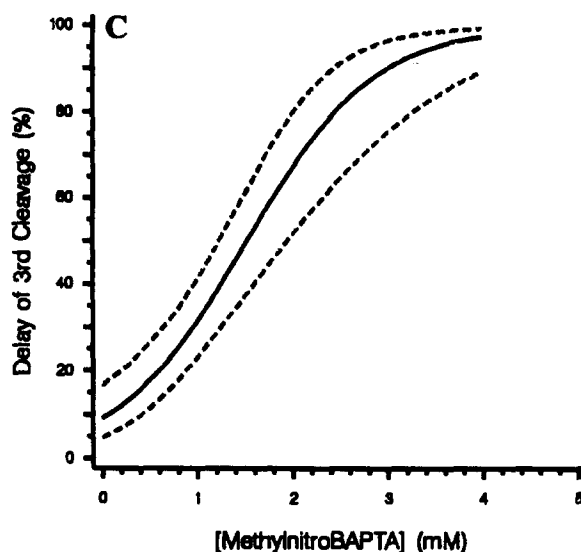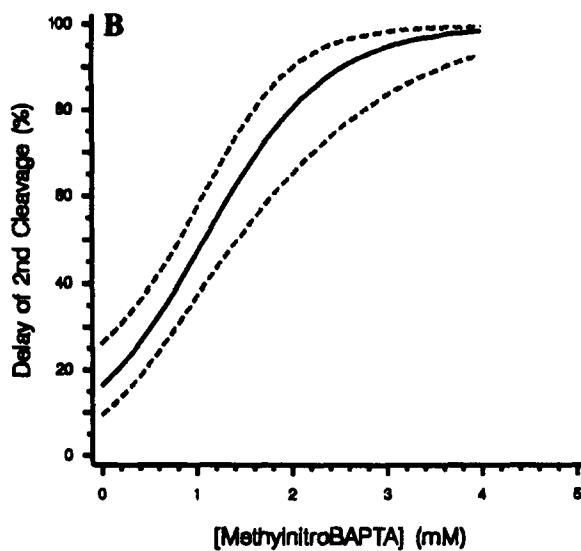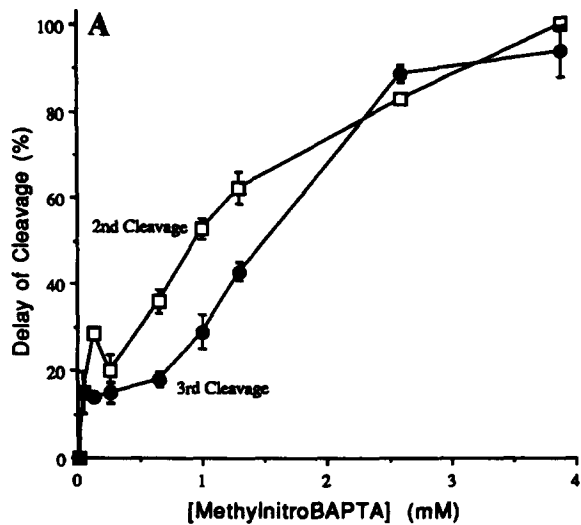
**Table I. Development of Injected Embryos**

| Group characteristics* | Percent delayed 4–6 h post-fertilization | Percent lysed 4–6 h post-fertilization | Percent tadpole |
|---|---|---|---|
| Embryos injected with concentrations of BAPTA-family buffer producing no delay of cleavage | 0 | 15 | 14 |
| Embryos injected with $DC_{50}$ concentrations of BAPTA-family buffers | 5 | 20 | 8 |
| Embryos injected with $DC_{100}$ concentrations of BAPTA-family buffer | 50 | 50 | 0 |
| KCl control injections 0.65–3.9 mM | 0 | 5 | 73 |

* Combined data from all BAPTA-family buffer injections.

their $Ca^{2+}$-binding affinity generates a U-shaped curve with dibromoBAPTA exhibiting inhibition of cleavage at the lowest concentration (Fig. 4). 50% delay of cleavage for difluoroBAPTA was observed over a wide concentration range and the lowest concentration resulting in 50% delay of cleavage was plotted (Fig. 4 A).

