Ca\(^{2+}\) released via IP\(_{3}\) receptors is required for furrow deepening during cytokinesis in zebrafish embryos

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ABSTRACT We have previously visualized three Ca\(^{2+}\) transients, generated by release from intracellular stores, which are associated with cytokinesis during the early cell division cycles of zebrafish embryos: the furrow positioning, propagation and deepening transients. Here we demonstrate the requirement of the latter for furrow deepening, and identify the Ca\(^{2+}\) release channels responsible for generating the deepening transient. The introduction of the Ca\(^{2+}\) buffer 5,5’-dibromo-BAPTA, at an appropriate time to challenge only the deepening transient, resulted in the dissipation of this transient and an inhibition of furrow deepening. Introduction of antagonists of the inositol 1,4,5-trisphosphate (IP\(_{3}\)) receptor (heparin and 2-aminoethoxydiphenylborate; 2-APB) at the appropriate time, blocked the furrow deepening transient and resulted in an inhibition of furrow deepening. In contrast, antagonists of the ryanodine receptor and the NAADP-sensitive channel had no effect on either the furrow deepening transient or on furrow deepening. In addition, microinjection of IP\(_{3}\) led to the release of calcium from IP\(_{3}\)-sensitive stores, whereas the introduction of caffeine or cADPR failed to induce any increase in intracellular Ca\(^{2+}\). Our new data thus support the idea that Ca\(^{2+}\) released via IP\(_{3}\) receptors is essential for generating the furrow deepening transient and demonstrate a requirement for a localized cytosolic Ca\(^{2+}\) rise for the furrow deepening process. We also present data to show that the endoplasmic reticulum and IP\(_{3}\) receptors are localized on either side of the cleavage furrow, thus providing the intracellular Ca\(^{2+}\) store and release mechanism for generating the deepening transient.

KEY WORDS: Ca\(^{2+}\), IP\(_{3}\) receptor, furrow deepening, cytokinesis, zebrafish

Introduction

The involvement of Ca\(^{2+}\) in cytokinesis has been the subject of much experimentation and debate over the last 30 years (reviewed in Rappaport, 1971; 1986; 1996; Mabuchi, 1986; Hepler, 1989; 1992; 1994; Salmon, 1989; Conrad and Schroeder, 1990; Satterwhite and Pollard, 1992; Fishkind and Wang, 1995; Silver, 1996). Its exact role, however, is still somewhat unclear even in the large embryos of certain fish and amphibians where the main cytokinetic steps are far easier to resolve (both temporally and spatially) than in smaller embryos and tissue culture cells.

It has been previously shown, using zebrafish (Danio rerio), medaka fish (Oryzias latipes) and frog (Xenopus laevis) embryos, that the sequence of events that are involved in cytokinesis include: (1) The transmission of the furrow positioning signal from the mitotic apparatus to the cell surface, (2) The propagation of the positioned cleavage furrow across the cell surface, (3) The deepening of the cleavage furrow in order to separate the daughter cells, and (4) The zipping-up or apposition of the daughter cells following separation. Several reports have suggested that these cytokinetic events may each involve distinct but perhaps interconnected localized Ca\(^{2+}\) transients. In fish embryos, furrow propagation, deepening and zipping transients were first visualized in dividing medaka embryos (Fluck et al., 1991). Following this, three separate groups reported furrow positioning, propagation and deepening signals during the first few cell divisions of zebrafish embryos (Chang and Meng, 1995; Webb et al., 1997; Créton et al., 1998). Localized increases in [Ca\(^{2+}\)]\(_{i}\) have also been observed during cytokinesis in Xenopus embryos. Mulo et al. (1996) visualized localized Ca\(^{2+}\) waves spreading along the first furrow after cleavage furrow formation. In addition, most recently Noguchi and Mabuchi (2002) reported the presence of not one, but two distinct Ca\(^{2+}\) waves during the first cleavage in Xenopus. They suggested that the first might be the counterpart of the furrow deepening transient observed in zebrafish and medaka, and that the...
second might be involved in the apposition of the two daughter blastomeres after furrow deepening has been completed.

In all the reports described above, the authors attempted to explore the function and developmental significance of the Ca$^{2+}$ transients, as well as their mechanism of generation, by introducing Ca$^{2+}$ buffers or antagonists of the various Ca$^{2+}$ release channels thought to be involved. However, the timing of their introduction as well as their rate of spread within an embryo is crucial to understanding what effect they have on the generation of a particular transient, and the developmental significance of blocking or modulating that transient. For example, it has been shown that when dividing *Xenopus* embryos are injected with members of the BAPTA family of Ca$^{2+}$ buffers (Pethig *et al*., 1989), the timing of the injection is critical; if the buffer is introduced too early then it blocks karyokinesis rather than cytokinesis, and the embryos fail to divide (Miller *et al*., 1993; Snow and Nuccitelli, 1993). If, however, it is introduced immediately after the completion of karyokinesis, but before the onset of cytokinesis, then furrow positioning is affected (Miller *et al*., 1993).

