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Protein kinase C acts downstream of calcium at entry into the first mitotic interphase of *Xenopus laevis*

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Transit into interphase of the first mitotic cell cycle in amphibian eggs is a process referred to as activation and is accompanied by an increase in intracellular free calcium ($[Ca^{2+}]_i$), which may be transduced into cytoplasmic events characteristic of interphase by protein kinase C (PKC). To investigate the respective roles of $[Ca^{2+}]_i$ and PKC in *Xenopus laevis* egg activation, the calcium signal was blocked by microinjection of the calcium chelator BAPTA, or the activity of PKC was blocked by PKC inhibitors sphingosine or H7. Eggs were then challenged for activation by treatment with either calcium ionophore A23187 or the PKC activator PMA. BAPTA prevented cortical contraction, cortical granule exocytosis, and cleavage furrow formation in eggs challenged with A23187 but not with PMA. In contrast, sphingosine and H7 inhibited cortical granule exocytosis, cortical contraction, and cleavage furrow formation in eggs challenged with either A23187 or PMA. Measurement of egg $[Ca^{2+}]_i$ with calcium-sensitive electrodes demonstrated that PMA treatment does not increase egg $[Ca^{2+}]_i$ in BAPTA-injected eggs. Further, PMA does not increase $[Ca^{2+}]_i$ in eggs that have not been injected with BAPTA. These results show that PKC acts downstream of the $[Ca^{2+}]_i$ increase to induce cytoplasmic events of the first *Xenopus* mitotic cell cycle.

Introduction

Dividing eukaryotic cells undergo a temporary arrest at metaphase which may last minutes in mitotic cells and hours in meiotic cells. What causes this arrest is unknown, but it is becoming increasingly apparent that rises in intracellular free calcium ($[Ca^{2+}]_i$) end the metaphase arrest and trigger transit into interphase. For example, rises in $[Ca^{2+}]_i$ have been shown to accompany exit from mitosis in plant cells (Hepler and Callaham, 1987), sea urchin embryos (Poenie *et al.*, 1985), and mammalian cells (Poenie *et al.*, 1986; Ratan *et al.*, 1986; Ratan *et al.*, 1988; Tombes and Borisy, 1989). In addition, inhibition of calcium increases blocks the metaphase/anaphase transition in plant cells, sand dollar embryos, and cultured mammalian cells (Izant, 1983; Hepler, 1985; Wolniak and Bart, 1985; Silver, 1989; Tombes and Borisy, 1989); and increasing $[Ca^{2+}]_i$ shortens the metaphase arrest in mammalian cells (Izant, 1983).

In eggs of many organisms, which are arrested at specific points in meiosis until fertilization, a calcium trigger for cell cycle resumption is especially well documented. Large, transient $[Ca^{2+}]_i$ rises are intrinsic to fertilization in sea urchins (Steinhardt *et al.*, 1977), fish (Gilkey *et al.*, 1978; Nuccitelli, 1987), amphibians (Busa and Nuccitelli, 1985; Kubota *et al.*, 1987; Nuccitelli *et al.*, 1988), and mammals (Cuthbertson *et al.*, 1981; Miyazaki *et al.*, 1986). In the case of amphibian fertilization, the evidence supporting a calcium trigger for entry into the first mitotic interphase ("egg activation") is compelling: 1) The wavelike rise in $[Ca^{2+}]_i$ is followed immediately by a wave of exocytosis and a wave of microvillar elongation, morphological changes characteristic of egg activation (Busa and Nuccitelli, 1985; Kubota *et al.*, 1987). 2) Experimental treatments that increase egg $[Ca^{2+}]_i$—such as application of calcium ionophore, pricking with a glass needle, microinjection of calcium, or microinjection of inositol 1,4,5 trisphosphate (IP_3)—trigger egg activation (Gingell, 1970; Schroeder and Strickland, 1974; Wolf, 1974a; Hollinger and Schuetz, 1976; Busa *et al.*, 1985; Picard *et al.*, 1985; Kubota *et al.*, 1987). 3) Treatments that prevent an increase in $[Ca^{2+}]_i$ abolish events associated with interphase entry, including cortical granule exocytosis, cortical contraction, chromosome decondensation, and nuclear envelope reformation in response to activating stimuli (Gingell, 1970; Schroeder and Strickland, 1974; Christensen *et al.*, 1984; Lohka and Masui, 1984; Kline, 1988).

Thus, an increase in $[Ca^{2+}]_i$ is apparently the critical trigger for transit into interphase in amphibian eggs, just as it is thought to be in other cell types. This observation provokes the following question: How is an increase in $[Ca^{2+}]_i$

transduced into the diverse cellular events characteristic of the M-phase/interphase transition, such as cytokinesis, chromosome decondensation, reformation of the nuclear envelope and golgi, and the resumption of endo- and exocytosis? One possibility is that these events are mediated by calcium-dependent enzymes. In support of this hypothesis, we have shown that activators of protein kinase C (PKC), a calcium, phospholipid-sensitive protein kinase, trigger cytoplasmic events of the M-phase/interphase transition, that is, cortical granule exocytosis, cortical contraction, and cleavage furrow formation, in oocytes and eggs of the amphibian, *Xenopus laevis* (Bement and Capco, 1989a).

PKC activation occurs as a result of increases in $[Ca^{2+}]_i$ in most systems (Kikkawa *et al.,* 1989) and indirect evidence suggested that this is also the case during *Xenopus* egg activation (Bement and Capco, 1989a). However, it is also possible that PKC activators somehow trigger a free-calcium increase within eggs. This is reported to occur in mouse oocytes (Cuthbertson and Cobbold, 1985; Colonna *et al.,* 1989), rat pituitary cells (Albert *et al.,* 1987), mouse pancreatic islet cells (Pace, 1984), and bag cell neurons of the mollusc *Aplysia* (DeRiemer *et al.,* 1985). It is therefore unclear whether PKC acts as the immediate stimulus for cortical granule exocytosis and cortical contraction in *Xenopus* eggs, or whether it acts indirectly by triggering an increase in $[Ca^{2+}]_i$.

If PKC mediates *Xenopus* egg activation events by causing a rise in $[Ca^{2+}]_i$, then three predictions follow. First, chelation of cytosolic calcium should prevent activation of eggs challenged with PKC agonists. Second, PKC agonists should increase $[Ca^{2+}]_i$. Third, PKC inhibitors should not affect activation of eggs challenged with calcium ionophore. If, however, PKC operates downstream of the $[Ca^{2+}]_i$ rise, the opposite predictions should hold true. PKC agonists should activate eggs even when cytosolic calcium has been chelated; PKC agonists should not increase $[Ca^{2+}]_i$; and PKC antagonists should inhibit calcium ionophore–induced egg activation. We have blocked increases in either $[Ca^{2+}]_i$ or PKC activity by treating eggs with a calcium chelator, or PKC antagonists, respectively, and then challenged treated eggs with either calcium ionophore or phorbol 12-myristate 13 acetate (PMA), a PKC activator. We have also measured $[Ca^{2+}]_i$ in eggs treated with PMA. As an indication of egg activation, we have monitored early morphological changes characteristic of this process: cortical granule exocytosis and cortical contraction. The results support a model in which PKC acts downstream of the $[Ca^{2+}]_i$ rise to trigger events of the M-phase/interphase transition of *Xenopus* eggs.