Cellular responses to microinjected calcium buffers have often been thought to be due to changes in the overall $[Ca^{2+}]_i$ in the cell. To address this issue dibromoBAPTA was injected with two different amounts of coinjected calcium which would provide two different levels of $[Ca^{2+}]_i$. The effectiveness of dibromoBAPTA changes only slightly when the amount of coinjected calcium is varied greatly (Fig. 5). The percentage of injected embryos delayed at second cleavage is shown as a function of concentration for dibromoBAPTA:calcium ratios of 11:1 and 1:1. Dibromo-BAPTA's effectiveness is similar at the two ratios of coinjected calcium. The 11:1 ratio is slightly more effective at very low concentrations of dibromoBAPTA, whereas the 1:1 ratio is more effective at higher concentrations.

### Effects of $Ca^{2+}$ Buffers on Cleavage Furrow Formation

Occasionally, buffer-injected embryos exhibited a partial

Figure 3. The concentration-dependent delay of second and third cleavage upon methylnitroBAPTA microinjection into one blastomere of two-cell stage *Xenopus* embryos. Intracellular concentrations of methylnitroBAPTA ranged from 0.01 to 3.9 mM and the methylnitroBAPTA:calcium ratio of the injectate was 11:1. The number of embryos scored for each concentration ranged from 12 to 24. (*A*) Error bars indicate SEM calculated as described in the Materials and Methods. If no error bar is shown, the error is smaller than the size of the symbol. Open squares indicate the delay of second cleavage whereas closed circles indicate the delay of third cleavage. (*B*) Logistic regression and the 95% confidence interval for the delay of second cleavage after methylnitroBAPTA injection is presented (goodness of fit, $P = 0.64$). (*C*) Logistic regression and the 95% confidence interval for the delay of third cleavage after methylnitroBAPTA injection (goodness of fit, $P = 0.79$). Confidence intervals and logistic regression calculated as described in the Materials and Methods.
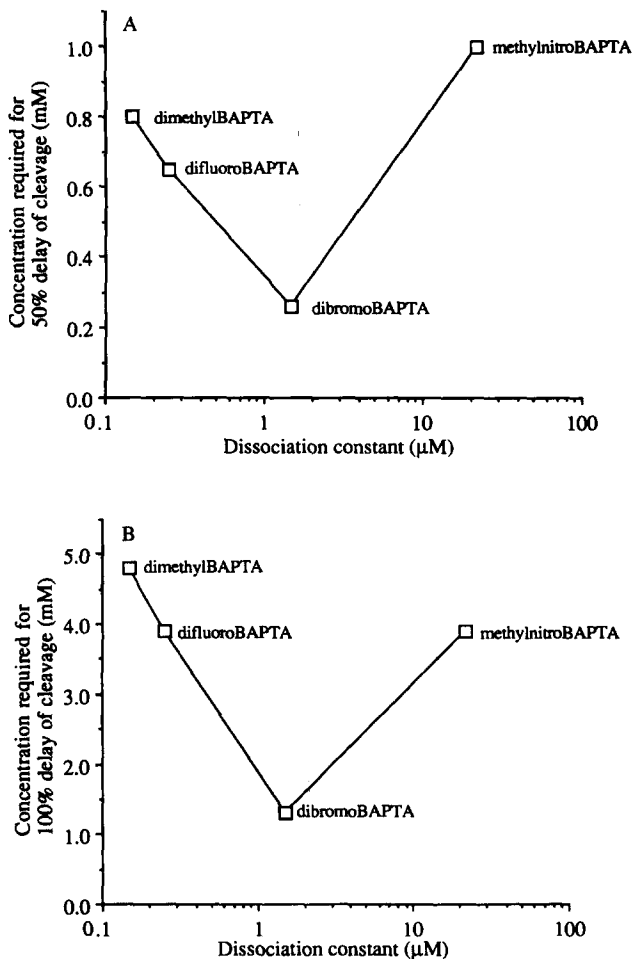
*Figure 4.* The concentration of buffer required to produce delay of cleavage is dependent upon buffer dissociation constant. (*A*) The concentration of BAPTA-type buffers which results in delay of second cleavage in 50% of the injected embryos is plotted against the buffer dissociation constant. A range of difluoroBAPTA concentrations produced 50% delay of cleavage, but here we plot the lowest concentration in this range. (*B*) The concentration of BAPTA-type buffers which results in 100% delay of cleavage is plotted versus the buffer dissociation constant. DibromoBAPTA-injected embryos require a higher concentration of buffer for 100% delay of third cleavage (2.6 mM) than is required for 100% delay of second cleavage (1.3 mM). 100% delay of second and third cleavage is achieved at the same buffer concentration for the other BAPTA-type buffers injected.