In many of these reports, however, the protocols followed with regards to when the buffer or antagonist was introduced, makes it hard to distinguish which of the possible downstream cytokinetic Ca$^{2+}$ transients were affected. Did just one or all the steps in the cytokinesis process (i.e., furrow positioning, propagation, deepening and apposition) have a Ca$^{2+}$-dependency? Using zebrafish embryos, Webb *et al*.* (1997) focussed their attention on the propagation of Ca$^{2+}$ transients during the first cell division. The embryo is in an axial orientation (Webb *et al*., 1997). The luminescent images (pseudocolor panels, labeled 'i' to 'vi') represent 60 s of accumulated light. A bright-field image (panels 'i' to 'vi') was grabbed just prior to each luminescent image. Images were obtained as follows: (i) during the furrow positioning signal (see asterisk); at the (ii) start and (iii) end of the furrow propagation signal; at the (iv) start and (v) end of the furrow deepening signal and (vi) during furrow apposition of the first cell division cycle. Color scale indicates luminescent flux in photons per pixel. Scale bar is 200 µm. Figure modified from Webb *et al*.* (1997). Arrowheads in panels 'i' and 'ii' indicate Ca$^{2+}$ elevations associated with ooplasmic segregation (Leung *et al*., 1998).
transient, and waited until the furrow had been positioned on the blastodisc surface (by observing either the appearance of the furrow on the surface or the Ca\(^{2+}\) transient associated with this event) before they introduced 5,5'-dibromo-BAPTA. These experiments clearly indicated a Ca\(^{2+}\)-requirement for furrow propagation in these embryos. However, without furrow propagation there can be no furrow deepening and so the Ca\(^{2+}\)-dependency of the subsequent deepening process was still unclear.

Here, in a continuation of our work on cytokinesis in zebrafish, we demonstrate that a localized elevation of cytosolic Ca\(^{2+}\) is also essential for furrow deepening and show that the Ca\(^{2+}\) generating the deepening transient is released via inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs). We also present data indicating that the endoplasmic reticulum (ER) and IP\(_3\)Rs are both localized on either side of the cleavage furrow, thus providing additional supporting evidence for the intracellular calcium source and release mechanism responsible for generating the deepening transient.

**Results**

**Sequence of Ca\(^{2+}\) transients accompanying cytokinesis in control embryos**

The normal sequence of Ca\(^{2+}\) transients that accompany cytokinesis of the first cell division are shown in Fig. 1. This representative embryo shows the transients from an axial view (Webb et al., 1997). A localized elevation of Ca\(^{2+}\), the furrow positioning signal (see asterisk in Fig. 1, panel i'), is observed just prior to the first physical sign of the cleavage furrow appearing on the blastodisc surface (see Fig. 1, panel i). After the furrow is positioned, two slow subsurface Ca\(^{2+}\) waves, (the furrow propagation signal; see Fig. 1, panels ii' and iii'), accompany the lateral extension of the furrow (see Fig. 1, panels ii and iii) and move outward toward the edge of the blastodisc. As the two wave fronts approach the edge of the blastodisc, the furrow deepening signal appears at the apex of the blastodisc where the positioning signal was first observed (see Fig. 1, panel iv'). The deepening signal moves both outward to the edge of the blastodisc and downward to the bottom of the blastodisc (see Fig. 1, panel v'). This deepening signal then begins to diminish from the apex outward (see Fig. 1, panel vi') and by the end of cytokinesis the localized Ca\(^{2+}\) elevation returns to precleavage resting levels. This is described in further detail in Webb et al. (1997).

**The effect of 5,5'-dibromo-BAPTA on furrow deepening and apposition**

A representative example (n = 3) to show the effect of the weak (K\(_D\) = 1.5 \(\mu\)M) Ca\(^{2+}\) buffer, 5,5'-dibromo-BAPTA (DBB), on the deepening Ca\(^{2+}\) signal is illustrated in Fig. 2A. Following the timing protocols described in Fig. 3A, DBB was injected into the blastodisc of the embryo to reach a final cytosolic concentration of ~260 \(\mu\)M. The luminescent images (see Fig. 2A, panels ii' and iii') clearly show that the buffer delocalizes the Ca\(^{2+}\) transient, and the corresponding bright-field images show that the deepening furrow (see arrowhead in panel ii) eventually regresses (see arrowhead in panel iii). When the same final concentration (i.e., ~260 \(\mu\)M) of 5,5'-dimethyl-BAPTA (DMB; K\(_D\) = 0.15 \(\mu\)M) is introduced, however, it has no effect on the deepening transient and embryos divide and develop normally (n = 3; data not shown). The effect of DBB on furrow apposition is also illustrated (see Fig. 2B). In this representative embryo (n = 4) the buffer was injected following the injection protocol illustrated in Fig. 3B. The luminescent images (see Fig. 2B, panels iii' to vii') again show that the buffer delocalizes the Ca\(^{2+}\) transients and the corresponding bright-field images show that although the furrow deepens (see arrowheads in Fig. 2B, panels iii and v), apposition of the daughter cells is abnormal and the furrow again eventually regresses (Fig. 2B, panel vii).
**Effects of Ca\(^{2+}\) channel antagonists on propagation and deepening transients**

The effects of different Ca\(^{2+}\) channel antagonists on the cytokinetic Ca\(^{2+}\) transients during the first cell division cycle are shown in Figs. 4 to 6.