Results

A23187 and PMA induce cortical contraction in the absence of external calcium

To determine the dependency of A23187 and PMA-induced egg activation on extracellular calcium, and to determine the appropriate concentrations of these agents to employ in later assays, we first generated dose-response curves by treating eggs with A23187 or PMA in the presence or absence of external calcium. Cortical contraction was used as a marker for egg activation. A23187 consistently induced cortical contraction in 100% of eggs when employed at a concentration of 100 nM (Figure 1A); treatment with higher concentrations was equally effective but often resulted in cell lysis. A23187-induced contraction was just as effective in the absence of extracellular calcium as it was in the presence of extracellular calcium. Likewise, PMA did not require extracellular calcium to induce cortical contraction and was also 100% effective at a concentration of 100 nM (Figure 1B).

BAPTA does not block cortical contraction in eggs challenged with PMA

Eggs in calcium-free medium have no access to extracellular calcium; however, the above experiments demonstrated that intracellular stores of calcium are sufficient to trigger events of activation. To clamp both $[Ca^{2+}]_i$ and extracellular calcium at low levels, eggs were microinjected with the calcium chelator 1,2,-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) and then challenged in calcium-free medium with either A23187 or PMA. BAPTA prevented cortical contraction in eggs challenged with 100 nM A23187 in calcium-free medium when the former was injected to a final intracellular concentration of 10 mM (Figure 2). This concentration of BAPTA also prevented cortical contraction in response to 1 μM A23187. In contrast, eggs injected with BAPTA and then challenged with 100 nM PMA in calcium-free medium underwent cortical contraction with the same frequency as eggs injected with water; that is, essentially 100% of the eggs challenged with PMA exhibited contraction (Figure 2). BAPTA-injected eggs also formed cleavage furrows when activated by PMA (not shown).

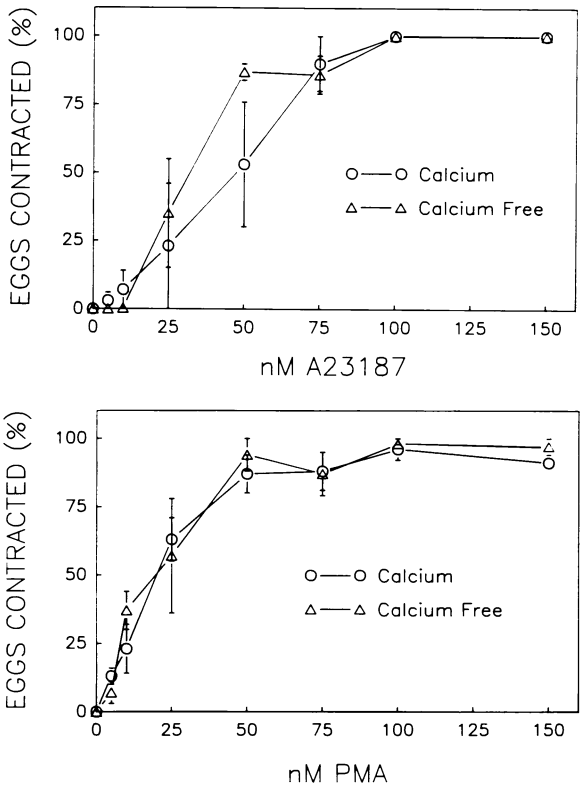

Figure 1. **A23187 and PMA induce cortical contraction in the absence of extracellular calcium.** Eggs were challenged with increasing concentrations of A23187 (A) or PMA (B) in the presence or absence of external calcium and then monitored for cortical contraction over a period of 20 min. Percentages were obtained by dividing the number of eggs that contracted within 20 min by the total number of eggs in the treatment group. (A) A23187 induces 100% cortical contraction when applied at a concentration of 100 nM, both in the normal and calcium-free medium (0.1× O-R2 without calcium and with 10 mM EGTA). (B) PMA also induces 100% cortical contraction at 100 nM in normal and calcium-free medium. Data expressed are the mean ± SE from three experiments. Where error bars are not visible, SE < 0.02.

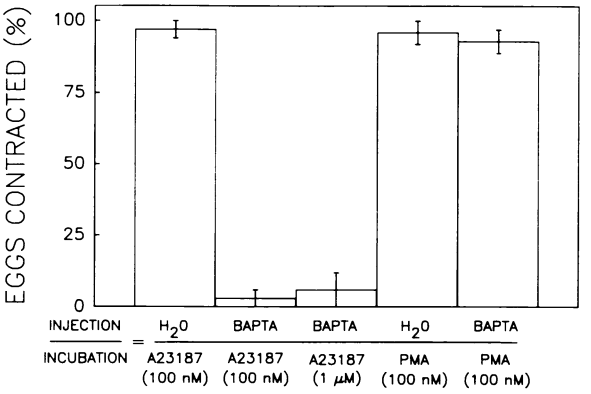

Figure 2. **BAPTA blocks contraction in response to A23187, but not PMA.** Eggs were injected with BAPTA to a final intracellular concentration of 10 mM and then challenged in calcium-free medium with A23187 or PMA at the concentrations indicated. Control eggs were injected with water. BAPTA blocks contraction in eggs challenged with 100 nM and 1 μM A23187, but does not block contraction in response to 100 nM PMA. Data were analyzed as in the legend to Figure 1.

In separate experiments, eggs were injected with calcium/BAPTA buffers formulated to contain decreasing concentrations of free calcium and then challenged with PMA to further characterize the minimum calcium sensitivity of PMA-induced activation (see Methods). Figure 3 shows that between a calculated free-calcium concentration of 2 and 0.5 nM, cortical contraction is inhibited. The inhibition was manifest as both a delay in the time to contraction and a reduction in the overall percentage of eggs that contracted. With decreasing free-calcium levels, an increase in the number of eggs that died after treatment with PMA also occurred; these eggs were not scored for cortical contraction. Because the $[Ca^{2+}]_i$ of BAPTA-injected eggs is dependent on the free-calcium concentration and calcium-buffering capacity of the egg (Kline, 1988), we do not know how closely the calculated free-calcium level corresponds to the actual intracellular calcium concentration (see below for direct measurement of $[Ca^{2+}]_i$). However, these data demonstrate that there is a

