INTRODUCTION

Ca²⁺ oscillations serve as intracellular signals that can have profound effects on cellular development and function. These oscillations first occur in the egg and may continue to regulate embryonic growth and differentiation throughout development, although the latter has not been demonstrated. In all mammalian species examined to date, the spermatozoon induces a series of repetitive Ca²⁺ transients (oscillations) in the egg during fertilization (Fissore et al., 1992; Fissore and Robl, 1993; Kline and Kline, 1992; Miyazaki et al., 1986; Miyazaki et al., 1993; Sun et al., 1992). These oscillations in the intracellular Ca²⁺ concentration, [Ca²⁺]ᵢ, are critical for the regulation of cytoplasmic maturation (Carroll and Swann, 1992), the production of maturation-promoting factor (Whitaker and Patel, 1985; Colonna et al., 1989; Epel, 1990; Nuccitelli, 1991) and the resumption of meiosis (Felici et al., 1991; Fissore et al., 1992; Homa, 1991; Sun et al., 1992; Vitullo and Ozil, 1992). The temporal pattern of Ca²⁺ oscillations that occurs in the egg following sperm binding could be critical for the completion of preimplantation embryogenesis.

Only a few studies have investigated the role of Ca²⁺ in development past the 1-cell stage. Ozil (1990) and later, Vitullo and Ozil (1992), demonstrated that mammalian oocytes could develop parthenogenetically to the blastocyst stage if intracellular Ca²⁺ oscillations were induced at a specific frequency. Depending on the frequency, induced Ca²⁺ transients caused either a failure to develop, several cleavage divisions or complete development to the blastocyst stage. These were the first studies demonstrating that Ca²⁺ signalling could affect preimplantation development, even several cell divisions after the Ca²⁺ oscillations occurred. Earlier work using ethanol or ionophores to activate oocytes parthenogenetically promoted...
only a few cleavage divisions before the eggs degenerated. The fact that ethanol and ionophores generate only a single intracellular Ca$^{2+}$ transient may help to explain these observations. Stricker (1995), in one of the few reported investigations of Ca$^{2+}$ signalling during early embryonic development, showed that repetitive Ca$^{2+}$ oscillations are prevalent for several cell cycles after fertilization of starfish oocytes. Furthermore, the initial post-fertilization Ca$^{2+}$ oscillations, originating from IP$_3$ stores, were necessary for normal development. In agreement, similar oscillations in the [Ca$^{2+}$]i occur in mammalian oocytes at fertilization and are critical for egg activation (Bement, 1992; Colonna et al., 1989; Cuthbertson and Cobbold, 1985). These Ca$^{2+}$ transients may also determine the developmental fate of embryos in mice and other mammals, but their exact role during development, and the biochemical pathways that they affect, are currently unresolved.

The temporal release of Ca$^{2+}$ from intracellular stores varies with cell type and is regulated by either (1) Ca$^{2+}$-induced Ca$^{2+}$ release acting through the ryanodine receptor (RR; e.g. skeletal muscle), (2) the binding of inositol 1,4,5-trisphosphate (IP$_3$) to the IP$_3$ receptor (IPR; e.g. Xenopus) or (3) both mechanisms (e.g. atrial cells, neurons, sea urchin eggs and mouse oocytes; Berridge, 1991, 1993; Galione et al., 1993; Lee et al., 1993; Miyazaki et al., 1993; Berridge and Dupont, 1994; Kline and Kline, 1994; Ayabe et al., 1995). The RR and IPR, in turn, serve as Ca$^{2+}$ channels (Berridge, 1993) that share both structural and functional homology, reflecting a common evolutionary origin (Mignery et al., 1989; Tsien and Tsien, 1990). However, they are functionally distinct (Clapper et al., 1987; Dargie et al., 1990; Feng et al., 1992; Galione et al., 1991; Lee et al., 1993; Worley et al., 1987).

In oocytes, the release of Ca$^{2+}$ through the IPR is regulated by the concentration of either Ca$^{2+}$ or IP$_3$. For example, Ca$^{2+}$ is most sensitizing over the 100-300 nM range, with higher concentrations leading to IPR inactivation (Amundson and Clapham, 1993). Therefore, the modulation of IPR activity by intracellular Ca$^{2+}$ and IP$_3$ levels provides a mechanism for the generation of Ca$^{2+}$ oscillations (Berridge, 1993; De Young and Kiezer, 1992; Missiaen et al., 1992; Swann, 1991; Tsien and Tsien, 1990). In the mammalian oocyte, the existence of the IPR is well documented (Miyazaki et al., 1992), but the role of the RR is controversial (Swann, 1991; Kline and Kline, 1994; Ayabe et al., 1995). Although it is likely that the receptors present in the mouse oocyte function in the same way during mouse embryogenesis, this hypothesis is currently untested.

While much is now understood about the mechanism of intracellular Ca$^{2+}$ release and the critical role that it plays in the events immediately following sperm-egg binding, it is unclear whether post-fertilization Ca$^{2+}$ transients regulate mammalian preimplantation growth and differentiation. Stricker (1995) provided some of the first evidence that Ca$^{2+}$ signals occur during early embryo development. We have recently demonstrated a functional role for Ca$^{2+}$ transients during murine embryogenesis. Pre- and peri-implantation development in vitro is accelerated after releasing Ca$^{2+}$ from intracellular stores using either ethanol or the Ca$^{2+}$ ionophore, A23187 (Stachecki et al., 1994a,b). The induction of a Ca$^{2+}$ transient in blastocysts during in vitro culture, followed by the transfer of these embryos to pseudopregnant dams 24 hours later, resulted in implantation rates that were twice the rate obtained with untreated embryos (Stachecki et al., 1994b). The observed developmental acceleration in vitro following ethanol or ionophore treatment does not surpass the rate of development in vivo, but clearly suggests an important regulatory role for Ca$^{2+}$ signalling during postfertilization development. Evidence to support the hypothesis that intracellular Ca$^{2+}$ signals occur naturally and regulate preimplantation embryogenesis (Collas et al., 1993; Ozil, 1990; Keating et al., 1994; Stricker, 1995) is still very preliminary, but could reveal critical biochemical mechanisms that control early mammalian development. To this end, the present study examines whether alterations of intracellular Ca$^{2+}$, either through naturally occurring mechanisms or the action of pharmacological agents, will specifically influence the rate of embryonic growth and morphogenesis in vitro.