**IP\(_3\)**-sensitive channel

Figure 4A illustrates a representative control embryo (n = 5) and shows the typical pattern of luminescence observed to accompany furrow positioning, propagation and deepening of the first cell division cycle. Figure 4B shows an example (n = 6) of an embryo injected with heparin, following the protocol described in Fig. 3C. The propagation transient (see Fig. 4B panel ii') extends normally to the edge of the blastodisc, but the deepening transient clearly does not progress down through the blastodisc to the yolk cell (compare the heparin-treated embryo, Fig. 4B panel iii' with the control, Fig. 4A, panel iii'). The corresponding bright-field images show that the extent of the furrow deepening in the

**Fig. 4. Effect of IP\(_3\) receptor antagonists on the propagation and deepening Ca\(^{2+}\) transients.** Representative sequence of images from aequorin-loaded embryos illustrating the changes in intracellular free Ca\(^{2+}\) during the first two cell division cycles when (A) raised under normal conditions; after injection with (B) heparin or (C) de-N-sulfated heparin, or incubated with 2-APB to challenge (D) the propagation and deepening transients and (E) the deepening transient alone following the protocols illustrated schematically in Fig. 3. The luminescent images (pseudocolor panels, labeled i' to iv') represent 60 s of accumulated light. A bright-field image (i to iv) was grabbed just prior to each luminescent image. In addition, a bright-field image (v) was grabbed at ~60 min post-fertilization when the embryos should be at the 4-cell stage. Images i to iii show the embryos in an axial orientation and were obtained as follows: (i and i') during the positioning signal that accompanies the first appearance of the furrow arc; then during (ii and ii') the propagation and (iii and iii') the deepening signals of the first cell division. Images iv and iv' show a bright-field facial view of the first cell division and a pseudocolor facial view of the deepening signal, respectively. The concentrations of the channel antagonists used are listed in Table 1. Color scale indicates luminescent flux in photons per pixel. Scale bar represents 200 \(\mu\)m.

**Fig. 5. Analysis of luminescence profiles during positioning, propagation and deepening of the first cell division in aequorin-loaded embryos, that were either (A) raised under normal conditions, or (B) injected with heparin during furrow positioning of the first cell division.**

With the embryos in an axial orientation, the profiles were obtained from 3 regions of the blastodisc shown in A': apex (green trace), middle (blue trace) and bottom (red trace) using the Count Rate Plot function in the IpdWin 95 software. Each profile shows the relative number of photons collected in a 120 s window.
heparin-treated embryo is also significantly less than in the untreated control (compare Figs. 4B, panel iv and 4A, panel iv). The limited extent of deepening that does occur in the heparin-treated embryos eventually regresses (see Fig. 4B, panel v). Figure 4C shows a representative example (n = 8) of an embryo injected with de-N-sulfated heparin. This inactive form of heparin has no effect on the transients and the embryo continues to divide normally during subsequent cell divisions (see Fig. 4C, panel v). Figure 4D shows a representative embryo (n = 14) incubated with the cell permeable IP₃-sensitive channel antagonist, 2-aminoethoxydiphenylborate (2-APB), following the protocol described in Fig. 3D, to challenge the propagation transient. The propagation transient (see Fig. 4D, panel ii') is completely abolished after incubation (compared to the control, Fig. 4A, panel ii'). The corresponding bright-field image shows that the furrow does not propagate to the edge of the blastodisc. Figure 4D panels iii' and iv' also clearly show that the deepening transient is also completely abolished. However, as mentioned previously, without furrow propagation there can be no furrow deepening. Thus in order to study the effect of 2-APB on the furrow deepening transient alone, 2-APB was applied after the furrow propagation transient was over, following the protocol described in Fig. 3E. Figure 4E shows an example (n = 7) of an embryo where incubation with 2-APB challenges the deepening transient. Figure 4E, panels iii' and iv' show that 2-APB completely inhibits the deepening transient, while the associated bright-field images (panels iii and iv) show that although deepening was initiated, the furrow does not progress down through the blastodisc to the yolk cell. The furrow eventually regresses and the embryo remains at the one-cell stage (Fig. 4E, panel v).

**Effect of heparin on propagation and deepening transient profiles**

We analysed the relative profiles of luminescence along the first cleavage furrow during furrow propagation and deepening. Figure 5A shows the profiles generated by the control embryo in Fig. 4A...
(panels i' to iii'). It indicates that whilst the positioning and propagation transients are restricted to the outer cortex of the blastodisc, the deepening transient spreads down through the blastodisc toward the non-dividing yolk cell. The ingressing furrow accompanies the slow moving Ca^{2+} wave thus serving to cut the blastodisc in two. Figure 5B shows the profiles generated by the heparin-treated embryo in Fig. 4B (panels ii' and iii'). Whereas the propagation transient appears to occur as normal in the blastodisc cortex, the deepening transient and its accompanying ingressing furrow are this time also restricted to the outer regions of the blastodisc and never penetrate down toward the yolk cell. This analysis supports the suggestion that Ca^{2+} release via IP_{3}Rs plays a crucial role in the latter stages of furrow deepening.