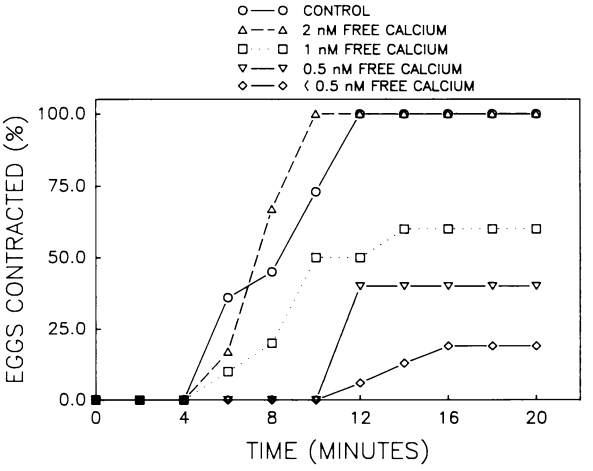

Figure 3. **PMA-induced contraction is inhibited only at extremely low free-calcium concentrations.** Eggs were injected with calcium buffers containing BAPTA and $CaCl_2$ at concentrations formulated to yield the indicated free-calcium levels (see Methods). Eggs were then challenged with 100 nM PMA and the percentage of eggs undergoing contraction was plotted against time. Between a calculated free-calcium concentration of 2 and 0.5 nM, a delay in the time to onset of contraction in response to PMA is evident, as is a reduction in the final percentage of eggs that undergo contraction.

lower limit of free calcium beyond which PMA-induced activation is inhibited.

BAPTA does not block cortical granule exocytosis in eggs challenged with PMA

To determine whether or not BAPTA treatment blocked cortical granule exocytosis, control and BAPTA-treated eggs were processed for electron microscopy after challenge with A23187 or PMA in calcium-free medium. Figure 4A shows the cortex of a control egg challenged with 100 nM A23187 in calcium-free medium. Cortical granule exocytosis has occurred, as evidenced by the lack of cortical granules. BAPTA injection blocks cortical granule exocytosis in eggs challenged with 100 nM A23187 in calcium-free medium, as demonstrated by the presence of intact cortical granules (Figure 4B). PMA treatment, in contrast, triggered cortical granule exocytosis in BAPTA-injected eggs. Eggs injected with sufficient BAPTA (final intracellular concentration 10 mM) to prevent exocytosis in response to A23187 underwent exocytosis in response to 100 nM PMA in calcium-free medium, as shown by the absence of cortical granules (Figure 4C).

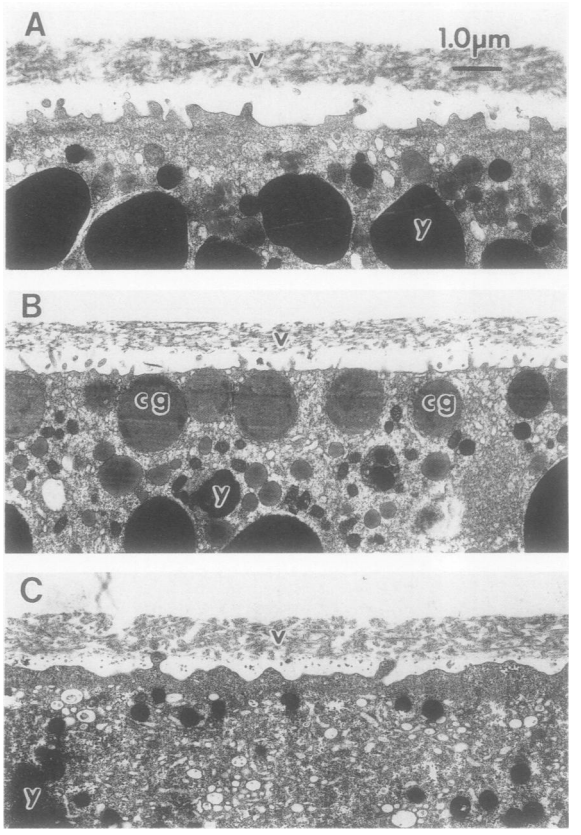

Figure 4. **BAPTA blocks cortical granule exocytosis in response to A23187 but not PMA.** (A) Electron micrograph of the cortex from a control egg challenged in calcium-free medium with 100 nM A23187. The egg has undergone cortical granule exocytosis, as demonstrated by the absence of cortical granules. y denotes yolk platelets; v marks the vitelline envelope. (B) Cortex of an egg injected with BAPTA (10 mM) and then challenged in calcium-free medium with 100 nM A23187. The presence of numerous cortical granules (cg) beneath the plasma membrane shows that cortical granule exocytosis has not occurred. (C) Cortex of an egg injected with BAPTA (10 mM) and then challenged in calcium-free medium with 100 nM PMA. Cortical granule exocytosis has occurred, as indicated by the absence of cortical granules.

PMA does not trigger a rise in $[Ca^{2+}]_i$

As a means to assess directly the effect of PMA treatment on egg $[Ca^{2+}]_i$, calcium-sensitive microelectrodes were employed to measure $[Ca^{2+}]_i$ in eggs injected with BAPTA to a final intracellular concentration of 10 mM. Eggs were impaled with microelectrodes, and, after the membrane potential and $[Ca^{2+}]_i$ had stabilized, PMA was applied at a final concentration of 100 nM. Figure 5A shows the results of a typical experiment. After BAPTA injection but before PMA treatment, the resting $[Ca^{2+}]_i$ was 40 nM. After PMA treatment, the pCa (negative log of $[Ca^{2+}]_i$) value decreased 11 mV by 435 s (7 min, 15 s) (as monitored by oscilloscope; see Methods), reflecting a $[Ca^{2+}]_i$ decrease of 24 to 16 nM. Measurements taken at intermediate time points revealed that $[Ca^{2+}]_i$ decreased immediately after PMA treatment, dropping 5 nM within the first 20 s, and continued to decrease steadily after that (Figure 5A). In this experiment, cortical contraction was apparent at 7 min. This experiment was repeated four times using eggs obtained from different frogs; in all cases PMA treatment resulted in cortical contraction and triggered a decrease in $[Ca^{2+}]_i$ (-17 ± 3.9 nM; mean ± SE), in one case to a level of 10 nM, demonstrating that PMA can trigger contraction even at this extremely low $[Ca^{2+}]_i$.

As a positive control for the sensitivity of our microelectrode measurements, in separate experiments untreated eggs were challenged with A23187 and changes in $[Ca^{2+}]_i$ were determined. As previously described (Busa *et al.*, 1985), A23187 treatment triggered an increase in $[Ca^{2+}]_i$ (data not shown).