**MATERIALS AND METHODS**

**Collection and treatment of embryos**

NSAxB6SJL oocytes and morulae were collected from superovulated mice as previously described (Leach et al., 1993; Stachecki et al., 1994a) and cultured at 37°C in Ham’s F10 medium supplemented with 4 mg/ml bovine serum albumin (BSA). Some embryos were pre-treated with BAPTA-AM (AM; acetoxymethyl ester) in Ham’s F10 medium for 20 minutes and/or 5 μM fluo-3-AM for 30 minutes and then washed in fresh medium. Morulae were also exposed to various agents including ethanol (Midwest Grain Company, Pekin, IL), ionomycin, A23187, thimerosal, dithiothreitol (DTT), lysophosphatidylcholine (LPC; a vital permeabilizing agent; Khidir et al., 1995), caffeine, (Sigma Chemical Co., St Louis, MO), W-7 HCl, W-12 HCl, IP$_3$ and/or ryanodine (Calbiochem, La Jolla, CA). These chemicals were diluted at least 1000-fold from stock solutions into Ham’s F10 medium or Ca$^{2+}$-free Biggers, Whitten and Whittingham medium (BWW; Biggers et al., 1971), supplemented with 4 mg/ml BSA, to achieve a working concentration. LPC was tested within the range 0.005% to 0.05% to determine the optimal working concentration that would allow permeabilization without decreasing embryo viability, as previously pointed out (Khidir et al., 1995). Each lot of BAPTA-AM produced slightly different results. The data in Tables 1 and 2 were produced using a different lot of BAPTA-AM from the data in all other experiments.

**Cavitation and blastocoel expansion**

Embryos were examined for cell number, the percentage cavitating and blastocoel expansion using epifluorescence microscopy and an image analysis system, as described previously (Stachecki et al., 1994). Cavitation and blastocoel expansion was observed using a Leitz (Wetzlar, Germany) Fluovert FU inverted microscope with Hoffman modulation contrast optics at 200x magnification, and recorded on a Panasonic AG-7350 video cassette recorder using a Dage CCD72 video camera (DAGE-MTI, Inc., Michigan City, IN). Video images of cavitating morulae were analyzed using MCID M4 image analysis software (version 4.12, Imaging Research, St Catherines, Ontario, Canada). The perimeter of the blastocoel cavity was traced using a computer mouse and, assuming spherical geometry, converted to volume using the formula:

\[ V = \frac{P^3}{(6\pi^2)}, \]

where P is the perimeter in μm, determined by the imaging software, and V is the volume in fl (×10$^{-15}$). Volumes were reported in pl (×10$^{-12}$).
Table 1. The effect of BAPTA-AM on [Ca^{2+}] and cavitation rates of murine morulae

<table>
<thead>
<tr>
<th>[BAPTA-AM] (µM)</th>
<th>[Ca^{2+}] (nM)</th>
<th>n1</th>
<th>28</th>
<th>48</th>
<th>56</th>
<th>60</th>
<th>n2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>220.0±21.0</td>
<td>20</td>
<td>53.3</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>0.1</td>
<td>179.2±10.2</td>
<td>15</td>
<td>28.5*</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>28</td>
</tr>
<tr>
<td>1.0</td>
<td>161.9±8.6*</td>
<td>15</td>
<td>33.3*</td>
<td>75.8*</td>
<td>100</td>
<td>100</td>
<td>66</td>
</tr>
<tr>
<td>10.0</td>
<td>91.0±11.6*</td>
<td>15</td>
<td>13.5*</td>
<td>29.7*</td>
<td>77.0*</td>
<td>100</td>
<td>74</td>
</tr>
<tr>
<td>100.0</td>
<td>82.15±15.6*</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42</td>
</tr>
</tbody>
</table>

Mean values±SEM are shown for [Ca^{2+}]. Data were combined from three separate experiments.†Morulae were loaded with BAPTA-AM for 20 minutes in Ham’s F10 medium at 37°C and then washed in BAPTA-AM-free medium. Some embryos (n2) were examined to determine the percentage cavitating during the next 60 hours, while others (n1) were loaded with 5 µM fluo-3-AM for 30 minutes to estimate the [Ca^{2+}]. *Values are lower (P<0.05) than controls.

Nuclear staining

In order to determine the number of blastomeres present in developing embryos, they were exposed to the nuclear stain 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) at 5 µM for 30 minutes, placed onto a clean slide, overlaid with mounting medium (Crystal/Mount, Biomeda Corp, Foster City, CA), coverslips placed on top, and allowed to air dry. DAPI-stained embryos were examined using a Leitz Diaplan microscope at 250-400X magnification. Cell number was determined in morulae and blastocysts at 24, 32 and 48 hours after morulae collection. The number of blastomeres was also determined for embryos in their first hour of cavitation.

Calcium measurement

The [Ca^{2+}] of fluo-3-AM-treated morulae was monitored using epi-fluorescence microscopy and an image analysis system, as described previously (Stachecki et al., 1994a). Images were printed using a Tektronix Phaser Hsdx color printer (Tektronix Inc., Beaverton, OR). For [Ca^{2+}] estimation, embryos were cultured in Ca^{2+}-free BWW. All measurements were made at 37°C using a digitally controlled stage warmer (Brook Industries, Lake Villa, IL) attached to a Leitz Fluorvert FU inverted microscope with a 20X fluorescence objective (EFL·fluorescence objective (EFL·fluorescence objective (EFL·fluorescence objective (EFL)). A Coherent Innova 90 Ion Laser (Coherent Laser Products; Palo Alto, CA) was used to excite Indo-1 and the results were analyzed using the Calcium Quantitation Program (v 3.22; Meridian).

Statistics

Differences in the number of embryos cavitating in the control and treatment groups were tested for significance using chi-square analysis. Differences in the [Ca^{2+}], blastocoel volumes and cell numbers in mouse embryos were assessed using a repeated measures ANOVA. The differences in the [Ca^{2+}] for embryos exposed to ryanodine or IP3 were examined separately by repeated measures ANOVA. For multisample ANOVA analyses, an overall significance was calculated before proceeding with secondary tests (Fisher PLSD and Dunnett) to determine the groups that were significantly different.

RESULTS

Culture conditions

We have previously cultured preimplantation embryos in Ham’s F10 medium for use in cavitation experiments (Stachecki et al., 1994a); however, recent studies have shown that the results were almost identical in the present study. MI). A Coherent Innova 90 Ion Laser (Coherent Laser Products; Palo Alto, CA) was used to excite Indo-1 and the results were analyzed using the Calcium Quantitation Program (v 3.22; Meridian).