**Ryanodine-sensitive channel**

Figure 6A illustrates the control embryo again for comparative purposes. Figures 6 B,C show representative embryos injected (n = 15) and incubated (n = 5) and with ryanodine, respectively following the protocols illustrated in Fig. 3 C,D, respectively. Figure 6D shows a representative embryo injected with ruthenium red (n = 14) following the protocol described in Fig. 3C. Neither of these antagonists have an effect on either the propagation or deepening transients, and furrows propagate and deepen normally during the early cell division cycles (see Figs. 6B to D, panels v).

**Range of elevated Ca^{2+} during propagation and deepening transients**

Figure 7 displays the mean fold Ca^{2+} rise (± s.e.m.) of the furrow propagation (7A) and deepening (7B) transients above the background resting level, comparing (using Student t-test) antagonist treated (for concentrations see Table 1) with control embryos. In the case of the propagation transient, only incubation in medium containing 2-APB resulted in a significantly lower (where P<0.05) fold increase in Ca^{2+}. Whereas in the case of the deepening transient, both incubation in 2-APB and injection of heparin resulted in a significantly lower fold increases in Ca^{2+} rise (where P<0.001 and P<0.01 respectively). Figure 7 also indicates the range of values calculated for the fold Ca^{2+} rises and the number of experiments carried out for each antagonist (or control).

**Release of calcium by various Ca^{2+} channel agonists**

Figure 8 shows the release of calcium in the blastodisc by introducing various calcium channel agonists into aequorin-loaded embryos. Figures 8 A,B show representative examples of embryos that were injected with IP_{3} (n = 15) and NAADP (n = 7), respectively, prior to the appearance of the cleavage furrow at the blastodisc surface, and show that Ca^{2+} is released immediately after agonist introduction. Figures 8 C,D show embryos either incubated in caffeine (n = 5) or injected with cyclic ADP ribose (n = 11),

**Fig. 7. Effect of Ca^{2+} channel antagonists** on the ratio of Ca^{2+} rise above resting level for (A) the propagation and (B) the deepening transients. The data presented in the histograms are expressed as means ± s.e.m., while the numbers above each bar show the range of values calculated and the number of imaging experiments carried out for each antagonist (or control). Student t-tests were performed and show that for the propagation transient (A), the ratio of Ca^{2+} rise above resting for the 2-APB-treated embryos (*) was significantly lower than the control at P<0.05, whilst for the deepening transient (B), the ratio of Ca^{2+} rise above resting for the heparin (*) and 2-APB (**) -treated embryos, was significantly lower than the control at P<0.01 and P<0.001, respectively. The concentrations of the channel antagonists used are listed in Table 1.
respectively. Neither of these agonists have an effect on releasing calcium via the ryanodine-sensitive calcium channel. The results shown in Figure 8 and the concentrations of agonists introduced during deepening of the first division cycle are summarized in Table 2. Scale bar represents 200 µm.

**TABLE 2**

<table>
<thead>
<tr>
<th>Ca²⁺ Agonist</th>
<th>Final Agonist Release</th>
<th>Agonist Concentration</th>
<th>Ref.</th>
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<td>Yes</td>
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<tr>
<td>Ryanodine</td>
<td><em>Caffeine</em></td>
<td>500 µM</td>
<td>No</td>
</tr>
<tr>
<td>Ruthenium red</td>
<td><strong>ADP Ribose</strong></td>
<td>10 - 30 µM</td>
<td>No</td>
</tr>
<tr>
<td>NADP-Sensitive Channel</td>
<td><strong>NADP</strong></td>
<td>60 - 330 nM</td>
<td>Yes</td>
</tr>
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Discussion

Requirement of elevated Ca\textsuperscript{2+} for both furrow deepening and apposition