Even in the absence of microinjected BAPTA, PMA failed to elicit an increase in egg $[Ca^{2+}]_i$. Uninjected eggs had higher resting $[Ca^{2+}]_i$ levels than BAPTA-injected eggs, but the response to PMA was qualitatively similar to that observed for BAPTA-injected eggs. A typical experiment is shown in Figure 5B. Before treatment with

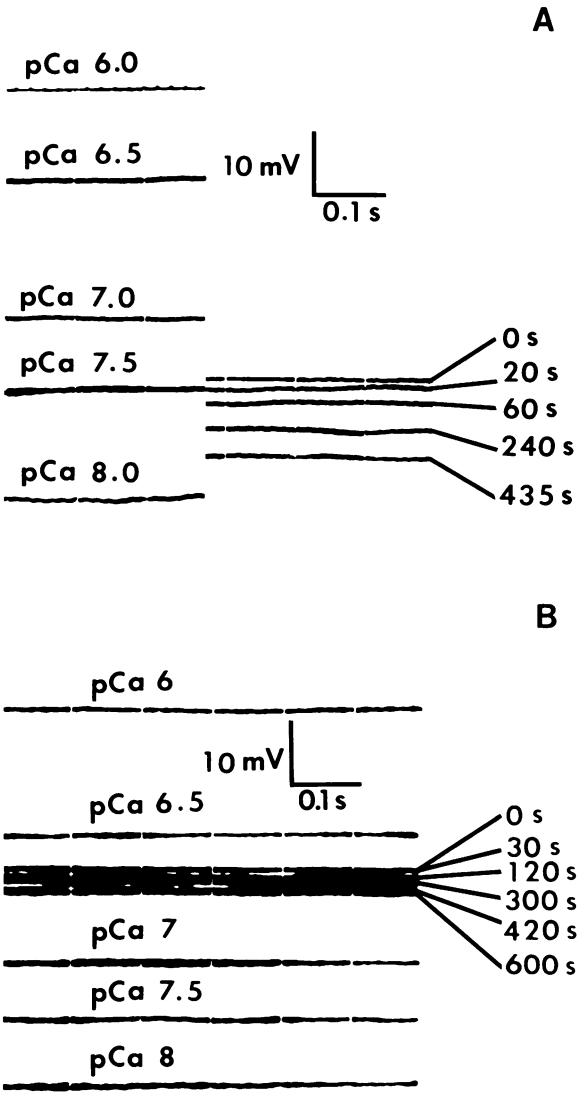

Figure 5. **PMA does not cause an increase in $[Ca^{2+}]_i$.** Photographs of oscilloscope tracings measuring $[Ca^{2+}]_i$ in response to 100 nM PMA in (A) an egg injected with BAPTA to a final intracellular concentration of 10 mM and (B) an uninjected egg. (A) The left half of the figure shows oscilloscope tracings, which represent the calibration performed immediately after the experiment was completed. For the calibration, pCa 6 = 1 μM free calcium, pCa 6.5 = 320 nM, pCa 7 = 100 nM, pCa 7.5 = 32 nM, and pCa 8 = 10 nM. The right half of the figure shows oscilloscope tracings demonstrating the $[Ca^{2+}]_i$ of a BAPTA-injected egg before PMA application (0 s, $[Ca^{2+}]_i$ = 40 nM), and $[Ca^{2+}]_i$ after PMA application for 20 s (35 nM), 60 s (32 nM), 240 s (24 nM), and 435 s (16 nM). (B) Oscilloscope tracings showing $[Ca^{2+}]_i$ in an uninjected egg before PMA application (0 s, $[Ca^{2+}]_i$ = 220 nM), and $[Ca^{2+}]_i$ after PMA application for 30 s (no change from 0 s, as demonstrated by the superimposition of the two tracings), 120 s (204 nM), 300 s (195 nM), 420 seconds (191 nM), and 600 seconds (178 nM). In this figure, the calibration tracings are on the same photograph as the experimental tracings and are labelled as in Figure 5A. In both 5A and 5B the calibrations were essentially the same both before and after egg impalement.

PMA, the resting $[Ca^{2+}]_i$ was 220 nM. Treatment with PMA induced a 4-mV decrease in the pCa value over the course of 600 s (10 min), corresponding to a 42-nM $[Ca^{2+}]_i$ decrease to a level of 178 nM. Measurements taken over the course of the experiment showed that no apparent $[Ca^{2+}]_i$ decrease occurred within the first 30 s, but by the first 120 s $[Ca^{2+}]_i$ dropped 16 nM, to 204 nM, and continued dropping steadily thereafter (Figure 5B). In this experiment, cortical contraction commenced at 300 s (5 min). Out of seven experiments, using eggs obtained from different frogs, in only one experiment was an increase in $[Ca^{2+}]_i$ observed, in this case from 110 to 123 nM, well below the reported increase in $[Ca^{2+}]_i$ that triggers egg activation at fertilization (Busa and Nuccitelli, 1985). In all other experiments, $[Ca^{2+}]_i$ decreased in response to PMA, although the magnitude of the decrease varied from experiment to experiment (-40 ± 25 nM; mean $\pm$ SE).

PKC antagonists inhibit cortical contraction

Sphingosine and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), two structurally unrelated PKC antagonists, were employed to inhibit PKC. To control for nonspecific effects, the activation response of eggs treated with H7 or sphingosine was compared with eggs treated with N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7). W7 is a drug with structural similarity to H7 (Hidaka *et al.,* 1984), but it does not inhibit PKC; rather it inhibits the calcium, calmodulin-dependent protein kinase. As an additional control, eggs treated with H7, sphingosine, or W7 were also compared with eggs treated with dimethyl sulfoxide (DMSO), the vehicle for these three agents. At 10 μM, a concentration where H7 is known to be specific for PKC (Hidaka *et al.,* 1984), H7 significantly ($p < 0.05$ when compared with DMSO controls) reduced the percentage of eggs that contracted in response to challenge with either A23187 (Figure 6A) or PMA (Figure 6B) in calcium-free medium. At 100 μM, H7 resulted in further inhibition of cortical contraction. Similarly, at 100 μM, a concentration reported to inhibit PKC in vivo (Hannun *et al.,* 1986), sphingosine sharply reduced the percentage of eggs that responded to either A23187 or PMA. Control agents DMSO and W7 had no apparent effect on the ability of eggs to contract when challenged with A23187. Moreover, even in those few cases where eggs treated with H7 or sphingosine were observed to contract in response to A23187 or PMA, the extent of contraction was less than that ob-