Fig. 1. The onset of blastocoel formation. Morulae were collected on gestation day 3 and cultured in Ham’s F10 medium for 24 hours. Embryos that are just beginning to form a blastocoel cavity (arrows), as well as those with partially expanded blastocoels, are shown. All of the embryos shown have initiated cavitation and would therefore be scored as such in assessing the percentage of embryos that have cavitated. Bar, 100 µm.
that KSOM, a newly developed culture medium, can enhance embryo development in vitro (Erbach et al., 1994). We have found that KSOM improves development through the 2-cell block, but development from the 8-cell morulae stage to the blastocyst stage was not significantly affected (unpublished data). Morulae cultured in Ham’s F10 medium cavitated at the same rate (31.4% at 24 hours; 65.7% at 29 hours; 97.1% at 48 hours; n=35) as those cultured in KSOM (23.3% at 24 hours; 56.7% at 29 hours; 96.7% at 48 hours; n=30; P>0.05). There was also no difference (P>0.05) in the number of cells in embryos cultured in Ham’s F10 medium (26.4±0.7; n=14; 51.4±3.2; n=10) or KSOM (24.9±1.3; n=13; 49.7±2.3; n=12) at 24 hours and 48 hours of incubation, respectively. Therefore, we continued to use Ham’s F10 medium as in our previous studies of preimplantation development in the mouse (Leach et al., 1993; Satchecki et al., 1994a,b).

**Measurement of [Ca\(^{2+}\)]\(_i\) in mouse embryos**

Accurate assessment of [Ca\(^{2+}\)]\(_i\) in morulae using the non-ratioable dye fluo-3-AM is difficult because of errors associated with loading concentration, dye leakage and bleaching. Because the [Ca\(^{2+}\)]\(_i\) in mouse oocytes has been well established (Carroll and Swann, 1992; Tombes et al., 1992; Kline and Kline, 1994), comparison of the fluorescence ratios of oocytes and morulae loaded with indo-1-AM allowed us to determine the [Ca\(^{2+}\)]\(_i\), more precisely. The ratio of bound to unbound indo-1 fluorescence intensities in murine morulae and oocytes was similar (0.423±0.011 and 0.421±0.006, respectively; P>0.05), indicating that the basal [Ca\(^{2+}\)]\(_i\) of morulae and oocytes are not significantly different. This information was used to verify the accuracy of [Ca\(^{2+}\)]\(_i\) estimations generated using fluo-3-AM.

**Effect of BAPTA-AM on [Ca\(^{2+}\)]\(_i\) and development**

To determine whether Ca\(^{2+}\) signalling is required during preimplantation embryonic development, the effect of the Ca\(^{2+}\) chelator, BAPTA-AM, on [Ca\(^{2+}\)]\(_i\), and the cavitation rate was assessed. Morulae treated with various concentrations of BAPTA-AM and examined using the fluorescent Ca\(^{2+}\) indicator, fluo-3 AM, exhibited a dose-dependent decrease in baseline [Ca\(^{2+}\)]\(_i\), (Table 1). The [Ca\(^{2+}\)]\(_i\) was suppressed for 2 to 3 hours following BAPTA-AM-exposure before increasing to a level slightly below the baseline concentration (Fig. 2). The [Ca\(^{2+}\)]\(_i\) equilibrated below the basal level because the K\(_d\) of BAPTA for Ca\(^{2+}\) (192 nM) is lower than the basal level of free Ca\(^{2+}\) in the cytoplasm (220 nM; Table 1). BAPTA should be a poor Ca\(^{2+}\) chelator below its K\(_d\) and an excellent one only when the [Ca\(^{2+}\)]\(_i\) exceeds K\(_d\); this is, in fact, what we observed. Chelating intracellular Ca\(^{2+}\) with BAPTA-AM also caused a dose-dependent delay in the onset of cavitation (Table 1). Embryos exposed to 10 or 20 µM BAPTA-AM cavitated significantly later than the control embryos. Exposure of mouse embryos to 0.1-20 µM BAPTA-AM was not toxic, as all embryos eventually cavitated. However, the majority of the embryos exposed to 100 µM BAPTA-AM degenerated 24 hours after treatment.

**Effect of BAPTA-AM on Ca\(^{2+}\) signalling**

We next investigated the effect of BAPTA-AM on Ca\(^{2+}\) signalling. BAPTA-AM-treated embryos were exposed to 1 µM ionomycin and the peak [Ca\(^{2+}\)]\(_i\) during the subsequent transient was measured. The ability of morulae to produce a Ca\(^{2+}\) transient after ionomycin treatment was inhibited by BAPTA-AM in a dose-dependent manner (Fig. 3). At the lowest concentration of BAPTA-AM (1 µM), a large increase in fluorescence, comparable to control embryos, was observed after

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**Fig. 2.** The effect of BAPTA-AM on the basal [Ca\(^{2+}\)]\(_i\), over time. Embryos were preloaded with 0 µM (filled circles), 1 µM (diamonds), 10 µM (squares), 20 µM (open circles) or 100 µM (triangles) BAPTA-AM for 20 minutes, washed in fresh Ham’s F10 medium, and treated with 5 µM fluo-3-AM for 30 minutes at 37°C before analysis. Morulae were then examined for alterations in [Ca\(^{2+}\)]\(_i\) at various times during the next 24 hours. There was no significant change in [Ca\(^{2+}\)]\(_i\) after the initial few hours, therefore only results for the first 5 hours are shown. The error bars represent the SEM of data combined from at least three separate experiments.

**Fig. 3.** Effect of BAPTA-AM on the timing of intracellular Ca\(^{2+}\) signalling. Embryos were preloaded with (A) 0 µM (circles) or 1 µM (diamonds) or (B) 10 µM (squares) or 20 µM (triangles) BAPTA-AM for 20 minutes, washed in fresh Ham’s F10 medium, and treated with 5 µM fluo-3-AM for 30 minutes at 37°C. Morulae were then exposed to 0.5 µM ionomycin at either 1, 2, 4, 5, 6 or 8 hours following the BAPTA-AM treatment. Morulae loaded with 10 µM BAPTA surpassed the threshold level of 365 nM Ca\(^{2+}\) by 4 hours whereas Ca\(^{2+}\) peaks in morulae loaded with 20 µM BAPTA remained below 365 nM until 8 hours. The data points represent an average of the maximal [Ca\(^{2+}\)]\(_i\) attained, as calculated from fluo-3 fluorescence intensity. The error bars represent the SEM of data combined from at least three separate experiments.
treatment with ionomycin at all times (Fig. 3A), but higher concentrations of BAPTA-AM (10 μM or 20 μM) chelated the majority of the Ca²⁺ released to the cytoplasm by ionomycin (Fig. 3B). Although ionomycin always released a large amount of Ca²⁺, the fluorescence intensity only increased when the concentration of BAPTA became low enough to free the Ca²⁺ for binding to fluo-3. Accordingly, this experiment reveals that the intracellular concentration of BAPTA decreased in a temporal manner, either by degradation or by export from the cell. It appears that the period of nonresponsiveness to ionomycin (Fig. 3), rather than the period of depressed baseline [Ca²⁺] (Table 1), better reflected the time required for the embryo to remove or degrade internalized BAPTA and regain its ability to utilize Ca²⁺-mediated signalling pathways.