Several groups have previously reported the presence of three distinct Ca\textsuperscript{2+} transients that accompany the first cell division in zebrafish embryos: the furrow positioning, propagation and deepening transients (Chang and Meng, 1995; Webb et al., 1997; Créton et al., 1998; Chang and Lu, 2000). In this current study we focused on the propagation and deepening Ca\textsuperscript{2+} transients in order to characterize further their mode of generation as well as the requirement of the latter in the cytokinetic process. The furrow propagation transient has been shown to take the form of two slow linear Ca\textsuperscript{2+} waves that propagate from the furrow-positioning transient at the apex of the blastodisc, out towards its edges at a velocity of approximately 0.5 µm/sec. The extending furrow accompanies these slow Ca\textsuperscript{2+} waves, and also propagates out towards the edges of the blastodisc at a similar velocity. The requirement of the slow Ca\textsuperscript{2+} waves for furrow propagation has been previously demonstrated by introducing the Ca\textsuperscript{2+} buffer 5,5’-dibromo-BAPTA (DBB) into the blastodisc as soon as the furrow is positioned (Webb et al., 1997). The buffer served to dissipate the slow linear Ca\textsuperscript{2+} waves and resulted in the arrest and eventual regression of the extending furrow. In these experiments, the timing of the introduction of the buffer was such that it could not be effecting a Ca\textsuperscript{2+} transient prior to the initiation of furrow propagation, such as furrow positioning (Webb et al., 1997; Chang and Lu, 2000) or some Ca\textsuperscript{2+}-dependent step during karyokinesis, such as nuclear envelop breakdown, or the metaphase anaphase transition (Créton et al., 1998). However, furrow deepening cannot occur if furrow propagation is arrested and the furrow then regresses. Thus, the requirement for elevated Ca\textsuperscript{2+} in the deepening process was still uncertain. Here we show that by careful timing (outlined in Fig. 3 A,B) of the introduction of an appropriate concentration of DBB, it is also possible to demonstrate the Ca\textsuperscript{2+} dependency of the deepening furrow as well as the subsequent apposition of the daughter cells. If DBB is introduced as the propagation Ca\textsuperscript{2+} wave is about to reach the edges of the blastodisc, the buffer gets to the deepening zone in time to dissipate the localized deepening transient, thus inhibiting furrow deepening. If the buffer is introduced later (i.e., injected when the deepening signal starts: see Fig. 3B), its introduction is too late to delocalize the early deepening Ca\textsuperscript{2+} transient and inhibit deepening, but it delocalizes the late deepening/apposition transient and prevents normal apposition of the daughter cells. Introducing a similar final cytosolic concentration of 5,5’-dimethyl-BAPTA (DMB), a buffer with a K\textsubscript{D} value some 10-fold lower than that of DBB (0.15 µM compared to 1.5 µM respectively) was ineffective at delocalizing the deepening Ca\textsuperscript{2+} transient or preventing the deepening of the cleavage furrow. These results suggest that the effect of DBB is not due to some indirect effect of the buffer unrelated to binding with Ca\textsuperscript{2+}. They also suggest that DBB is acting to facilitate the diffusion of Ca\textsuperscript{2+} away from the elevated domain in the deepening furrow and thus supports previous suggestions that such injected chelators act as Ca\textsuperscript{2+} shuttle buffers (Speksnijder et al., 1989; Miller et al., 1993; Snow and Nuccitelli, 1993). These experiments support the proposal that localized domains of elevated Ca\textsuperscript{2+} as well as being essential for furrow propagation are also a critical requirement for furrow deepening and subsequent apposition.

Source of Ca\textsuperscript{2+} generating propagation and deepening transients

Once again, paying particular attention to the timing of the pharmacological challenge with respect to the stage of the cytokinetic process, we determined that neither the furrow propagation or deepening Ca\textsuperscript{2+} transient could be inhibited by antagonists of either ryanodine receptors (RyRs; Figs. 6B-D) or NAADP-sensitive channels (Figs. 6E-F). The furrow propagation and deepening processes were likewise unaffected by the application of these antagonists. However, introduction of the non-specific antagonists of IP\textsubscript{3} receptors (IP\textsubscript{3}R; heparin and 2-APB, suggested that these receptors play a significant role in generating the cytokinetic Ca\textsuperscript{2+} transients. Heparin injection (following the experimental protocol indicated in Fig. 3C) had no apparent effect on the propagation transient or furrow propagation across the blastodisc surface (Figs. 4B, 5B and 7A) but significantly inhibited the deepening Ca\textsuperscript{2+} transient and halted the deepening of the cleavage furrow (Figs. 4B, 5B and 7B). On the other hand, 2-APB incubation inhibited both the propagation transient and furrow propagation (Figs. 4D and 7A) as well as the deepening transient and furrow deepening (Figs. 4E and 7B).

The conflicting results regarding the effects of heparin and 2-APB on the generation of the furrow propagation transient might be due to several factors. Clearly, the site of localized cytokinetic Ca\textsuperscript{2+} release during furrow propagation is restricted mainly to the blastodisc cortex (i.e., close to the external source of membrane permeable 2-APB). Whereas, the slow diffusion rate of injected heparin (Taylor and Broad, 1998) might result in insufficient antagonist being present in the cortex during the time-window selected to challenge just the generation of the propagation transient (i.e., through following the protocol illustrated in Fig. 3C). Such factors might explain the discrepancy between the results obtained using these two IP\textsubscript{3}R antagonists. On the other hand, our results with regards to the inhibitory effects of heparin and 2-APB on the Ca\textsuperscript{2+} deepening transient (and the accompanying furrow deepening) are complimentary. We suggest that sufficient heparin is present within the deeper regions of the blastodisc through which the ingressing furrow must pass during the furrow deepening process. This inhibits the release of Ca\textsuperscript{2+} and thus the generation of the deepening transient, which in turn stops the deepening process and leads to eventual furrow regression.

