Zygote **7** (November), pp 285–292. © 1999 Cambridge University Press Printed in the United Kingdom

Microinjection of cyclic ADP-ribose triggers a regenerative wave of Ca^{2+} release and exocytosis of cortical alveoli in medaka eggs

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Date submitted: 8.5.99. Date accepted: 17.6.99

Summary

Medaka (*Oryzias latipes*) eggs microinjected with the Ca^{2+}-mobilising messenger cyclic adenosine diphosphate ribose (cADPR) underwent a wave of exocytosis of cortical alveoli and were thus activated. The number of eggs activated was sharply dependent on the concentration of cADPR in the pipette, the threshold concentration was approximately 60 nM. After injection, a pronounced latency preceded the onset of cortical alveoli exocytosis; this latency was independent of the concentration of cADPR but decreased markedly with increasing temperature. Heat-treated cADPR, which yields the inert non-cyclised product ADP-ribose, was ineffective in activating eggs. When cADPR was injected into aequorin-loaded eggs, a wave of luminescence arose at the site of cADPR injection and then swept out across the egg with a mean velocity of approximately 13 μm/s; the velocity was independent of the concentration of injected cADPR. In such a large cell (diameter of around 1 mm), this is considerably faster than that possible by simple diffusion of cADPR, which unambiguously demonstrates that cADPR must activate a regenerative process. cADPR has been demonstrated to modulate Ca^{2+}-induced Ca^{2+} release (CICR) via ryanodine receptors (RyRs) in many cell types, and consistent with this was the finding that microinjection of the pharmacological RyR modulator, ryanodine, also activated medaka eggs. These results suggest that a cADPR-sensitive Ca^{2+} release mechanism is present in the medaka egg, that cADPR is the most potent activator of medaka eggs described to date, and that it activates eggs by triggering a wave of CICR from internal stores that in turn stimulates a wave of exocytosis.

Keywords: Calcium, Cyclic ADP-ribose, Medaka, Ryanodine, Wave

Introduction

Egg activation at fertilisation is accompanied by regenerative Ca^{2+} waves emanating from the site of sperm–egg fusion. These waves propagate by an autocatalytic mechanism involving Ca^{2+}-induced Ca^{2+} release (CICR) from intracellular stores (Jaffe, 1991). This process was first directly visualised in aequorin-loaded medaka eggs, in which an explosive rise in luminescence occurred upon activation of the eggs with either sperm or Ca^{2+} ionophore (Ridgway *et al.*, 1977; Gilkey *et al.*, 1978). Since the injected aequorin was confined to the cytoplasm of the egg, the Ca^{2+} wave was clearly cytoplasmic. Moreover, the initial rise in luminescence was exponential (Ridgway *et al.*, 1977) and could be also triggered by elevating Ca^{2+} locally above a threshold level by injecting Ca^{2+} or by treating eggs with a Ca^{2+} ionophore (Gilkey *et al.*, 1978). Also, the Ca^{2+} wave could be excluded from regions of cytoplasm that had been treated with Ca^{2+} buffers (Gilkey, 1983). Thus, it was proposed that the Ca^{2+} wave was propagated by an autocatalytic CICR reaction.

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The site of the CICR has been assumed to be intracellular Ca^{2+} stores because propagation of the waves is not dependent on extracellular Ca^{2+}. Two families of Ca^{2+}-release channels – inositol 1,4,5-triphosphate receptors ($InsP_3R$) and ryanodine receptors (RyRs) – have been characterised in the endoplasmic reticulum (ER) of cells (Berridge, 1993; Furuichi *et al.*, 1994), including eggs and oocytes (Galione *et al.*, 1993*a*; Stricker *et al.*, 1994; Ayabe *et al.*, 1995; Jones *et al.*, 1995; Yue *et al.*, 1995). Both families of Ca^{2+}-release channels can give rise to CICR (Galione *et al.*, 1993*a*). For example, in medaka eggs, injection of $InsP_3$ triggers an immediate Ca^{2+} release, which propagates as a wave across the egg (Iwamatsu *et al.*, 1988*b*) even in the absence of extracellular Ca^{2+}. The injection of $InsP_3$ also activates a wave of activation current (Nuccitelli, 1980) and a wave of exocytosis of cortical alveoli (Iwamatsu, 1989). Taken together, these results strongly suggest the presence of $InsP_3Rs$ in intracellular Ca^{2+} stores of the medaka egg.