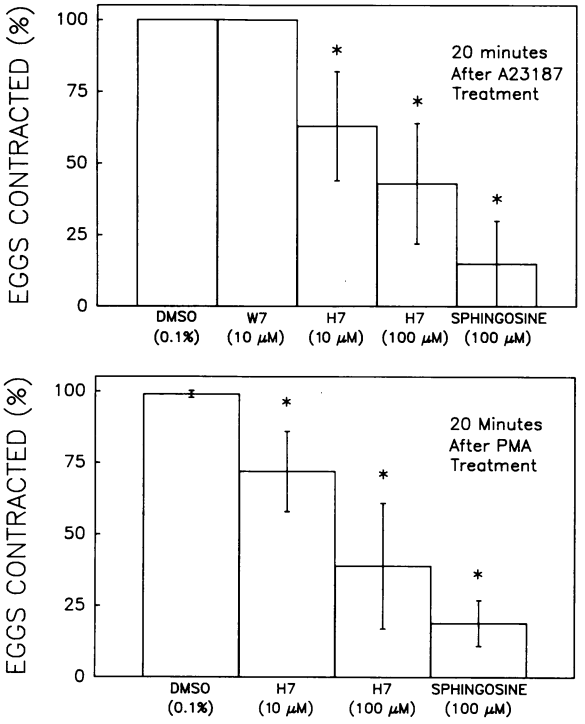

Figure 6. **PKC antagonists inhibit cortical contraction.** Eggs were treated with the indicated concentrations of DMSO, W7, H7, or sphingosine and then challenged in calcium-free medium with 100 nM A23187 (A) or PMA (B). (A) Sphingosine and H7 significantly reduce the percentage of eggs that contract in response to challenge with A23187 when compared with DMSO controls, whereas W7 does not. (B) Sphingosine and H7 also reduce the percentage of eggs that contract in response to challenge with PMA. Data were analyzed as in legend to Figure 1. Asterisk indicates significant difference from DMSO controls ($p < 0.05$; $n = 3$) as described in the Methods. When error bars are not visible, SE < 0.02.

served in eggs treated with DMSO or W7. That is, whereas control eggs generally underwent a very marked contraction when challenged with A23187 or PMA, sometimes forming a small cap of pigment on the top of the animal hemisphere, contraction of eggs treated with sphingosine or H7 was much less obvious.

Sphingosine and H7 inhibit cortical granule exocytosis

To determine whether sphingosine and H7 inhibit cortical granule exocytosis, eggs were treated with sphingosine, H7, W7, or DMSO as described above and then challenged with 100 nM A23187 in calcium-free medium. Six minutes after A23187 treatment, eggs from each of these treatment groups were fixed for electron microscopy and examined for the presence of intact cortical granules. Eggs treated with either H7 (Figure 7A) or sphingosine (Figure 7B) did not undergo cortical granule exocytosis within

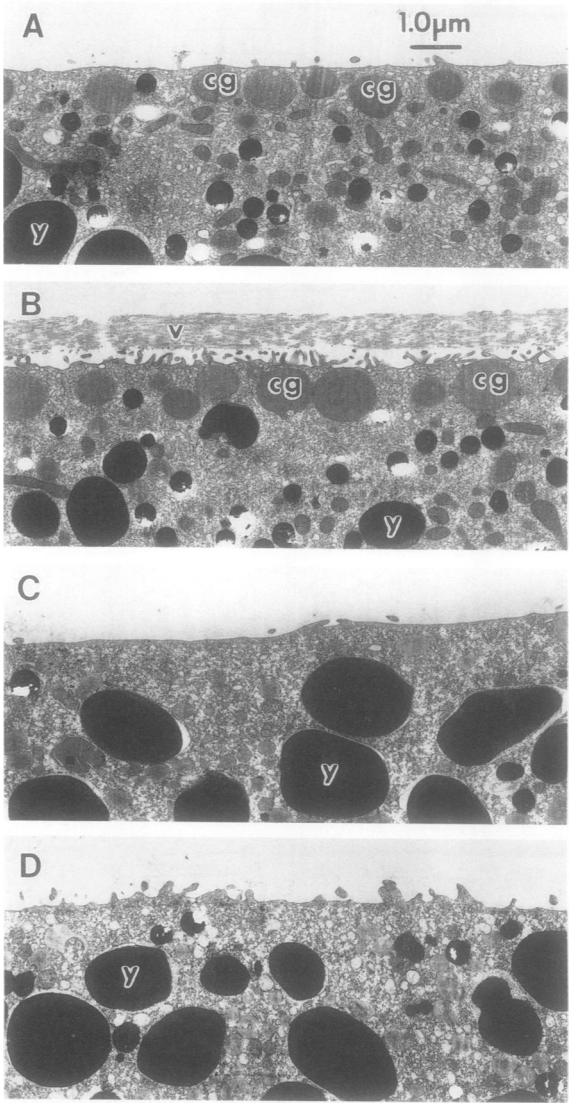

Figure 7. **PKC antagonists inhibit cortical granule exocytosis.** Eggs were treated with 100 μM H7 (A), sphingosine (B), W7 (C), or 0.1% DMSO (D) and then challenged in calcium-free medium with 100 nM A23187 for 6 min. (A) Cortex of an egg treated with H7 and then challenged with A23187. The presence of numerous cortical granules reveals that exocytosis has not occurred. (B) Cortex of an egg treated with sphingosine and then challenged with A23187. Again, the row of intact cortical granules beneath the plasma membrane shows that exocytosis has not occurred. (C) Cortex of an egg treated with W7. The lack of intact cortical granules beneath the plasma membrane demonstrates that the egg has undergone exocytosis. (D) Cortex of an egg treated with DMSO and then challenged with A23187. The absence of cortical granules shows that exocytosis has occurred.

6 min, as demonstrated by the presence of intact cortical granules. Eggs treated with either W7 (Figure 7C) or DMSO (Figure 7D), however, did undergo cortical granule exocytosis within 6 min, as revealed by the absence of intact cortical granules.

As a means to quantify the amount of exocytosis that occurred in eggs treated with H7 or sphingosine, protein release from eggs was measured spectrophotometrically (Wolf, 1974a; Bement and Capco, 1989a). After treatment with H7, sphingosine, or W7, eggs were placed in quartz cuvettes and challenged with 100 nM A23187 or PMA in calcium-free medium. Exocytosis was measured for 16 min, and the amount of protein released by eggs treated with sphingosine, H7, or W7 relative to the amount of protein released from DMSO-treated controls was determined by comparing the final absorbance readings from the different treatment groups. Figure 8A demonstrates that H7 significantly reduced cortical granule exocytosis at concentrations of 10 μM (40% reduction) and 100 μM (50% reduction). Sphingosine was an even more potent inhibitor of exocytosis, with a concentration of 100 μM resulting in an 80% reduction of the amount of protein released. W7, on the other hand, did not significantly inhibit exocytosis in response to challenge with A23187. In keeping with their roles as PKC inhibitors, sphingosine and H7 also inhibited cortical granule exocytosis in eggs challenged with PMA (Figure 8B).

The effects of H7 and sphingosine on cleavage furrow formation varied considerably. In those eggs that failed to contract after H7 or sphingosine treatment, cleavage furrows did not form. On the other hand, eggs that contracted slightly after H7 or sphingosine treatment occasionally formed cleavage furrows.