**Effect of Ca²⁺ signalling on cavitation**

Consistent with our previous findings (Stachecki et al., 1994a), exposure of mouse morulae to 0.1% ethanol or 1 μM ionomycin caused a transient increase in [Ca²⁺], and accelerated the onset of cavitation (% cavitation) and expansion of the blastocyst (blastocoe volume; Table 2). To establish a direct relationship between the elevation of [Ca²⁺] and the acceleration of preimplantation development, we examined the ability of morulae to cavitate after chelating the intracellular Ca²⁺ released by exposure to ethanol or ionomycin. Exposure of morulae to 0.5 μM BAPTA-AM neither decreased [Ca²⁺], nor altered the cavitation rate (Table 2). When morulae were pre-treated with 0.5 μM BAPTA-AM before the addition of 0.1% ethanol, the increase in the [Ca²⁺] that occurs with ethanol exposure alone was attenuated (Table 2). Consistent with the hypothesis that [Ca²⁺] elevation is responsible and necessary for the acceleration of development, cavitation following treatment with BAPTA-AM and ethanol progressed at a rate similar to that of control embryos (Table 2). In contrast, the same concentration of BAPTA-AM was unable to chelate all of the Ca²⁺ released following the addition of 1 μM ionomycin and, as expected, an accelerated rate of blastocoe formation occurred (Table 2). These results, together with previously published data (Stachecki et al., 1994a), establish that the generation of Ca²⁺ transients is, in fact, essential during cavitation and can have profound effects on progression of development throughout the preimplantation period. Furthermore, the ability of ethanol and ionophores to induce a Ca²⁺ transient is central to their ability to accelerate development.

Although fluo-3 also chelates Ca²⁺, more than 90% of the embryos cultured overnight in 5 μM fluo-3-AM cavitated at a rate similar to control embryos (P=0.5; P>0.05). This suggests that the constant chelation of Ca²⁺ by fluo-3 does not impair development and thus, does not inhibit the production of a Ca²⁺ transient large enough to trigger division to the 16-cell stage and promote cavitation. This is possible because the Kᵦ of fluo-3 for Ca²⁺ (316 nM) is higher than that of BAPTA (192 nM). Therefore, fluo-3 efficiently binds Ca²⁺ at a much higher [Ca²⁺], permitting the cell to generate a Ca²⁺ transient above the minimum required for continuation of the cell cycle.

**Effect of BAPTA-AM and ionomycin on cell number**

To further explore the role of Ca²⁺ in blastocoe formation and determine whether cavitation is linked to cell number, we induced or blocked Ca²⁺ signalling using ionomycin or BAPTA-AM, respectively (Table 3). As above (Table 1), morulae exposed to BAPTA-AM exhibited a dose-dependent delay in cavitation, whereas those treated with ionomycin cavitated sooner than controls. Note that different lots of BAPTA-AM were used in Tables 1 and 3, and these differed in their inhibitory potency. BAPTA-AM also caused a dose-dependent delay in cell division (Table 3). The number of cells in morulae cultured for 24 or 48 hours decreased as the concentration of BAPTA-AM increased. Individual embryos exposed to 10-40 μM BAPTA-AM that did not cavitate also failed to cleave to the 16-cell stage by 48 hours, whereas all embryos that reached the 16-cell stage by 24 hours contained a blastocoe. Furthermore, all embryos reaching the 16-cell stage had a high probability (>90%) of cavitating, regardless of prior treatment. In every group, embryos beginning to cavitate had, on average, at least 16 cells (16-24, range; 21±1, mean±SEM; n=50) and no morulae reached the 32-cell stage without first beginning to cavitate. Only two morulae (4%) began cavitating before reaching the 16-cell stage. All embryos that began to cavitate continued to divide (cell number at 24 hours versus 48 hours) regardless of previous treatment. In contrast to embryos treated at the 8-cell stage, 16-cell morulae exposed to 20 μM BAPTA-AM cavitated at a rate similar to control embryos (P<0.05; data not shown).

**Detection of the IP₃ receptor**

Exposure of murine morulae to the IPR-specific agonist, thimerosal (Bootman et al., 1992; Fissore et al., 1992; Fissore...
and Robl, 1993; Galione et al., 1993; Kline and Kline, 1994; Missiaen et al., 1991, 1992; Miyazaki et al., 1992, 1993), induced intracellular Ca\(^{2+}\) oscillations (Fig. 4A). Coaddition of dithiothreitol, a sulfhydryl antagonist, specifically blocked the action of thimerosal, inhibiting the release of intracellular Ca\(^{2+}\) (Fig. 4A). Instead, 0.01-10 mM caffeine, a methylxanthine that releases intracellular Ca\(^{2+}\) from ryanodine-sensitive storage sites (Feng et al., 1992; Galione et al., 1993; Kline and Kline, 1994; Lechleiter and Clapham, 1992; Malgaroli et al., 1990), did not alter the [Ca\(^{2+}\)] (Fig. 4B).