In the present study we looked for the other family of Ca^{2+}-release channel in medaka eggs, RyRs, by microinjecting cyclic ADP-ribose (cADPR), which has been proposed to be an endogenous activator of RyRs in sea urchin eggs (Galione *et al.*, 1991) and several other types of egg and somatic cells (Galione & White, 1994). We have shown that cADPR can mobilise intracellular Ca^{2+}, trigger the generation of Ca^{2+} waves and thus activate medaka eggs. Based on these data, we propose that medaka eggs contain functionally operational $InsP_3Rs$ and RyRs and that either or both of these receptors may be involved in the initiation and propagation of CICR-based Ca^{2+} waves at fertilisation.

Materials and methods

Biological material

Methods for removing gonads have been previously described (Yamamoto, 1967; Kirchen & West, 1976; Fluck, 1978; Abraham *et al.*, 1993). Gonads and gamates were placed in the same BSS used in other studies of the fertilised medaka egg (Fluck *et al.*, 1991, 1992; Abraham *et al.*, 1993): 111 mM NaCl, 5.37 mM KCl, 1.0 mM $CaCl_2$, 0.6 mM $MgSO_4$, 5 mM HEPES pH 7.3. To prepare unfertilised eggs for microinjection, we transferred them through five successive washes of Ca^{2+}/Mg^{2+}-free BSS over a period of 1 h. The experiments were done at room temperature (19.0–25.4 °C).

Preparation of micropipettes

Glass micropipettes (filamented, 1 mm diameter, Narashige USA, Greenvale, NY) were made with a Narashige PN-3 microelectrode puller. The tip of each micropipette was bevelled at an angle of 20° with a Narashige EG-4 microgrinder; this process was monitored with an audio system (Miller *et al.*, 1993). Tip diameter was approximately 5 μm at the widest part of the bevel. Each pipette was calibrated by injecting small droplets of injectate into vegetable oil and measuring their diameter.

Microinjection

A stock of 20 μM cADPR or heat-inactivated cADPR was made in medium containing 150 mM KCl, 10 μM EGTA, and 5 mM HEPES pH 7.3. Dilutions of the stock solution were made with a medium containing 150 mM KCl, and 5 mM HEPES pH 7.3. Control eggs received injections of either (a) 150 mM KCl, 5 mM HEPES pH 7.3; or (b) a solution of heat-inactivated cADPR (Dargie *et al.*, 1990; 200 nM in a medium containing 150 mM KCl, and 5 mM HEPES pH 7.3).

Batches of three to six eggs were transferred to closely fitting holes drilled in a transparent plastic holder, and a high-pressure method (Narashige IM-200 microinjection system) was used to inject fluid into the thin, peripheral layer of ooplasm of the egg (Ridgway *et al.*, 1977; Gilkey, 1983; Fluck *et al.*, 1991, 1992, 1994). Approximately 1.4–1.9 nl of fluid was injected into the vegetal hemisphere of the egg, approximately 60° arc from the vegetal pole. The accessible cytosolic volume of the medaka egg is approximately 28 nl (Fluck *et al.*, 1992), but previous studies have shown that the bolus of injectate spreads no more than about 50° arc in all directions from the injected site within 5 min (Fluck *et al.*, 1994). Thus the injectate was diluted relatively little during the experiments described in the present study. After each injection we noted whether the egg was activated, as evidenced by the exocytosis of cortical vesicles. If the egg was activated we recorded the latency (the elapsed time between injection and egg activation) and the transit time (the elapsed time between the beginning of the cortical vesicle reaction at the injection site and its end on the opposite side of the egg).

To determine whether cADPR triggers a calcium wave in medaka eggs, we injected both aequorin and cADPR into some eggs. We first microinjected recombinant *h*-aequorin (1% w/v in 100 mM KCl, 5 mM MOPS, 0.05 mM EDTA pH 7.05: Shimomura *et al.*, 1993) into eggs and waited at least 1 h for the aequorin to diffuse around the egg. We then transferred the egg to a modified injection chamber that permitted us to microinject cADPR while the egg was on the stage of a Zeiss Axiovert 100 TV inverted microscope. With the microscope, we could either view the egg via a video camera or monitor aequorin luminescence via an imaging photon detector (IPD), which consists of a