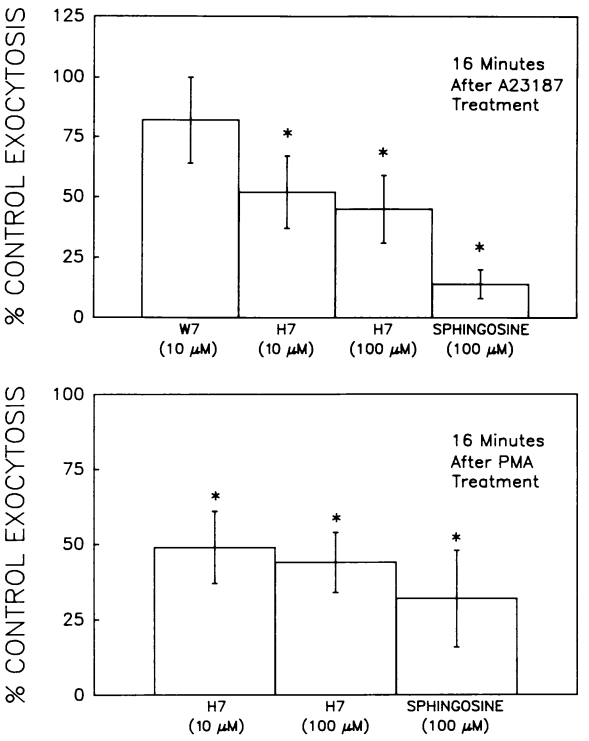

Figure 8. **Quantification of exocytosis inhibition by PKC antagonists.** Eggs were treated with the indicated concentrations of W7, H7, or sphingosine and then challenged in calcium-free medium with 100 nM A23187 (A) or PMA (B). Exocytosis was monitored spectrophotometrically and compared with DMSO-treated controls as described in the Methods. (A) Sphingosine and H7 significantly reduce the amount of exocytosis in eggs challenged with A23187 when compared with DMSO controls, whereas W7 does not have a significant effect. (B) Sphingosine and H7 also reduce the amount of exocytosis in eggs challenged with PMA relative to the amount in DMSO-treated controls. Data were analyzed as in the legend of Figure 6.

Discussion

Increases in $[Ca^{2+}]_i$ are thought to trigger egg activation in most, if not all, animal species (Steinhardt *et al.*, 1974; Jaffe, 1983) and are also thought to be the major signal for exit from mitosis and entry into interphase in embryonic and somatic cells (Poenie *et al.*, 1985; Ratan *et al.*, 1988; Tombes and Borisy, 1989). Thus, an increase in levels of cytosolic calcium must somehow be transduced into a diverse array of cellular events, including anaphase A and B, nuclear reformation, cortical granule exocytosis, cortical contraction, and cytokinesis. Although the cellular entities responsible for these events remain obscure, calcium-dependent enzymes are likely candidates (Dinsmore and Sloboda, 1988; Bement and Capco, 1989a).

The principal aim of this study was to determine whether one such calcium-dependent enzyme, PKC, mediates events characteristic of *Xenopus* egg transit into interphase (i.e., egg activation) by a direct or indirect mechanism. In particular, we wished to clarify whether PKC triggers activation events—that is, cortical granule exocytosis, cortical contraction and cleavage furrow formation—by acting upstream or downstream of an increase in cytosolic free calcium. Two lines of evidence from our results indicate that PKC mediates egg activation events as a downstream result of the calcium wave.

The first line of evidence is derived from activation of eggs in which the availability of free

calcium was experimentally curtailed. PMA, a potent PKC agonist, rescues activation events when applied to BAPTA-injected eggs in calcium-free medium. That calcium-free medium and BAPTA injection is sufficient to prevent significant calcium increases is demonstrated by the fact that calcium ionophore fails to activate eggs under these conditions. Thus, PMA cannot be activating eggs by triggering an increase in $[Ca^{2+}]_i$ unless it is a more effective calcium ionophore than A23187, a possibility that was excluded in this study by direct measurement of $[Ca^{2+}]_i$. Although it may appear paradoxical that a calcium-dependent enzyme such as PKC can be activated in the virtual absence of free calcium, it has been demonstrated that PMA can activate PKC in vivo at $[Ca^{2+}]_i$ concentrations of 8 nM (Di Virgilio *et al.,* 1984). Thus, treatment of eggs with PMA after clamping intracellular and extracellular calcium levels provides a useful and direct means to distinguish PKC-mediated cellular events from events mediated by other calcium-dependent enzymes.

Direct measurement of $[Ca^{2+}]_i$ in eggs injected with BAPTA demonstrates that PMA does not trigger an increase in cell free calcium. Indeed, even under conditions of freely available intracellular and extracellular free calcium (e.g., in the absence of BAPTA and the presence of extracellular calcium), PMA treatment triggered no increase in $[Ca^{2+}]_i$ that could be detected by calcium-sensitive microelectrodes. Similar results have recently been obtained by ratio-imaging with the use of the calcium-sensitive dye Fura-2 (C.A. Larabell and R. Nuccitelli, personal communication). We found that $[Ca^{2+}]_i$, rather than increasing in response to PMA, actually decreased in response to PMA and that, in spite of this decrease, activation events nevertheless occurred. Thus, PKC cannot be triggering these events by acting as an upstream stimulus for an increase in $[Ca^{2+}]_i$. Based on our results with microinjection of the calcium buffers and measurement of $[Ca^{2+}]_i$ in BAPTA-injected eggs, we conclude that PMA is capable of triggering egg activation events at free-calcium concentrations of 2–10 nM, well below the $[Ca^{2+}]_i$ required for other known calcium-dependent processes (Cheung, 1980). The results therefore also exclude the possibility that PKC activators merely lower the calcium requirement of cortical granule exocytosis and cortical contraction such that resting levels of $[Ca^{2+}]_i$ trigger these events.

The second line of evidence supporting a downstream site of PKC action is based on the ability of PKC inhibitors to suppress activation events under conditions that should result in the direct release of calcium from intracellular stores. The PKC inhibitors H7 and sphingosine antagonize both cortical granule exocytosis and cortical contraction in eggs challenged with A23187 in calcium-free medium. This result is not consistent with a model wherein PKC acts to trigger a calcium increase, because, if this were the case, direct calcium release by A23187 should bypass the inhibitor-imposed blockade. This result *is* consistent with a model wherein the calcium increase triggers PKC activation, which, in turn, mediates cortical granule exocytosis and cortical contraction.

On the basis of the evidence above, we propose the following model for *Xenopus* egg activation (Figure 9). Fertilization (Busa and Nuccitelli, 1985) or artificial activation—by treatment with calcium ionophore (Schroeder and Strickland, 1974), microinjection of IP_3 (Busa *et al.,* 1985; Picard *et al.,* 1985), or pricking the egg in calcium-containing medium (Wolf, 1974a; Kubota *et al.,* 1987)—triggers an increase in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increase then results in PKC activation, which can be mimicked in the absence of a $[Ca^{2+}]_i$ increase by treatment of eggs with PKC agonists such as PMA, phorbol 12,13-didecanoate (PDD), 1-oleoly-2-acetyl-sn-glycerol (OAG) (Bement and Capco, 1989a), or 1,2-dioctanoylglycerol (DiC8) (Bement and Capco, unpublished results). Upon activation, PKC mediates cortical granule exocytosis, cortical contraction, and cytokinesis, presumably by phosphorylating endogenous substrates.