Although heparin has traditionally been used as one of the main pharmacological inhibitors of IP\textsubscript{3}Rs (Chang and Meng, 1995; Stricker, 1995; Muto et al., 1996; Groigno and Whitaker, 1998) it does have limitations due to multiple actions. These include activating RyRs (Bezprozvanny et al., 1993), uncoupling the receptors that stimulate IP\textsubscript{3} formation from their G-proteins, inhibition of IP\textsubscript{3} 3-kinase, which normally stimulates the phosphorylation of IP\textsubscript{3} to IP\textsubscript{4}, and blocking the binding of IP\textsubscript{4} (reviewed in Taylor and Richardson, 1991; Taylor and Broad, 1998). However, there appear to be no functional RyRs in the zebrafish blastodisc during the early cleavage period (see Figs. 8C and D) thus eliminating at least this possible complication. Unfortunately, like heparin, 2-APB is not a specific inhibitor of IP\textsubscript{3} receptors and is also known to have multiple actions inside cells (Bootman et al., 2002). Indeed, its main antagonistic effect seems to be on Ca\textsuperscript{2+} entry via store-operated channels (SOCs; Gregory et al., 2001) rather than on Ca\textsuperscript{2+} release via IP\textsubscript{3}R. In addition, only Types 1 and 3 IP\textsubscript{3}Rs appear to be sensitive to 2-APB while Type 2 IP\textsubscript{3}Rs are reported to be insensitive (Bilmen and Michelangeli, 2002; Bootman et al., 2002). How-
ever, we have demonstrated previously that zebrafish embryos can generate a regular series of cytokinetic Ca\textsuperscript{2+} transients and divide normally (for at least the first few cell division cycles) in Ca\textsuperscript{2+}-free medium (Webb et al., 1997). This indicates that extracellular Ca\textsuperscript{2+} is not directly involved (even if it is refilling the ER via SOCs) in generating the cytokinetic Ca\textsuperscript{2+} transients and that the Ca\textsuperscript{2+} generating the transients is being released from intracellular stores. Furthermore, by immunohistochemistry we show that Type 1 IP\textsubscript{3}R (i.e., one of the isoforms known to respond to 2-APB; Bootman et al., 2002), and the ER are co-localized within the elevated level of intracellular free Ca\textsuperscript{2+} on either side of the cleavage furrow (see Fig. 9). Although the lateral extension of the aequorin-generated signal appears to be greater than that of the ER and IP\textsubscript{3}R staining (compare Fig. 9A with Figs. 9B and C), this might be explained by aequorin-generated light scattering from the deepening furrow. We suggest, therefore, that in the case of the zebrafish cytokinetic Ca\textsuperscript{2+} transients, 2-APB was more likely to be inhibiting Ca\textsuperscript{2+} release via IP\textsubscript{3}Rs, rather than inhibiting Ca\textsuperscript{2+} entry.

When these results are combined with those obtained from applying the antagonists of the other two Ca\textsuperscript{2+} release channels (i.e., the NAADP-sensitive channels and RyRs), it suggests that IP\textsubscript{3}Rs may indeed be the release channels involved in generating these transients. A role for Ca\textsuperscript{2+} release via IP\textsubscript{3}Rs during cytokinesis has also been suggested in dividing newt (\textit{Cynops pyrrhogaster}) eggs. Microinjection of Ca\textsuperscript{2+} store-enriched microsomal fractions (derived from mouse cerebella and CHO cells) into these eggs induced extra cleavage furrows at the site of injection via IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release (Mitsuyama et al., 1999).

To further support our proposition that IP\textsubscript{3}Rs are, but RyRs and NAADP-sensitive channels are not, involved in generating the cytokinetic Ca\textsuperscript{2+} transients in zebrafish, we also introduced a variety of channel agonists into the blastodisc (see Fig. 8) to see if they could induce the release of calcium from their respective receptors. It is clear that injected IP\textsubscript{3}Rs released Ca\textsuperscript{2+} via IP\textsubscript{3}Rs and NAADP released Ca\textsuperscript{2+} from NAADP-sensitive channels. However, caffeine and cADP ribose did not induce any response suggesting that RyRs are either not present or are non-functional at this stage of zebrafish development. Along with the results from the antagonist injections, this provides additional compelling evidence that IP\textsubscript{3}Rs play a key role in releasing calcium during both furrow propagation and deepening in zebrafish embryos. Introducing NAADP into the zebrafish blastodisc clearly resulted in the release of Ca\textsuperscript{2+} (see Fig. 8B) indicating that NAADP-sensitive channels are present in this embryo. However, the introduction of the NAADP-sensitive channel antagonists, verapamil and nifedipine, (Genazzani et al., 1996, 1997) had no effect on the cytokinetic Ca\textsuperscript{2+} transients or cytokinesis itself, suggesting that although the NAADP-sensitive channels are present, they do not play a role in generating these Ca\textsuperscript{2+} signals.