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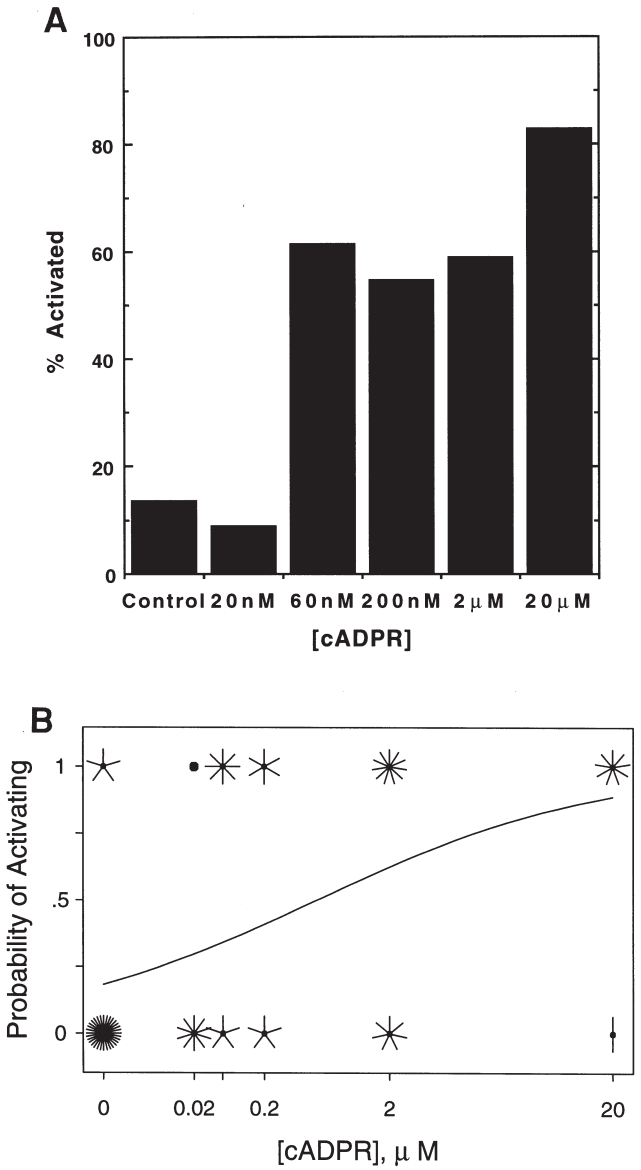

Figure 1 Dose-dependence of the activation of medaka eggs by injected cADPR. Control eggs (Control) on the ordinate in (*A*) and 0 on the ordinate in (*B*) received 150 mM KCl, 5 mM HEPES pH 7.3. $N \geq 10$ at each concentration. (*A*) The threshold concentration of cADPR appears to be between 20 nM and 60 nM. (*B*) The data shown are displayed in a sunflower plot, in which the number of eggs that did (upper row of flowers) or did not activate (bottom row of flowers) is shown for each injectate. Each egg is shown as a petal, except that a single egg is shown by a point. For example, 200 nM cADPR was injected into 11 eggs; 6 of them activated, and five did not. The estimated probability of egg activation, obtained by the logistic regression against the fourth root of [cADPR], increased from about 0.3 for control medium to almost 1.0 for a pipette concentration of 20 μM cADPR.

microchannel plate intensifier with a resistive anode as the positional encoder (Miller *et al.*, 1994). The raw data from the IPD consist of a sequential record of photon positions and times, measured one at a time. Images were generated by accumulating data over any desired interval and representing multiple photons per pixel with a colour scale. Computer-smoothed profiles of photon density were made at several consecutive 5 s intervals as the calcium wave traversed the egg. The velocity of a calcium wave was then estimated by measuring the time between the half-maximum points on the profiles at successive intervals. The experiments in which we co-injected aequorin and cADPR into eggs were performed at 22 °C.

To determine whether injected ryanodine activates medaka eggs, we dissolved ryanodine (Sigma) in injection buffer (150 mM KCl, 10 μM EGTA, 5 mM HEPES pH 7.3) to a final concentration of 10 mM or 1 mM and then injected approximately 1.45 nl of the solution into unfertilised medaka eggs. Control eggs received a similar volume of injection buffer. Unfertilised eggs were also incubated in BSS containing either 25 mM caffeine (Sigma) or 1 mM ryanodine for at least 2 h.