This hypothesis prompts several testable predictions about intracellular signalling events during *Xenopus* egg activation. 1) Because PKC activity is associated with translocation of PKC to the plasma membrane (Hirota *et al.,* 1985; Ito *et al.,* 1988), and because cortical granule exocytosis occurs as a wave during fertilization

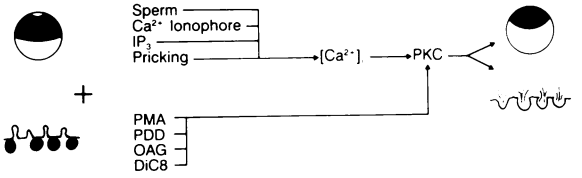

Figure 9. **Model of the signalling sequence that triggers cytoplasmic events of the first *Xenopus* cell cycle.** Fertilization, calcium ionophore, IP_3, or pricking all trigger an increase in egg $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ increase results in PKC activation, which, in turn, triggers cortical granule exocytosis and cortical contraction. Activators of PKC such as PMA, PDD, OAG, or DiC8 can bypass the $[Ca^{2+}]_i$ rise by directly activating PKC, thereby triggering cortical granule exocytosis and cortical contraction in the absence of an increase in $[Ca^{2+}]_i$.

(Grey *et al.,* 1974), the calcium wave should be accompanied by a wave of PKC translocation. 2) Because PKC activity requires one of several forms of diacylglycerol as well as calcium (Kikkawa *et al.,* 1989), the calcium wave should be accompanied by an increase in diacylglycerol formation. 3) Because diacylglycerol used in intracellular signalling is generally formed as a result of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis (Berridge, 1987) or as a result of phosphatidylcholine (PC) hydrolysis (Loffelholz, 1989), an increase in the activity of phospholipases that hydrolyze PIP_2 or PC should be observed during egg activation.

Is the model proposed above for *Xenopus* likely to be relevant for calcium-regulated cell cycle transitions in other systems? Several reports suggest that PKC might mediate events of mammalian egg activation (Cuthbertson and Cobbold, 1985; Endo *et al.,* 1987; Colonna *et al.,* 1989), but it is unknown whether PKC acts directly or indirectly in this system (see Introduction). For other cell types, even less information on the role of PKC at entry into interphase is available, although one report has implicated PKC as a downstream effector of calcium at the metaphase/anaphase transition in stamen hair cells (Larsen *et al.,* 1989). Evidence from sea urchin eggs suggests that the calcium wave that accompanies fertilization is the result of PIP_2 hydrolysis (Swan and Whitaker, 1986), an event that would provide the diacylglycerol required for PKC activation. Furthermore, measurement of diacylglycerol levels has shown that an increase does occur upon sea urchin fertilization (Ciapa and Whitaker, 1986), and evidence also suggests that PKC mediates the pH increase that accompanies sea urchin fertilization (Shen and Burgart, 1986). On the other hand, treatment of sea urchin eggs with PMA does not appear to induce cortical vesicle exocytosis (Ciapa *et al.,* 1988) as it does in *Xenopus* eggs, and previous conclusions regarding the role of PKC in the pH increase have recently been questioned (Shen, 1989). Comparisons between sea urchin eggs and those of amphibians are further complicated by the fact that sea urchin eggs are arrested in interphase, whereas amphibian eggs are arrested in M-phase.

Nevertheless, two characteristics of PKC make the proposed model attractive as a general paradigm for calcium-controlled cell cycle transitions. First, PKC activation is associated with translocation of PKC to intracellular membranes (Hirota *et al.,* 1985; Ito *et al.,* 1988; Leach *et al.,* 1989), thus providing it with access to potential substrates that might control membrane events associated with interphase entry, such as resumption of endocytosis and exocytosis and reformation of the nuclear envelope and the golgi. Second, multiple isoforms of PKC exist (Coussens *et al.,* 1986; Housey *et al.,* 1987), each with different sensitivities to calcium and phospholipids, including some forms that apparently require little or no calcium for activity (Kikkawa *et al.,* 1989). Different PKC isoforms could mediate different cellular events, thereby accounting for the variety of events that accompany the transit into interphase. Using the *Xenopus* system as an example, one isoform might be responsible for triggering exocytosis and another for cortical contraction. The differing sensitivities of these isoforms to calcium and diacylglycerol would also allow the cell to sequentially regulate different events. By way of illustration, the isoform responsible for mediating cortical granule exocytosis could be activated at calcium and diacylglycerol concentrations characteristic of the early part of the calcium wave, whereas that isoform responsible for cortical contraction could be activated at calcium and diacylglycerol concentrations achieved during the late part of the calcium wave. Regardless of the actual mechanisms involved, the heterogeneity and site(s) of action of PKC make it an attractive candidate as a widespread regulator of events keyed to cell cycle transitions.

Methods

Egg procurement

Eggs were obtained from adult female *X. laevis* purchased from Nasco Biologicals (Fort Atkinson, WI). Mature females were primed 3–5 d before use with 42 IUs of pregnant mares' serum gonadotropin (Sigma Chemical Co., St. Louis, MO) injected into the dorsal lymph sac. Frogs were anesthetized by hypothermia, and ovarian fragments were removed from an incision in the body wall. Full-grown oocytes were isolated after collagenase treatment as previously described (Bement and Capco, 1989a) and stored in 1× O-R2 medium (Wallace *et al.,* 1973). Eggs were obtained by incubating full-grown oocytes in 2 μg/ml progesterone (Larabell and Capco, 1988) (Sigma Chemical Co., St. Louis, MO) until 2 h after appearance of a white spot on the animal pole, at which point essentially all eggs challenged with calcium ionophore undergo activation.