cleavage furrow at second cleavage. The partial furrow was a small arc of a cleavage furrow (generally extending through less than one-fifth of the animal hemisphere) located at either the top of the animal hemisphere or at the equator between the animal and vegetal hemispheres. These embryos were classified as unilaterally delayed or bilaterally cleaving depending upon whether the furrow had regressed or had progressed through the animal hemisphere by the time of third cleavage. We found that we could predict where a partial furrow would be located by moving the injection site slightly off-center. For example, if the injection site was shifted towards the animal pole a partial furrow would be observed at the equator. In each of six embryos in which the injection site was moved off center and a partial furrow was
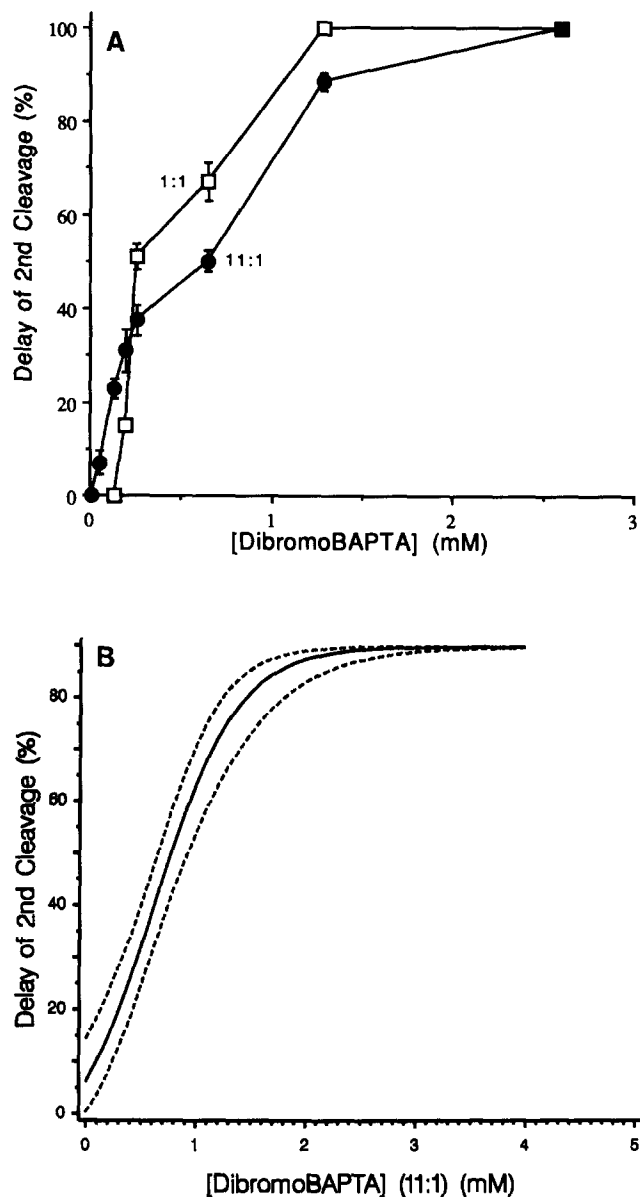


*Figure 5.* The dose-dependent delay of second cleavage after the microinjection of dibromoBAPTA with two different amounts of coinjected calcium. Intracellular concentrations of the 1:1 ratio of dibromoBAPTA:calcium ranged from 0.13 to 2.6 mM and from 0.01 to 2.6 mM for the 11:1 ratio. The number of embryos scored for each concentration ranged from 11 to 78 for the 1:1 ratio and 12 to 68 for the 11:1 ratio. Error bars indicate SEM calculated as described in the Materials and Methods. If no error bar is shown the error is smaller than the size of the symbol. Open squares indicate the delay of cleavage for embryos receiving the 1:1 ratio whereas closed circles indicate the delay of cleavage for embryos receiving the 11:1 ratio of dibromoBAPTA:calcium. (*B*) Logistic regression and a 95% confidence interval for the delay of second cleavage when dibromoBAPTA (11:1) is injected (goodness of fit, $P = 0.61$). Confidence intervals and logistic regression calculated as described in the Materials and Methods.

observed, the furrow occurred opposite to the injection site (dibromoBAPTA, 0.65 mM). We believe that the partial furrow appears because the BAPTA-type buffer had not completely diffused into the distal portions of the blastomere so

the cell was able to maintain a sufficient calcium gradient for furrow formation in that region.