Results

Microinjection of cADPR activates medaka eggs

Injected cADPR activated medaka eggs (Fig. 1*A*). The threshold concentration (at the tip of the micropipette) was between 20 nM and 60 nM. The estimated probability of activation, obtained by the logistic regression against the fourth root of [cADPR], increased from about 0.3 following the injection of control medium (150 mM KCl and 5 mM HEPES pH 7.3) to almost 1.0 at 20 μM cADPR (Fig. 1*B*). Only 1 of 13 eggs into which we injected heat-inactivated cADPR was activated. Temperature did not have a statistically significant effect on the probability of activation (date not shown).

The latency between injection and the beginning of the cortical vesicle exocytosis (range 30–90 s) did not vary significantly with the concentration of cADPR ($p = 0.4276$; Fig. 2*A*) but did vary significantly with temperature ($p \leq 0.0001$; Fig. 2*B*). At 23.0–23.8 °C the latency was 45 ± 14 s (mean ± SD, $n = 7$). Similarly, transit time (range 67–187 s) did not vary significantly with concentration of cADPR ($p = 0.0892$; Fig. 3*A*) but did vary significantly with temperature ($p \leq 0.001$; Fig. 3*B*). At 23.0–23.8 °C the transit time was 131 ± 19 s (mean ± SD, $n = 7$).

Aequorin imaging of medaka eggs reveals that cADPR microinjection triggers a Ca^{2+} wave

All six eggs into which we injected both aequorin and cADPR (20 μM, three eggs; 200 nM, three eggs) were activated, as evidenced by the exocytosis of cortical vesicles, whereas none of the three eggs into which we injected aequorin and heat-inactivated cADPR (20 μM) were activated. Cytosolic [Ca^{2+}] began to rise within

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15 s after the injection of cADPR (i.e. by the time we opened the shutter of the IPD and began to collect photons). Cytosolic [Ca^{2+}] rose first at the injection site, and then a wave of elevated cytosolic [Ca^{2+}] spread over the egg at a speed of 12.7 ± 1.9 μm s^{-1} (mean ± SD, n = 5) towards the side of the egg antipodal to the injection site (Fig. 4, Table 1).

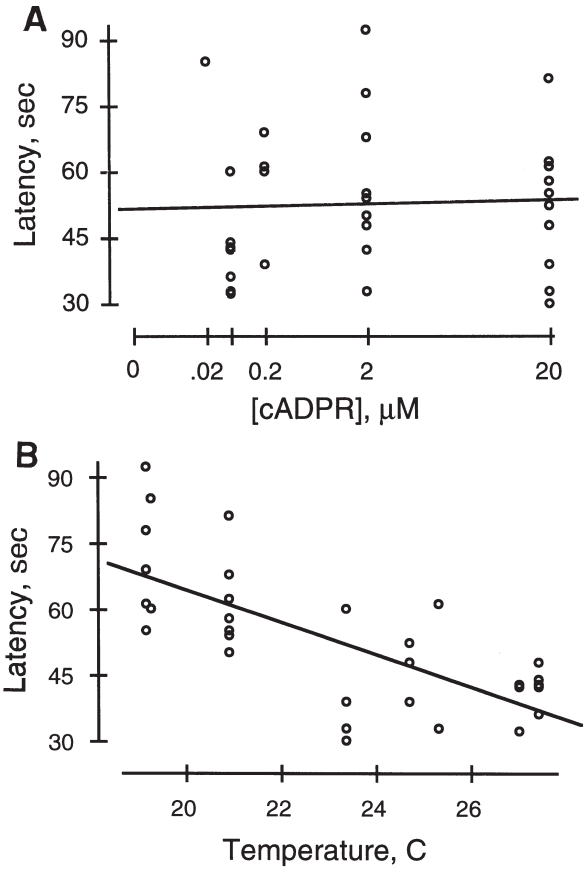

Figure 2 (above) Latency of cortical vesicle exocytosis after injection of cADPR. The latency (in seconds) between injection of cADPR and the initiation of cortical vesicle exocytosis was not significantly affected by the concentration of cADPR in the pipette (*A*) but was by temperature (*B*). The lines were obtained by linear regression.