Experimental treatments

Eggs were challenged with calcium ionophore A23187 (Sigma) or phorbol 12-myristate 13-acetate (Sigma) at the concentrations indicated in the Results section. Eggs were challenged in either 0.1× O-R2 (pH 7.8) or calcium-free 0.1× O-R2 (0.1× O-R2 without $CaCl_2$ and with 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid [EGTA]; pH 7.8). Eggs challenged in calcium-free medium

were washed three times in calcium-free medium before treatment with activating agents. The PKC inhibitor H7 (Molecular Probes, Corvalis, OR), the calmodulin-dependent protein kinase inhibitor W7 (Molecular Probes), and DMSO were all applied by incubation of eggs for 3 h in 1× O-R2 containing the indicated concentrations of these agents. Sphingosine (Sigma) was applied by incubation of eggs for 1 h in 1× O-R2 containing 100 μM sphingosine, which was sonicated immediately before addition of eggs to the medium. Cell impermeant BAPTA (Molecular Probes) was microinjected into eggs to a final intracellular concentration of 10 mM (Figures 2, 4, and 5a) or 40 mM (Figure 3). The concentration of 10 mM was established in preliminary experiments as the minimum required to consistently prevent cortical granule exocytosis and cortical contraction in eggs challenged with 100 nM A23187. Although the egg has total volume of 900 nL (Kline, 1988), much of the cell interior is occupied by yolk, thus, calculations of intracellular concentrations were based on an egg volume of 300 nL. Free-calcium concentrations were calculated according to the equation

$$[Ca^{2+}] = K_d[Ca^{2+}BAPTA]/[BAPTA]$$

taken from Kline (1988), where $[Ca^{2+}]$ is the free-calcium concentration, K_d is the calcium dissociation constant for BAPTA (0.1 μM), $[Ca^{2+}BAPTA]$ is the concentration of calcium-bound BAPTA, and [BAPTA] is the total BAPTA concentration (calcium bound + unbound). Microinjections were performed by positive pressure with the use of a Drummond micropipette and glass needles pulled on a vertical needle puller (Narishige, Greenvale, NY) and broken to a tip diameter of 20 μm; injection volumes ranged from 5 to 50 nL. Eggs were injected in 1.5× O-R2 and then allowed to heal for 20 min after injection. BAPTA injections did not trigger egg activation in most eggs; those eggs that did activate, or that appeared unhealthy after injection, were discarded. Control injections of water included EGTA (400 nM) to prevent impalement from activating eggs (Karsenti *et al.*, 1984). In separate experiments, eggs were injected with water alone in calcium-free medium. Neither procedure significantly altered the ability of eggs to activate in response to calcium ionophore or PMA. Of the two procedures, control injections of water containing EGTA produced a higher percentage of healthy, unactivated eggs; consequently, this procedure was employed preferentially.

Measurement of intracellular calcium

Calcium electrodes were prepared as described by Busa and Nuccitelli (1985) with modifications described by Busa (1986). Microcapillary tubes of borosilicate glass were chromerged and pulled with a horizontal needle puller (Industrial Science Associates Inc., Ridgewood, NY) to a tip diameter of 5–10 μm. Microelectrodes were beveled on an aluminum silicate disk and then treated with tributylchlorosilane (Fluka, Ronkonkoma, NY) in a closed chamber at 200°C for 1 h. Subsequently the chamber was opened to allow the silane vapor to disperse. Calcium electrodes were filled with pCa 7 buffer and the tip subsequently filled with calcium cocktail (Fluka). Electrodes were then coated with polyvinyl chloride spiked with calcium cocktail and stored under pCa 7 buffer until use. Electrodes were used within 4 h of their construction. Each cell was impaled with a calcium electrode and a membrane potential electrode; measurements from the latter were subtracted from the former to determine the levels of $[Ca^{2+}]_i$ calcium. These subtractions were performed electronically on an oscilloscope (Tektronix, Beaverton, OR) with a dual differential channel. Data shown in figures represent recordings from the oscilloscope screen after subtraction of the membrane potential. Recordings of individual electrodes were also made to ensure that changes in $[Ca^{2+}]_i$ that might have occurred at intermediate time points were not missed. Calibrations were conducted before and after the cell was impaled, using buffers of the following compositions: pCa 6 = 10 mM EGTA, 5 mM $CaCl_2$, 10 mM PIPES, 45 mM KOH, 15 mM KCl (pH 6.77); pCa 6.5 = 10 mM EGTA, 5 mM $CaCl_2$, 10 mM PIPES, 47 mM KOH, 12 mM KCl (pH 7.02); pCa 7 = 10 mM EGTA, 5 mM $CaCl_2$, 10 mM MOPS, 35.5 mM KOH, 29.3 mM KCl (pH 7.27); pCa 7.5 = 10 mM EGTA, 5 mM $CaCl_2$, 10 mM HEPES, 35 mM KOH, 29.5 mM KCl (pH 7.53); pCa 8 = 10 mM EGTA, 5 mM $CaCl_2$, 10 mM HEPES, 36.9 mM KOH, 27.5 mM KCl (pH 7.78). The calibrations shown in the figures were performed after the electrodes were removed from the cell; these were virtually identical to those obtained before impalement.

Electron microscopy

Eggs were fixed overnight as previously described (Bement and Capco, 1989b) in either 0.1× O-R2 or calcium-free 0.1× O-R2 containing 2.5% glutaraldehyde and 0.1% tannic acid (Charbonneau and Grey, 1984). Samples were then rinsed and osmicated for 3.5 h in 0.1 M sodium cacodylate buffer (pH 7.9) containing 1% osmium tetroxide. After osmication, samples were rinsed and then dehydrated in a graded series of ethanol. After dehydration, the ethanol was replaced with acetone, the samples were embedded in Spurr embedding medium and polymerized, and ultrathin sections were cut on glass knives with the use of a Porter-Blum ultramicrotome. Sections were poststained with lead citrate and uranyl acetate and then viewed and photographed on a Philips EM 201.

Spectrophotometry

Spectrophotometric analysis was performed with the use of the technique of Wolf (1974b) modified as previously described (Bement and Capco, 1989a). Five or 10 eggs were transferred with a wide-bore pipette into a 1-ml quartz cuvette containing 0.4 ml of the experimental media described in the Results section. The assay was started by the addition of A23187 or PMA and absorbance readings were then taken at 280 nm on a Beckman Du-64 spectrophotometer. As the eggs released cortical granule contents, the protein content of the medium increased, resulting in increased absorbance readings. Readings were taken every 2 min after gentle agitation of the cuvette. Extreme care was required during the agitation to prevent lysis of the eggs, particularly in the case of treatment with A23187. After the assay, eggs were inspected to confirm that cell lysis had not occurred. To determine the relative amount of exocytosis, the final absorbance readings of experimentals were divided by the readings of DMSO-treated controls. Spectrophotometric analysis could not be used on eggs injected with BAPTA or water, because such treatments tended to result in eggs that leaked small amounts of cytoplasm into the medium; leakages were detected as increases in absorbance. Exocytosis readings were taken for no longer than 16 min after treatment with A23187, because A23187 treatment for longer periods often induced nonspecific cytoplasmic leakage into the medium.

Analysis of cortical contraction and exocytosis

Cortical contraction and cleavage furrow formation were monitored with a dissecting microscope equipped with a measuring reticule. Eggs were considered to have con-

tracted when the amount of surface area occupied by the pigmented animal hemisphere decreased >10% relative to the amount occupied before experimental treatment. Data obtained from cortical contraction and cortical granule exocytosis assays were analyzed by a one-tailed Mann-Whitney *U* test and p values of <0.05 were considered significant.

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