Embryos were injected throughout the two-cell stage, so occasionally the second cleavage furrow would appear during the injection. These embryos were not included in the experiment because they were not injected before cleavage began but their development was sometimes monitored. We observed that the furrow forming at the animal pole did not progress toward the equator after the calcium buffer injection and sometimes the oncoming cleavage furrow receded. We have observed a cleavage furrow being arrested as it approached the area containing the injected buffer in at least five different embryos. In contrast, control KCl injections never interfered with furrow formation.

## Discussion

We have conducted the first direct comparison of the effectiveness of four different $Ca^{2+}$ buffers at inhibiting cleavage in an animal system. Our results indicate that the ability of such buffers to inhibit cleavage is not a simple function of their $Ca^{2+}$ affinity. Both high affinity and low affinity buffers are less effective than those with moderate affinities. This helps to clear up many conflicting observations in the literature. For example, two previous studies using EGTA as a $Ca^{2+}$ chelator reported that very high levels (we estimate up to 30 mM) were required to inhibit cleavage (2, 32). Our data suggest that this is exactly what one would expect given the very high affinity of EGTA ($K_d = 0.02$ $\mu$M) in 110 mM ionic strength frog cytoplasm at pH 7.7. We have found that the microinjection of calcium buffers into two-cell stage *Xenopus laevis* blastomeres delays cleavage in the injected blastomere, and we have identified dibromoBAPTA as the most effective of the four BAPTA-family buffers tested at delaying cleavage based on the intracellular concentration of buffer required. Buffers with either lower or higher affinity for $Ca^{2+}$ than dibromoBAPTA required higher concentrations to produce a similar delay of cleavage (Fig. 4). These results support the model of calcium buffers acting within the cell to dissipate a calcium gradient necessary for cleavage (27). The most efficient shuttle molecule should exhibit a calcium affinity that would nearly saturate it when it entered the high $[Ca^{2+}]_i$ region and allow it to rapidly lose the calcium as it diffused away into a region with lower $[Ca^{2+}]_i$. A buffer with a higher than optimum calcium affinity would be completely saturated in the high $[Ca^{2+}]_i$ region and would not lose the calcium as quickly when it diffused away into lower $[Ca^{2+}]_i$ regions. It would therefore be less effective as a shuttle molecule that reduces the calcium gradient. Similarly, a buffer with a lower than optimum calcium affinity would not bind calcium as well in the high $[Ca^{2+}]_i$ region so more of this molecule would be required to give the same reduction in the calcium gradient.

Our data do not support the common notion that these calcium buffers act by changing the global calcium concentration. If that were the case, there should be a roughly linear relationship between the dissociation constant and the concentration of buffer needed to delay cleavage (17):

$$[Ca^{2+}]_i = K_d [Ca-BAPTA]/[BAPTA]. \qquad (2)$$

These data do not show a linear increase in buffer effectiveness as the affinity for calcium is increased. Instead we ob-

serve a U-shaped curve when buffer effectiveness is compared to the buffer dissociation constant. In addition, two dibromoBAPTA solutions with very different free calcium concentrations were found to exhibit similar cleavage inhibition ability. We used MaxChelator (version 6.5) written by Chris Patton of Hopkins Marine Station (Pacific Grove, CA) to calculate the $[Ca^{2+}]$ of these two buffer solutions to be 40 and 0.17 $\mu$M. We therefore conclude that the critical target for $Ca^{2+}$ buffer action is not likely to be the overall $[Ca^{2+}]_i$ in the egg but is more likely to be a localized region of increased $[Ca^{2+}]_i$ where the concentration reaches levels near the $K_d$ of dibromoBAPTA (1.5 $\mu$M).

These same conclusions were made by Speksnijder et al. (27) who found no difference in the effectiveness of dibromoBAPTA at inhibiting fucoid egg development when it was coinjected with three different amounts of calcium. In addition this model predicts that the global $[Ca^{2+}]_i$ remains unchanged by the calcium buffers and that was observed by Kao et al. (15) who reported that the measured basal calcium level in Swiss 3T3 cells loaded with BAPTA is not significantly different from cells without BAPTA. These results support the hypothesis that calcium gradients are necessary for cellular events and that BAPTA-type buffers disrupt these regulatory calcium gradients without altering the overall calcium concentration.