Direct evidence for the existence of IPRs and RRs was obtained by the introduction of IP3 and ryanodine into morulae permeabilized with LPC, a vital permeabilizing agent (Khidir et al., 1995). Morulae exposed to LPC and 50 mM IP3 exhibited a significant increase in [Ca\(^{2+}\)] (Fig. 5A). Treatment with 100 nM to 100 mM IP3 produced a significant Ca\(^{2+}\) transient, whereas 10 nM IP3 did not (Table 4). Addition of LPC alone had no effect on baseline [Ca\(^{2+}\)] (Fig. 5B, first arrow); however the [Ca\(^{2+}\)] increased immediately after addition of IP3 to embryos that had first been treated with LPC (Fig. 5B, second arrow). Coaddition of LPC and ryanodine caused a small, nonsignificant alteration of baseline [Ca\(^{2+}\)] (Fig. 5C, first arrow), whereas the subsequent addition of IP3 (Fig. 5C, second arrow) produced a rapid and marked elevation in the [Ca\(^{2+}\)]. We tested a range of ryanodine concentrations from 1 nM to 100 mM and observed a small, but significant, increase in the [Ca\(^{2+}\)] at 10 nM (Table 4). Morulae pretreated with 1 or 10 mM BAPTA-AM and exposed to LPC and IP3 exhibited a significantly reduced Ca\(^{2+}\) transient (Fig. 5D). The [Ca\(^{2+}\)] did not immediately increase in Fig. 5A,D, because of the time necessary for cellular permeabilization (85 seconds, on average) by LPC (Khidir et al., 1995). These results, similar to those generated with mouse oocytes (Ayabe et al., 1995; Kline and Kline, 1992; Xu et al., 1994), suggest that IPRs, and possibly RRs, are present in murine preimplantation embryos, and further indicate that the Ca\(^{2+}\) transients produced by ethanol and ionophores are predominantly derived from IP3-mediated stores.

### Critical role of calmodulin

It is well known that Ca\(^{2+}\) signalling has widespread developmental effects. This is due, in part, to the Ca\(^{2+}\)-binding protein calmodulin, which transduces Ca\(^{2+}\) signals for a plethora of biochemical reactions that affect the cell cycle, microtubule formation and the activation of protein kinases and many other enzymes (Lu and Means, 1993; Chafoules et al., 1982). To determine whether calmodulin serves as a downstream transducer of Ca\(^{2+}\) signals during mouse preimplantation embryogenesis we treated morulae with the calmodulin antagonist W-7, or the less active, structurally related analog, W-12, and monitored the rate of blastocoel formation. Treatment with W-7 for 18 hours inhibited cavitation in a dose-dependent manner (Fig. 6A). At a concentration of 10 mM, W-7 completely inhibited cavitation while 10 mM W-12 had no effect (Fig. 6A). Embryos treated with 5 mM W-7 or 10 mM W-7 had fewer (P<0.05) cells after 24 hours of culture than stage-matched controls (14.8±1.1, 11.4±1.6 and 19.5±1.5, respectively).

To determine more precisely whether the interaction of Ca\(^{2+}\) with calmodulin was necessary to accelerate cavitation, we inhibited calmodulin during the period of Ca\(^{2+}\) signalling. Morulae were pretreated for 2 hours in 10 mM W-7 before elevating intracellular Ca\(^{2+}\) with 1 mM A23187. After ionophore treatment, morulae were incubated for an additional 2 hours in W-7 to maintain the inactivation of calmodulin throughout the period of elevated intracellular Ca\(^{2+}\). Treatment

### Table 3. Altering intracellular Ca\(^{2+}\) release affects cell number and cavitation rate

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Time (hours)</th>
<th>% Cavitation</th>
<th>Cell number‡</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Morulae</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>30.40 (148)</td>
<td>17±1 (24)</td>
</tr>
<tr>
<td></td>
<td>28-32</td>
<td>60.87 (115)</td>
<td>15±2 (14)</td>
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<tr>
<td></td>
<td>48</td>
<td>93.91 (115)</td>
<td>10±1 (7)</td>
</tr>
<tr>
<td>1 µM iomycin</td>
<td>24</td>
<td>53.40 (58)*</td>
<td>14±1 (22)</td>
</tr>
<tr>
<td></td>
<td>28-32</td>
<td>94.29 (58)*</td>
<td>13±1 (22)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>100.00 (58)</td>
<td>ND</td>
</tr>
<tr>
<td>1 µM BAPTA-AM</td>
<td>24</td>
<td>21.18 (85)</td>
<td>16±2 (11)</td>
</tr>
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<td></td>
<td>28-32</td>
<td>59.70 (67)</td>
<td>17±3 (11)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>97.00 (67)</td>
<td>22±9 (4)*</td>
</tr>
<tr>
<td>10 µM BAPTA-AM</td>
<td>24</td>
<td>21.24 (113)</td>
<td>12±1 (11)*</td>
</tr>
<tr>
<td></td>
<td>28-32</td>
<td>52.08 (96)</td>
<td>12±2 (9)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>91.67 (96)</td>
<td>14±2 (17)</td>
</tr>
<tr>
<td>20 µM BAPTA-AM</td>
<td>24</td>
<td>3.30 (91)*</td>
<td>10±1 (13)*</td>
</tr>
<tr>
<td></td>
<td>28-32</td>
<td>10.13 (79)*</td>
<td>10±1 (9)*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>44.30 (79)*</td>
<td>10±1 (25)</td>
</tr>
<tr>
<td>40 µM BAPTA-AM</td>
<td>24</td>
<td>1.22 (82)*</td>
<td>10±1 (8)*</td>
</tr>
<tr>
<td></td>
<td>28-32</td>
<td>3.66 (72)*</td>
<td>9±1 (7)*</td>
</tr>
</tbody>
</table>

Values for cell numbers are mean±SEM. Data were combined from three separate experiments. The number of embryos analyzed is indicated in parentheses. *Values are significantly different (P<0.05) from controls. **Values for cell numbers are mean±SEM. Data were combined from three separate experiments. The number of embryos analyzed is indicated in parentheses.
with 10 μM W-7 for 4 hours without A23187 exposure had no significant effect on cavitation, as compared to controls. The acceleration of cavitation following A23187 exposure was completely attenuated when morulae were coexposed to W-7 (Fig. 6B). These results provide evidence that calmodulin is required for cell division and cavitation, and that the stimulation of preimplantation development through Ca2+ signalling is, at least in part, due to the activation of calmodulin.

**DISCUSSION**

In this study, we examined the critical role that Ca2+ signalling plays in regulating cavitation. The ability of the cell to transduce signals using intracellular Ca2+ is of central importance for cell division and differentiation (Baitinger et al., 1990; Grandin and Charbonneau, 1991; Poenie et al., 1985). Artificial induction of a Ca2+ transient accelerated the ability of blastomeres to cleave and form a blastocoel. The delay or inhibition of Ca2+ signalling or calmodulin activation caused a subsequent delay or complete inhibition of cell division and blastocyst differentiation. We also showed that Ca2+ signalling can occur through the IPR, suggesting a central role for IP3 in transducing Ca2+ signals that control preimplantation development. Our results suggest that Ca2+ signalling, the biochemical mechanism that regulates oocyte development and egg activation at fertilization, may also play an important role during embryonic growth and differentiation.