Ryanodine-induced egg activation

Unfertilised eggs incubated for up to 2 h in BSS containing either 25 mM caffeine or 1 mM ryanodine were not

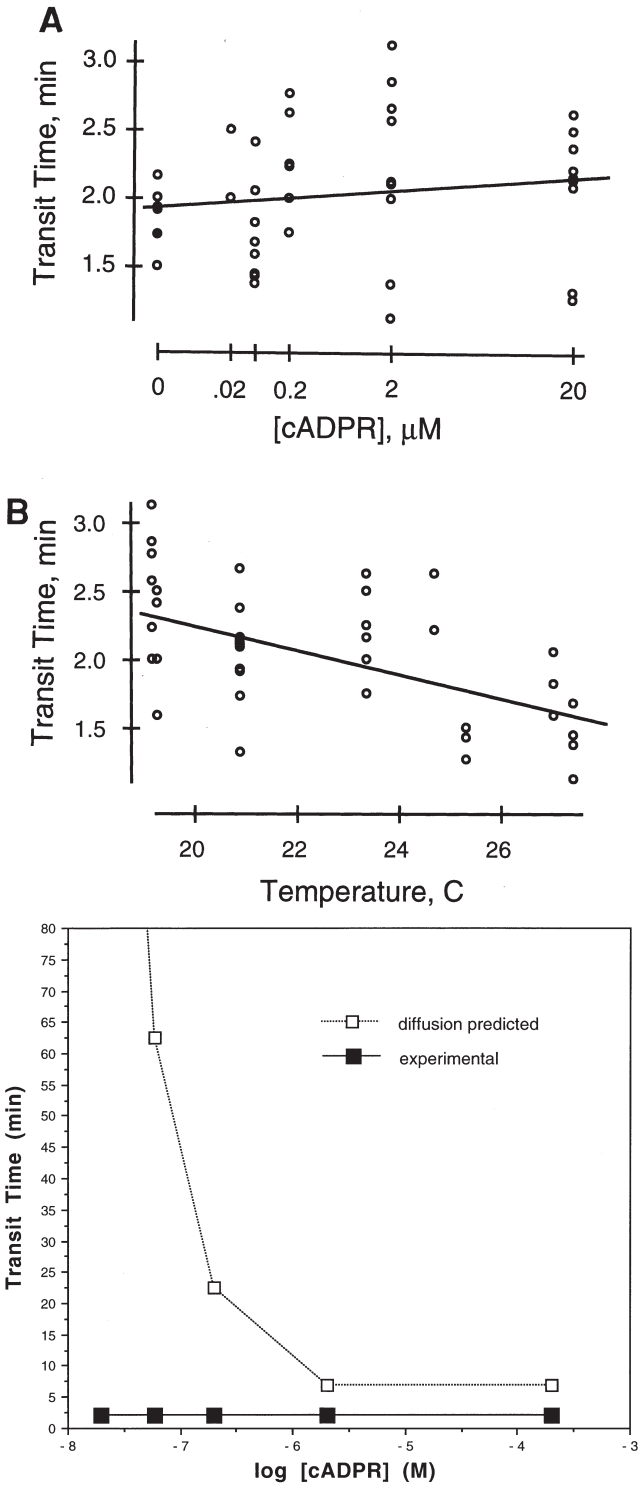

Figure 3 (right) Transit times of the wave of cortical vesicle exocytosis after injection of cADPR. The time required for the wave of cortical granule exocytosis to move from the injection to the antipode of the egg was not significantly affected by the concentration of cADPR (*A*) but it was by temperature (*B*). The lines were obtained by linear regression. (*C*) An approximate predicted relation between transit time and concentration of cADPR in the pipette (dotted line) which can be compared with the experimental data in (*A*). It was assumed that diffusion occurred from a point source at the pipette tip (after a maximal 10% injection v/v) into an infinite medium with first-order degradation of cADPR (Segel, 1984; Whitaker & Irvine, 1984) and that a threshold concentration of 20 nM cADPR is required to trigger Ca^{2+} release and exocytosis. The value of the diffusion coefficient for cADPR in cytoplasm was arbitrarily set at 300 $\mu m^2/s$, similar to that for IP_3, a molecule of similar size (Kasai & Petersen, 1994). A path length of 500 π μm was assumed, since the wave travels a hemi-circumference in the thin layer of cytoplasm under the plasma membrane.