**Materials and Methods**

**Egg collection**

Zebrafish (\textit{Danio rerio}) were maintained and their fertilized eggs collected as described in detail elsewhere (Webb et al., 1997). To enhance optical clarity during imaging, embryos were dechorionated by treatment with pronase (Sigma; 1 mg/ml in 30% Danieau’s solution; 19.3 mM NaCl, 0.23 mM KCl, 0.13 mM MgSO\textsubscript{4}, 2H\textsubscript{2}O, 0.2 mM Ca(NO\textsubscript{3})\textsubscript{2}, 1.67 mM Hepes pH 7.2) for several minutes at room temperature and then washed several times in 30% Danieau’s solution. They were then placed in custom-made holding/viewing chambers (described in Webb et al., 1997) for microinjection and subsequent imaging.

**Microinjection of aequorins**

The microinjection pipettes, the pressure injection system and the other protocols used for injecting embryos with aequorin are described in detail elsewhere (Webb et al., 1997). Approximately 1.2 nl of recombinant f-or h-aequorin (supplied by Dr. O. Shimomura, The Photoprotein Laboratory, Falmouth, MA, USA; at 0.5-1.0% in 100 mM KCl, 5 mM MOPS, and 50 µM EDTA) was injected into the center of the embryo’s yolk cell. Following microinjection, embryos were manipulated to provide a particular viewing orientation (i.e., facial, axial or top views; Webb et al., 1997) and then transferred to our photon imaging microscope (PIM; Science Wares, East Falmouth, MA, USA) for data acquisition.

**Data acquisition and review**

Aequorin-generated and bright-field images were acquired using our custom-designed PIM, which is described in detail elsewhere (Webb et al., 1997; Lee et al., 1999). Images were collected using either Zeiss Fluar 10x/0.5 NA or Plan Neofluar 10x/0.3 NA objectives. At the end of data acquisition, files were downloaded into Corel DRAW 8 and PHOTO PAINT 8 (Corel Corp.) for figure preparation and presentation.

**Determination of challenge time**

It was important to establish protocols to ensure that the Ca\textsuperscript{2+} buffers, channel antagonists and agonists introduced into the blastodisc of a dividing embryo reached the propagating or deepening furrow in time to have an effect directly on the particular aspect of cytokinesis under consideration. From our previous imaging study (Webb et al., 1997), we were able to obtain a good estimation of the timing of the various stages in the cytokinetic process (i.e., furrow positioning, propagation and deepening) by monitoring the appearance and progression of the associated Ca\textsuperscript{2+} transients. We used these, therefore, to time when the embryo should be removed from the PIM for injection. Thus, to challenge furrow propagation, deepening and apposition, embryos were removed for injection or incubation: as soon as the positioning signal appeared at the blastodisc surface (see Figs. 3C and D); as the propagation signal approached the edges of the blastodisc (see Figs. 3A and E); and when the deepening signal appeared at the apex of the blastodisc (see Fig. 3B), respectively. By following these protocols, we were confident that a particular Ca\textsuperscript{2+} transient was being challenged directly, rather than indirectly via an upstream Ca\textsuperscript{2+}-sensitive step.

**Calibration of injectate volume and cytosolic concentration**

Micropipettes were calibrated by injecting solution under Wesson vegetable oil and measuring the diameter of the droplets produced with an eyepiece reticle (Miller et al., 1994). This was always done before (and sometimes after) each injection to ensure that a known volume had been delivered to the embryo. In order to estimate the final cytosolic concentration of injectate, the volume of each zebrafish embryo was assumed to be ~128 nl (from a dechorionated egg diameter of ~625 µm). We have previously determined the water percentage of single cell zebrafish embryos to be ~68% (Leung et al., 1998). The approximate final cytosolic concentrations of the antagonists and agonists used during the experiments are given in Tables 1 and 2 respectively.

**Microinjection of BPATa buffers during furrow deepening and apposition**

The need for a localized elevation of intracellular free Ca\textsuperscript{2+} in furrow propagation has been demonstrated previously (Webb et al., 1997). Here, experiments were carried out to explore whether localized increases in intracellular free Ca\textsuperscript{2+} are also required for both furrow deepening and apposition of the daughter blastomeres at the end of cytokinesis. Embryos were injected with aequorin and imaged using our PIM as described above. Following the protocols described in the previous section (and see Figs. 3A and 3B), 5,5′-dibromo-BAPTA (DBB, K\textsubscript{D} = 1.5 µM; ~0.9 nl of a 25 mM solution in 18.25 mM CaCl\textsubscript{2}, 5 mM Hepes pH 7.0) or 5,5′-dimethyl-BAPTA (DMB, K\textsubscript{D} = 0.15 µM; ~0.9 nl of a 25 mM solution in 10.5 mM CaCl\textsubscript{2}, 5 mM Hepes pH 7.0) was injected into the
blastodisc and imaging then resumed. Buffers were injected to reach a final cytosolic concentration of ~260 µM (see calibration factors in previous section). In the case of DBB and DMB, enough CaCl₂ (i.e., 18.25 mM and 10.5 mM, respectively) was added to each buffer to set its free [Ca²⁺] at ~400 nM, a level well above that reported for the resting level in zebrafish embryos (i.e., ~60 nM; Créton et al., 1998). Both dibromo- and dimethyl-BAPTA were obtained from Molecular Probes.