**Releasing Ca2+ from intracellular stores stimulates development**

We have previously observed that treatment with ethanol or ionophores can enhance pre- and peri-implantation development (Stachecki et al., 1994a,b). This phenomenon appears to be associated with the ability of these agents to transiently elevate the [Ca2+]i. Using Ca2+-free medium, exposure of mouse embryos to ethanol or ionomycin produced a transient elevation in the [Ca2+]i, accelerated the onset and progression of cavitation, and stimulated cell division. These data support the hypothesis that Ca2+, through its release from intracellular stores, regulates the rate of early embryonic development.

**Evidence for a Ca2+ threshold**

We have previously shown that exposure of morulae to 1 μM ionophore elevates [Ca2+]i to a greater level than does treatment with 0.1% ethanol (Stachecki et al., 1994a). However, the rate of cavitation was similar for ethanol- and ionophore-treated morulae, suggesting that the effect on development is not proportional to the magnitude of the Ca2+ transient. Other studies (Berridge and Galione, 1988; Vitullo and Ozil, 1992) have suggested that Ca2+ signalling during development is frequency-modulated and not amplitude-dependent, as long as a threshold concentration of Ca2+ is reached. Just as FM radio waves contain more information than AM waves, a frequency modulated system of Ca2+ signalling can potentially encode more information. This paradigm helps to explain Ozil’s (1990) finding that oocytes most readily develop parthenogenetically to the blastocyst stage when Ca2+ transients are induced at a frequency similar to those occurring naturally at fertilization. In this communication, every treatment that produced a significant increase in [Ca2+]i, (ethanol, 365 nM; ionomycin, 847 nM; and BAPTA-AM + ionomycin, 569 nM) accelerated cavitation to a similar extent. Because ethanol treatment produced the smallest rise in [Ca2+]i, that stimulated development, it appears that an intracellular

**Table 4. Ca2+ release through the RR or IPR in mouse morulae**

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>[Ca2+]i (nM)‡</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>241±7</td>
<td>22</td>
</tr>
<tr>
<td>1 nM ryanodine</td>
<td>215±5</td>
<td>5</td>
</tr>
<tr>
<td>10 nM ryanodine</td>
<td>307±18*</td>
<td>10</td>
</tr>
<tr>
<td>50 nM ryanodine</td>
<td>243±42</td>
<td>5</td>
</tr>
<tr>
<td>100 nM ryanodine</td>
<td>205±7</td>
<td>5</td>
</tr>
<tr>
<td>1 μM ryanodine</td>
<td>236±10</td>
<td>7</td>
</tr>
<tr>
<td>10 μM ryanodine</td>
<td>224±11</td>
<td>5</td>
</tr>
<tr>
<td>10 nM IP3</td>
<td>300±26</td>
<td>8</td>
</tr>
<tr>
<td>100 nM IP3</td>
<td>611±76*</td>
<td>8</td>
</tr>
<tr>
<td>1 μM IP3</td>
<td>686±74*</td>
<td>5</td>
</tr>
<tr>
<td>10 μM IP3</td>
<td>663±100*</td>
<td>6</td>
</tr>
<tr>
<td>50 μM IP3</td>
<td>734±74*</td>
<td>14</td>
</tr>
</tbody>
</table>

†Morulae were loaded with fluo-3-AM for 30 min in Ham’s F10 medium at 37°C to estimate the [Ca2+]i. Embryos were treated with Ham’s F10 medium containing 0.005% lysophosphatidylcholine (LPC) with or without ryanodine or IP3.

‡Values are mean±SEM. Data were combined from three separate experiments, n, number of embryos.

*Values are significantly greater (P<0.05) than controls.
Ca$^2+$ transient equal to or exceeding that produced by ethanol (baseline+increase≥365 nM), has the ability to accelerate development.

**Chelation of intracellular Ca$^2+$ inhibits cavitation**

BAPTA lowered the [Ca$^{2+}$], and inhibited Ca$^2+$ signalling, cavitation and cell division in a dose-dependent manner. Because the $K_d$ of BAPTA for Ca$^{2+}$ (192 nM) is lower than the $K_d$ of fluo-3 for Ca$^{2+}$ (316 nM), Ca$^{2+}$ will be preferentially chelated by BAPTA. Therefore, as Ca$^{2+}$ is released within cells containing BAPTA, the fluo-3 fluorescence intensity will increase only when the existing BAPTA is saturated with Ca$^{2+}$ and additional free-Ca$^{2+}$ exists to bind to fluo-3. It was initially unclear whether the delay in development following BAPTA treatment was due to the decrease in the basal [Ca$^{2+}$], or a temporal inhibition of the ability of the embryo to generate a suprathreshold (≥365 nM) intracellular Ca$^{2+}$ transient, necessary for development to continue. To answer this question, we examined the changes in the [Ca$^{2+}$], and the rate of cavitation following BAPTA treatment. The highest concentration of BAPTA (40 μM) suppressed the [Ca$^{2+}$], significantly below the baseline level of 210 nM for only 3 hours following treatment, whereas cavitation was delayed for a much longer period of time. Kao et al. (1990) also found that the [Ca$^{2+}$], decreased briefly after exposing 3T3 cells to BAPTA. Embryos treated with 10 or 20 μM BAPTA were delayed in their ability to produce a Ca$^{2+}$ transient beyond the threshold level of 365 nM, for ≥4 hours and ≥8 hours, respectively. These data suggest that Ca$^{2+}$ signalling was impaired in BAPTA-AM-treated embryos and that the temporal inability to elevate the [Ca$^{2+}$] beyond the threshold level following treatment resulted in a corresponding delay in blastocoe formation. The transient nature of the inhibition of development at 10-20 μM BAPTA-AM illustrates that Ca$^{2+}$ signalling eventually resumed. Indeed, the time- and dose-dependent patterns of recovery of Ca$^{2+}$ signalling mirrored the delay in cavitation following BAPTA-AM treatment (see Fig. 3 and Table 3).

To dissociate any pleotropic effects of the pharmacological agents used in this study from their effects on the [Ca$^{2+}$], we examined the effect of ethanol on cavitation when no intracellular Ca$^{2+}$ signal was produced. Titration of BAPTA-AM revealed that 0.5 μM did not inhibit the development of morulae, but blocked the increase in [Ca$^{2+}$], following ethanol exposure and attenuated the ethanol-induced acceleration of cavitation. Therefore, without the subsequent increase in [Ca$^{2+}$], following ethanol exposure, development was not accelerated. Attenuation of ethanol-accelerated development by 0.5 μM BAPTA-AM was not due to BAPTA-AM toxicity, because BAPTA-AM alone did not inhibit development and ionomycin was capable of accelerating the rate of cavitation.