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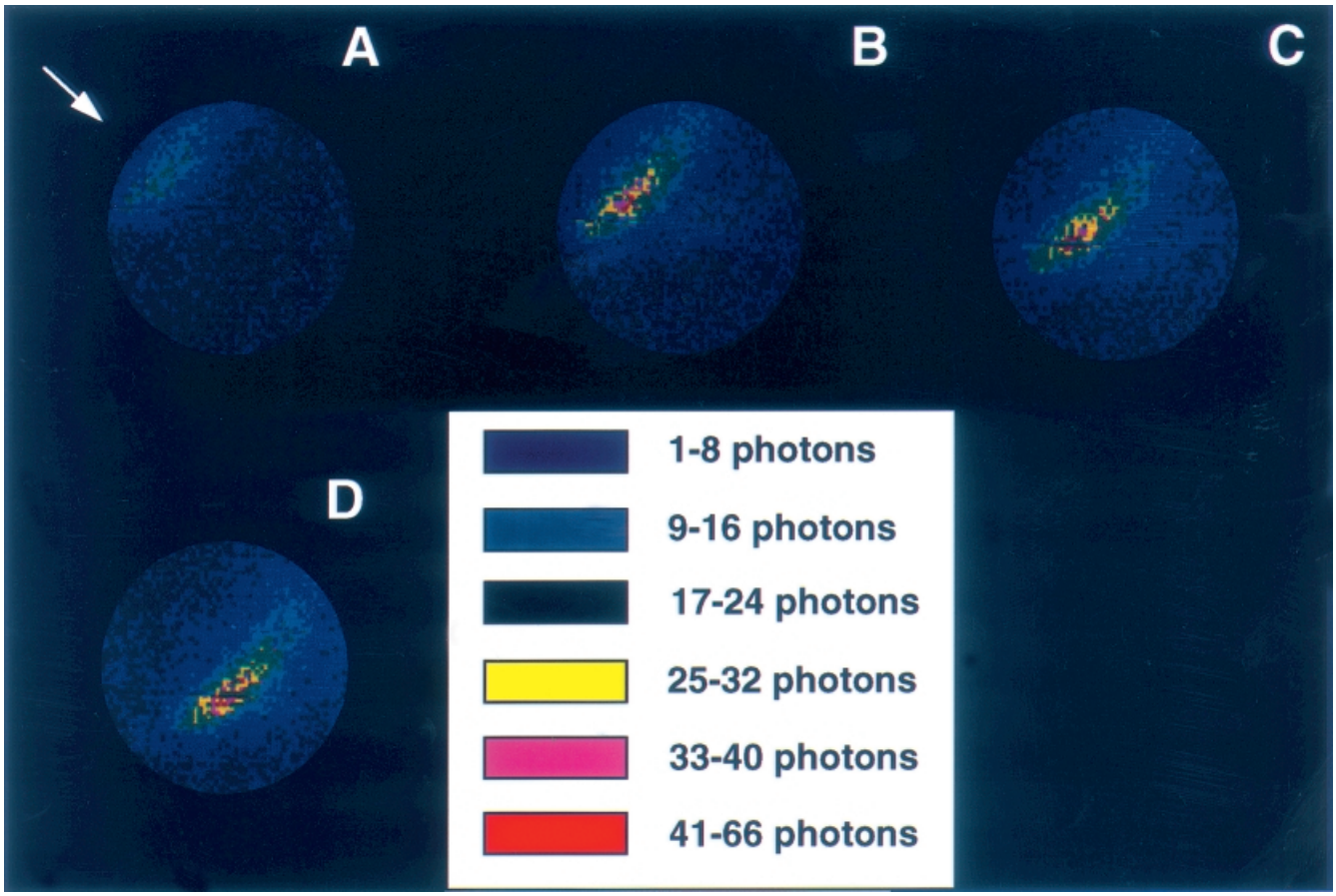

Figure 4 Injection of cADPR initiates a wave of elevated cytosolic [Ca^{2+}]. Aequorin luminescence was recorded in an egg (Table 1; egg no. 3) into which 20 μM cADPR had been injected. The four images were made at four successive 10 s intervals using a pseudocolour scale to represent multiple photons, with the non-egg background (which was generally less than 8 photons per time interval) masked for clarity. The calcium wave began near the injection site (*A*, upper left edge of the egg) and traversed the egg at a velocity of about 13 μm s^{-1}. The diameter of the egg is approximately 1 mm.

activated. However, injection of 10 mM ryanodine (tip concentration) activated eggs (10/10) within 30 s. Injection of 1 mM ryanodine (tip concentration; 5 eggs) or injection buffer (5 eggs) did not activate the eggs.

Table 1 Quantitative data from individual eggs co-injected with aequorin and cADPR[a]

Egg no.	[cADPR][b] (μM)	Latency (s)[c]	Wave velocity (μm s^{-1})
1	20	< 20	10.5
2	20	< 20	13.6
3	20	< 20	12.2
4	0.2	< 15	11.8
5	0.2	< 19	15.6
Mean ± SD			12.7 ± 1.9

[a] These experiments were performed at a room temperature of 22 °C.
[b] Tip concentration.
[c] The elapsed time between injection of cADPR and when the first increase in aequorin luminescence was observed.