Treatment with Ca²⁺ channel antagonists

In order to identify the source and the mechanism of Ca²⁺ release used to generate the cytosolic transients, embryos were treated with a variety of Ca²⁺ channels antagonists. The antagonists of: (1) the inositol 1,4,5-trisphosphate receptor (heparin and 2-APB), (2) the ryanodine receptor (ryanodine and ruthenium red), and (3) NAADP-sensitive calcium channel (nifedipine, and verapamil) were utilized during the study. In addition, the inactive form of heparin (de-N-sulfated heparin) was used as a heparin control. It has been reported that at low concentrations, (<10 µM) ryanodine activates the ryanodine receptor, whilst at concentrations of >10 µM it acts as an antagonist (Ehrlich et al., 1994). Here, ryanodine was used at ~120 µM for microinjecting into the blastodisc and at ~500 µM for incubation. Common L-type channels antagonists (i.e., nifedipine and verapamil) when used at high concentrations have been reported to inhibit the calcium release from NAADP-sensitive channels in sea urchin embryos (Genazzani et al., 1996; 1997). As it has been previously established that the Ca²⁺ generating the cytosolic transients was released from intracellular rather than extracellular Ca²⁺ stores (Webb et al., 1997), these antagonists could be used to determine the possible contribution of Ca²⁺ released via NAADP-sensitive channels to the generation of cytosolic Ca²⁺ transients without the complication of their effect on inhibiting L-type voltage-gated channels. The ruthenium red was obtained from Fluka whereas the other antagonists and the inactive heparin were obtained from Sigma. Stock solutions of heparin (H-5027; bovine intestinal mucosa sodium salt; MW 13,000; at 10 mg/ml), de-N-sulfate heparin (10 mg/ml) and the ryanodine used for the injection experiments (5 mM) were prepared in injectate dilution buffer (150 mM KCl, 5 mM Hepes, pH 7.2). The ryanodine used for the incubation experiments was prepared at 500 µM in 30% Danieau’s solution. Stock solutions of 2-APB (10 mM) and nifedipine (50 mM) were prepared in dimethyl sulfoxide (DMSO), while a stock solution of verapamil (25 mM) was prepared in 30% Danieau’s solution. Embryos were either injected with or incubated in the appropriate concentrations (see Table 1) of these antagonists, following the protocols described in the previous section and illustrated schematically in Fig. 3. Some embryos were also injected with injectate dilution buffer or incubated with the appropriate amount of DMSO in 30% Danieau’s solution as controls.

The extent of the Ca²⁺ rise during the generation of the propagation transient in the presence or absence of channel antagonists was estimated in the following manner. The light output from a selected area of the blastodisc was measured at rest (i.e., before the occurrence of a transient) then measured again during the peak passage of the transient. This form of “self-calibration” makes experience generally available. This was obtained from Molecular Probes.

Immunohistochemical labeling of the ER and IP₃ Receptors

Embryos were dechorionated as described above and fixed during furrow deepening of the first cell division cycle with 4% paraformaldehyde in phosphate buffered saline (PBS: Westerfield, 1994) overnight at 4°C. Fixed embryos were washed initially with PBS (>5 washes) to remove excess fixative, then with PBS containing 0.1% Tween-20 (PBST) for four 5-min washes, and finally with PBST containing 1% DMSO (PBSTD) for 5 min. Embryos were then incubated in blocking buffer (PBSTD containing 10% bovine serum albumin, BSA) for 2 hours after which they were incubated for one hour with either anti-calnexin (Stressgen Biotechnologies Corp.) diluted 1:100 in blocking buffer to label the ER, or else anti-IP₃Rs (Type 1; Sigma), diluted 1:200 in blocking buffer. They were then washed extensively with PBSTD containing 1% BSA (PBSTD/BSA) and incubated for one hour with FITC-conjugated goat anti-rabbit IgG (H+L; Zymed Laboratories Inc.) diluted 1:100 (for the ER) or 1:500 (for the IP₃Rs) in blocking buffer, after which they were washed extensively again with PBSTD/BSA. FITC-labelled ER and IP₃Rs were visualized using a Bio-Rad MRC-600 laser scanning confocal microscope, equipped with a krypton/argon laser, mounted on a Zeiss Axioskop upright microscope. Images were collected using a Zeiss water immersion Achroplan 20X/0.5NA objective. Embryos were observed from an animal pole view and a z-series of confocal sections were scanned through the blastodisc to the top of the yolk cell. Figures 9B and 9C represent projected images of 21 and 25 confocal sections, respectively, to show each blastodisc from the animal pole, whereas Figs. 9D and 9F represent projected images of the confocal stacks rotated 90° to show them from a facial orientation.

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References


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