**Fig. 5.** Characterization of receptor-mediated Ca$^2+$ release from intracellular stores. Morulae were loaded with 5 μM fluo-3-AM, permeabilized in Ham’s F10 medium containing 0.05% lyssolecithin (LPC; a vital permeabilizing phosphatidylcholine; Khidir et al., 1995), treated with IP$_3$ or ryanodine, and monitored for changes in [Ca$^{2+}$], as in Table 1. (A) Pseudocolor representation of [Ca$^{2+}$], in a typical murine embryo exposed to IP$_3$. The color bar at the right indicates the [Ca$^{2+}$], from low (top) to high (bottom) values. A representative morula illustrating a characteristic response is shown periodically (in seconds) after the addition of 0.05% LPC and 50 μM IP$_3$ (n=14). (B) Morulae were exposed to 0.05% LPC alone (first arrow) followed by 50 μM IP$_3$ (second arrow), or (C) 0.05% LPC and 100 nM ryanodine (first arrow) followed by 50 μM IP$_3$ (second arrow). (D) Morulae pretreated with 0 (yellow line), 1 μM (red line) or 10 μM (blue line) BAPTA-AM were exposed to 0.05% LPC and 100 nM IP$_3$. Representative profiles of three morulae shown as colored lines are displayed in B (n=15), C (n=10) and D (n=15) as [Ca$^{2+}$], (nM).
under these conditions. Collectively, our results suggest that these pharmacological agents alter cellular growth and differentiation by specifically modulating intracellular Ca\(^{2+}\) signalling.

Ca\(^{2+}\) signalling regulates preimplantation cell division

Chelation of intracellular Ca\(^{2+}\) by BAPTA inhibited cell division in a dose-dependent manner. Exposure to high levels of BAPTA-AM (10-40 \(\mu M\)) produced both an inhibition of cleavage to the 16-cell stage and a failure to cavitate. In a previous study, Kline and Kline (1992) found that exposure of mouse eggs to \(\geq 1 \mu M\) BAPTA-AM abolished fertilization-induced intracellular Ca\(^{2+}\) oscillations and prevented cleavage to the 2-cell stage. Similarly, our data reveal that the inhibition of Ca\(^{2+}\) signalling with BAPTA-AM prevented cavitation by blocking cleavage to the 16-cell stage. Furthermore, if and when the embryo reached the 16-cell stage, cavitation and additional cell divisions occurred, providing indirect evidence that Ca\(^{2+}\) signalling resumed. We have shown that treatment with BAPTA can transiently delay or completely inhibit blastocoeel formation, whereas inducing the release of intracellular stores of Ca\(^{2+}\) can accelerate embryo development. These data demonstrate that an intracellular Ca\(^{2+}\) transient is essential for cell division to the 16-cell stage, which in turn is required for cavitation. BAPTA may therefore indirectly inhibit cavitation by blocking cell division. Although a more direct effect of BAPTA on morphogenesis is plausible, BAPTA-treated 16-cell morulae cavitated at a rate similar to control embryos, suggesting that calcium transients mediate cell division to the 16-cell stage, but may not be directly involved in cavitation. Ca\(^{2+}\) transients mediate cell division by activating calmodulin (Lu and Means, 1993), and calmodulin activity appears to be critical for preimplantation cell division and blastocoeel formation. Several studies have observed a direct correlation between Ca\(^{2+}\) transients and the timing of cellular division in embryos (Yoshimoto et al., 1985; Ozil, 1990; Collas et al., 1993; Kubota et al., 1993; Stricker, 1995). Yoshimoto et al. (1985) and Kubota et al. (1993) revealed that periodic oscillations of intracellular Ca\(^{2+}\) correlated exactly with the cell division cycle. In a more recent study, Stricker (1995) showed that Ca\(^{2+}\) oscillations coincided directly with starfish embryo cleavage. We therefore suggest that intracellular Ca\(^{2+}\) transients may also control cellular growth in preimplantation mouse embryos.

The inhibitory effect of BAPTA on cell division is consistent with previous work demonstrating that the cell cycle is linked to Ca\(^{2+}\) signalling (Baitinger et al., 1990; Grandin and Charbonneau, 1991; Poenie et al., 1985). According to other authors (Wiley and Eglitis, 1981; Watson and Kidder, 1988; Manejwala et al., 1989; Manejwala and Schultz, 1989; Gardiner et al., 1990), cavitation begins in the mouse between the 16-cell and 32-cell stages, suggesting that the ability to cavitate is related to the number of cell divisions. Although Spindle et al. (1985) reported that inhibition of DNA synthesis by aphidicolin at the early morula stage resulted in a significant decrease in blastocoeel formation, an earlier study (Dean and Rossant, 1984) obtained the opposite result, perhaps due to procedural differences. Other studies reveal that compaction and cavitation are not dependent on cytokinesis, but are instead linked to the number of nuclear divisions (Kimber and Surani, 1981; Pratt et al., 1981; Surani et al., 1980). Our data support these studies since cavitation occurred only after the embryos reached the 16-cell stage and BAPTA did not prevent 16-cell morulae from cavitating. In this respect, cavitation is linked to the cell cycle and begins after the fourth nuclear division.

Receptors on intracellular Ca\(^{2+}\) channels in mouse morulae

We have shown that mouse embryos are capable of generating intracellular Ca\(^{2+}\) fluctuations specifically through a Ca\(^{2+}\) release mechanism involving the IPR. This result was anticipated from earlier reports (Kline and Kline, 1994; Xu et al., 1994) showing that mouse oocytes possess an IP\(_3\)-mediated Ca\(^{2+}\) release mechanism. There was a slight elevation of \([Ca^{2+}]_i\) after treatment with 10 nM ryanodine, supporting previous work that ryanodine releases Ca\(^{2+}\) from mouse oocytes in a biphasic manner; low concentrations are insufficient to release Ca\(^{2+}\) and high concentrations downregulate the receptor (Ayabe et al., 1995; Xu et al., 1994). Because the level of Ca\(^{2+}\) released was small following treatment with 10 nM ryanodine, it is unclear whether IP\(_3\)-independent Ca\(^{2+}\) release plays a significant role in Ca\(^{2+}\) signalling during preimplanta-
tion development. It is possible that ryanodine would not alter preimplantation development because the amount of Ca\textsuperscript{2+} released was below our hypothesized threshold for stimulation, and according to the observation that although RRs are present in mouse oocytes (Ayabe et al., 1995), they do not play a significant role in either the early or late stages of egg activation.