Discussion

In this study we have shown that injecting cADPR into unfertilised medaka eggs initiates a cascade of responses similar to that seen at fertilisation. We showed first that cADPR activated medaka eggs as indicated by the exocytosis of the prominent cortical alveoli. The threshold concentration of cADPR for egg activation was extremely low, at between 20 and 60 nM (Fig. 1). This is significantly lower than the pipette tip concentration of $InsP_3$ required for egg activation, which was in excess of 1–10 μM (Table 2; Iwamatsu *et al.*, 1988*b*; Iwamatsu, 1989). This is similar to the situation in sea urchin eggs, where the effective concentration of cADPR for half-maximal Ca^{2+} release (EC_{50}) from egg homogenates is approximately 17 nM, which is approximately 5 times lower than that for $InsP_3$ (100 nM) (Dargie *et al.*, 1990). In an important control, we showed that injecting a solution of heat-inactivated cADPR did not activate medaka eggs. Heat inactivation of cADPR breaks the *N*-glycosidic bond, thus cleaving the ring and yielding ADP-ribose, which is inactive in

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Table 2 Latencies following activation of medaka eggs with various agents

Activating agent	Temperature (°C)	Region of egg	Latency (s)		Reference
			Rise in [Ca^{2+}]	Cortical granule exocytosis	
Sperm	21–23	Animal pole		47 ± 8	Uwa (1967)
Needle	21–23	Animal pole		20 ± 3	Uwa (1967)
$CaCl_2$	25–26	Animal pole		7.7–10.8[a]	Iwamatsu & Ito (1986)
$CaCl_2$	25–26	Vegetal pole		16.7–18.3[b]	Iwamatsu & Ito (1986)
$CaCl_2$	?	Animal pole	13.3–18.8[c]		Iwamatsu *et al.* (1988*a*)
$InsP_3$	?	Animal pole	0.4–15[d]		Iwamatsu *et al.* (1988*a*)
A23187	?	Animal pole	8.1 ± 1.6		Iwamatsu *et al.* (1988*a*)
cGMP	?	Animal pole	15.7 ± 3.9		Iwamatsu *et al.* (1988*a*)
cGMP	?	Animal pole		15.0–16.0[e]	Iwamatsu (1989)
$InsP_3$	?	Animal pole		4.7–5.7[f]	Iwamatsu (1989)
$InsP_3$	?	Vegetal pole	10.1		Iwamatsu (1989)
Sperm	23–25	Animal pole		9–10	Iwamatsu *et al.* (1991)
Sperm	20–22	Animal pole		4–9[g]	Nuccitelli (1980)
Sperm	23–25.5	Animal pole		9.2 ± 2.1[h]	Gilkey *et al.* (1978)

[a] Depended on the concentration of $CaCl_2$, which ranged from 5 μM to 10 mM.
[b] Depended on the concentration of $CaCl_2$, which ranged from 0.1 mM to 10 mM.
[c] Depended on the volume of 0.5 mM $CaCl_2$ injected.
[d] Depended on the concentration of $InsP_3$, which ranged from 1 μM to 10 μM.
[e] Depended on the concentration of cGMP, which ranged from 0.1 mM to 1.0 mM.
[f] Depended on the concentration of $InsP_3$, which ranged from 1 μM to 10 μM.
[g] Depolarisation of the membrane potential began 1–5 s after sperm entered the micropyle, and the exocytosis of cortical vesicles began 10 ± 2 s after sperm entered the micropyle.
[h] Measured as the time after sperm entry.

sea urchin homogenates in mobilising Ca^{2+}, even at high nanomolar concentrations (Dargie *et al.*, 1990). Injection of 1.4–1.9 nl of a 20 μM solution of cADPR that had been heat-treated for 50 min and shown to be ineffective at mobilising Ca^{2+} in sea urchin egg homogenates (data not shown) did not activate medaka eggs. Thus, it is unlikely that contaminating Ca^{2+} in the solutions of cAPR was the factor responsible for activating the eggs. Moreover, because activation occurred in a Ca^{2+}/Mg^{2+}-free medium, the Ca^{2+} release induced by cADPR is independent of external Ca^{2+}; thus activation results from Ca^{2+} liberation from internal stores.