Difficulties in reproducibly measuring such $[Ca^{2+}]_i$ changes have also led some investigators to suggest that calcium gradients or localized $[Ca^{2+}]_i$ increases rather than global $[Ca^{2+}]_i$ increases are responsible for the regulation of these events. Kao et al. (15) observed calcium transients at nuclear envelope breakdown, the metaphase-to-anaphase transition, and cell division in Swiss 3T3 fibroblasts. These transients, however, were measured only in cells cultured in serum-containing media. They demonstrated that although no transients were detected in cells cultured without serum, calcium does indeed still regulate nuclear envelope breakdown in these cells because BAPTA inhibited nuclear envelope breakdown and an increase in $[Ca^{2+}]_i$ caused by photolysis of nitr-5 elicited precocious nuclear envelope breakdown. These investigators concluded that nuclear envelope breakdown is regulated by a localized calcium increase which is not detected in global measurements of calcium levels.

Similarly, Grandin and Charbonneau (9) measured very weak $[Ca^{2+}]_i$ oscillations using $Ca^{2+}$-selective microelectrodes in cleaving *Xenopus laevis* embryos with a 30 min periodicity, the length of the cell cycle. These investigators were unable to detect oscillations in $[Ca^{2+}]_i$ in the earliest cell divisions, but detected oscillations in the later cell divisions. They suggested that the large volume of the *Xenopus* blastomeres at the earliest divisions makes the oscillations more difficult to detect because the increase in $[Ca^{2+}]_i$ may be occurring only in localized areas.

How are these suspected $[Ca^{2+}]_i$ gradients generated and what are their likely targets? Our earlier work (10) suggested that, in addition to $[Ca^{2+}]_i$ gradients, $PIP_2$ turnover and $Ins(1,4,5)P_3$-induced $Ca^{2+}$ release were required for normal cleavage. Thus, components of the inositol cascade may well be involved in this $[Ca^{2+}]_i$ gradient generation. Since *Xenopus laevis* eggs cleave perfectly normally in distilled water, it is unlikely that $Ca^{2+}$ influx contributes to any $[Ca^{2+}]_i$ gradients required for cleavage. We must emphasize that no localized $[Ca^{2+}]_i$ gradients have been reported thus far in

cleaving eggs, and it will be important to determine if these $Ca^{2+}$ buffers suppress any gradients that are observed in the future.

The most likely target for such $[Ca^{2+}]_i$ gradients is the cleavage furrow because some of the observations made during the course of our experiments suggest that calcium gradients are necessary at the time of furrow formation and progression. The occurrence of partial cleavage furrows and the disappearance of an oncoming cleavage furrow upon buffer injection indicate an immediate requirement for a calcium gradient rather than calcium acting earlier to indirectly regulate cell division. Miller et al. (20) also report that progressing cleavage furrows can be arrested by dibromo-BAPTA injection and conclude that there is an immediate requirement for the calcium gradient for furrow formation during cleavage.

Cao and Wang (3, 4) have shown that the contractile ring is formed chiefly from the migration of actin filaments into the furrow. A localized increase in $[Ca^{2+}]_i$ in the region of furrow formation may serve to recruit actin filaments into the contractile ring, and Fluck et al. (8) have detected a localized increase in $[Ca^{2+}]_i$ in the region of furrow formation with aequorin in the medaka embryo. Another possible target for a regulatory calcium gradient would be the phosphorylation of myosin by myosin light chain kinase. Contraction of actomyosin gels isolated from *Xenopus* embryos has been associated with phosphorylation of myosin (7) and phosphorylation has been found to be triggered by calcium via a calmodulin-dependent protein kinase.

The inhibition of development to tadpole stage by $Ca^{2+}$ buffer concentrations too low to delay cleavage is quite interesting. Embryos injected with as little as 0.1 mM dibromoBAPTA usually cleave normally but exhibit patches of white (dead?) cells at gastrula and neurula stage and do not survive to the tadpole stage. We do not know the cause of this inhibition but suggest that it might be due to enhanced net $Ca^{2+}$ influx resulting from a reduced efflux due to lower subsurface $[Ca^{2+}]$. Enhanced influx might strain the cell's ability to pump $Ca^{2+}$ out so that cells might become overloaded with $Ca^{2+}$ and die as proposed for similarly injected seaweed eggs (27).

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