**The role of calmodulin in transducing the Ca\textsuperscript{2+} signal**

We investigated whether calmodulin was required for blastocoelem formation and cell division through its ability to transduce Ca\textsuperscript{2+} signals in preimplantation embryos. Cavitation and cell division were inhibited in the presence of the calmodulin antagonist W-7. The inhibition of calmodulin during a Ca\textsuperscript{2+} transient attenuated the ionophore-induced acceleration of cavitation. Therefore, calmodulin activity is critical for the developmental acceleration that follows Ca\textsuperscript{2+} signalling. Ca\textsuperscript{2+} alters cellular events through its activation of Ca\textsuperscript{2+}-dependent proteins, in particular calmodulin (Sasaki and Hidaka, 1982; Lu and Means, 1993). Calmodulin is essential for cellular growth and division, and has been shown to be the primary downstream receptor for Ca\textsuperscript{2+} (Chafouleas et al., 1982; Cheung, 1980; Lu and Means, 1993; Rasmussen and Means, 1987, 1989; Means and Dedman, 1980; Means et al., 1991). Calmodulin regulates the activity of many enzymes, including myosin light chain kinase, nitric oxide synthase and calcineurin (Yagi et al., 1978; Klee et al., 1979; Chafouleas et al., 1982; Lu and Means, 1993; Hepler, 1989; Mayer et al., 1990; Silver, 1990; Means and Dedman, 1980). Through the activation of these and other enzymes, calmodulin controls a variety of cellular processes including protein phosphorylation, glycogen regulation, cyclic nucleotide metabolism, microtubule formation, cellular motility, secretion and cell division (Cheung, 1980; Chafouleas et al., 1982; Lorca et al., 1993).

**Ca\textsuperscript{2+} signalling in preimplantation embryos**

Although ethanol, A23187 and ionomycin release Ca\textsuperscript{2+} from intracellular stores in mouse embryos (Stachecki et al., 1994a; this communication), the mechanism by which this occurs is unknown. Ethanol can induce the mobilization of intracellular Ca\textsuperscript{2+} stores in hepatocytes by activation of the phosphoinositide-specific phospholipase C and the elevation of IP\textsubscript{3} (Hock et al., 1987). It is therefore possible that ethanol works through this mechanism to mediate the observed developmental effects in murine embryos. Alternatively, ethanol may elevate the [Ca\textsuperscript{2+}]\textsubscript{i} by directly inducing the IP\textsubscript{3} to open and release sequestered Ca\textsuperscript{2+}. In ongoing studies, we have found that mouse preimplantation development is dependent on active phospholipase C (J.J.S. and D.R.A., unpublished observations). In addition to producing IP\textsubscript{3}, phospholipase C produces diacylglycerol, resulting in the activation of protein kinase C. It will therefore be important to assess the role of protein kinase C during embryonic cell division and cavitation. In vivo, cell growth and development may be regulated through a Ca\textsuperscript{2+}-mediated pathway that is stimulated by natural agonists, possibly growth factors, that generate Ca\textsuperscript{2+} oscillations within cells (Fu et al., 1991; Hess et al., 1993; Tanaka et al., 1992). Growth factors are known to be produced by preimplantation embryos (Werb, 1990) and stimulate development in vitro through paracrine and autocrine regulation (Dardik and Schultz, 1991; Paria and Dey, 1990; Wood and Kaye, 1989).

Our data are therefore consistent with the hypothesis that intracellular Ca\textsuperscript{2+} is a critical component of a signal transduction pathway, existing in both oocytes and early embryos, that regulates embryonic development.

Ca\textsuperscript{2+} oscillations are not necessarily confined to the activated cell, but can spread in a wave-like manner to adjacent cells (Berridge, 1993; Allbritton and Meyer, 1993; Boitano et al., 1992; Ospichuck et al., 1992). This may occur by the diffusion of either Ca\textsuperscript{2+} or IP\textsubscript{3} into neighboring cells through gap junctions. Gap junctions are formed during compaction and play an important role in cavitation (Ducibella et al., 1975; Goodall, 1986; Goodall and Johnson, 1982, 1984; Lo and Gilula, 1979; Ziomek and Johnson, 1980). Signals generated in one cell can be transmitted via gap junctions to other cells, thereby synchronizing the developmental rate of the blastomeres within the embryo. Cell-to-cell signal transmission following the coaddition of LPC and IP\textsubscript{3} to morulae may be responsible for the sequential release of Ca\textsuperscript{2+} in adjacent blastomeres observed in Fig. 5A, although no direct evidence for cell to cell transfer of IP\textsubscript{3} or Ca\textsuperscript{2+} was sought in these experiments.

The release of intracellular Ca\textsuperscript{2+} stores accelerates development in vitro by no more than 8 hours, as compared to control embryos (Leach et al., 1993; Stachecki et al., 1994a,b; this communication). Therefore, we hypothesize that the experimentally induced release of Ca\textsuperscript{2+} could mimic naturally occurring Ca\textsuperscript{2+} transients that regulate the transition of cells into M-phase (Charbonneau and Grandin, 1992; Lorca et al., 1994). Artificial induction of M-phase would cause precocious division and could thus accelerate cavitation or other morphogenetic events. Since the cell cycle is approximately 12 hours in vitro (Streffer et al., 1980), we predict that induction of a Ca\textsuperscript{2+} transient will accelerate development by a maximum of 12 hours. As most embryos are not completely synchronous, experimentally induced Ca\textsuperscript{2+} release could occur 0-12 hours prior to a natural Ca\textsuperscript{2+} transient and potentially accelerate development by 12 hours, with an average shift of 6 hours. We have observed that the development in vitro of ethanol- or ionophore-treated embryos is approximately 6 hours ahead of control embryos (Leach et al., 1993; Stachecki et al., 1994a,b). Therefore, we hypothesize that the release of intracellular Ca\textsuperscript{2+} beyond a threshold concentration, either by natural or artificial means, will stimulate embryonic progression into M-phase and provide the necessary stimulus for continued development. Whether preimplantation development is regulated by Ca\textsuperscript{2+} transients that occur during each cell cycle, at fertilization, or both, remains to be elucidated.

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2496 J. J. Stachecki and D. R. Armantr