A pronounced latent period (15–90 s) occurred between the injection of cADPR and the onset of the exocytosis; the length of the latent period did not vary significantly with the concentration of cADPR in the pipette but was markedly temperature-dependent (Fig. 2). The range of this latent period is greater than that reported for $InsP_3$, which varied from 5–10 s and was inversely dependent on the tip concentration of $InsP_3$ (1–100 μM) (Iwamatsu, 1989). However, latencies as long as those reported in the present study occur at fertilisation (5–16 s) (Iwamatsu *et al.*, 1991) and after injection of cGMP and other guanine nucleotides (Iwamatsu *et al.*, 1991) or Ca^{2+} itself (13–28 s) (Iwamatsu, 1989).

The transit time for the cortical wave of exocytosis ranged from 67 to 187 s and was longer at lower temperatures (Fig. 3*B*). However, there was no clear relationship between this parameter and tip concentration of cADPR – a result consistent with a model in which cADPR triggers an all-or-nothing regenerative wave of CICR (Fig. 3*A*). The transit time of the wave of exocytosis in sea urchin eggs is also independent of the concentration of injected $InsP_3$ over a 5-fold range of concentrations below which simple diffusion is an order of magnitude too slow to be the major determinant – a result taken to indicate that this agent is triggering an all-or-none regenerative process such as CICR (Whitaker & Irvine, 1984). In the case of the medaka egg, with a diameter 10 times that of the sea urchin egg, it appears that simple diffusion is far too slow to account for experimental transit times (Fig. 3*C*). The Q_{10}s for both the latent period and transit times in medaka eggs were near 2, consistent with a similar

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reaction being responsible for the initiation and propagation of the Ca^{2+} wave, namely CICR (Berridge, 1994). The values for the transit times can be converted into velocities by assuming that the Ca^{2+} wave takes a cortical path. The transit time would thus represent the time for the wave to travel across a hemispherical shell (Cheer *et al.*, 1987): so that $c = \pi d/2t$, where c is wave velocity, d is egg diameter and t is the transit time. This analysis gives a velocity range of 4–12 μm/s for cADPR-induced waves, which is similar to that seen in medaka eggs for fertilisation, or activation by Ca^{2+} injection, $InsP_3$ or guanine nucleotides (Iwamatsu *et al.*, 1991). Both the remarkable constancy of wave velocities initiated by several different activators and the independence of wave velocities from the magnitude of the initiating stimulus, with only temperature differences causing appreciable differences in velocity, are characteristic of regenerative Ca^{2+} waves (Jaffe, 1991). Thus the results of the present study are consistent with a model in which cADPR triggers a reaction-diffusion controlled reaction, i.e. CICR.

Given that cADPR caused a wave of exocytosis in medaka eggs, we predicted that cADPR would also trigger a Ca^{2+} wave. Such a wave, which underlies the exocytotic wave, was postulated by Yamamoto (1964) and later visualised in fertilised aequorin-injected medaka eggs by Jaffe and his colleagues (Ridgway *et al.*, 1977). We tested this prediction by microinjecting cADPR into eggs which we had previously injected with aequorin and then imaging changes in luminescence with an IPD. The spatial and temporal dynamics of the Ca^{2+} signal elicited by cADPR (Fig. 4, Table 1) revealed a non-decrementally propagating wave of free cytosolic Ca^{2+} that started at the injection site and then spread out from this site at a velocity of around 13 μm/s, a velocity similar to that of the cADPR-induced waves of cortical alveolar exocytosis. The latency between cADPR injection and the rise in $[Ca^{2+}]_{cyt}$ was less than 15 s, the minimum time between completing the injection and opening the shutter for data collection; $[Ca^{2+}]_{cyt}$ had always begun to rise by the time we opened the shutter. This latency is shorter than the latency for cADPR-induced exocytosis – a result that is consistent with a rise in free $[Ca^{2+}]_{cyt}$ causing the exocytotic reaction.

Since cADPR has been demonstrated to regulate a CICR mechanism via RyRs in many cell types (Galione & White, 1994), we tested whether two pharmacological activators of RyRs, caffeine and ryanodine, would mimic the effects of cADPR in activating medaka eggs. High extracellular concentrations of both agents, which are sufficient to cause activation of sea urchin eggs (Buck *et al.*, 1992; Sardet *et al.*, 1992), failed to induce appreciable activation of medaka eggs. A possible reason for this discrepancy is that unlike sea urchin eggs, medaka eggs are surrounded by a chorion punctuated only by a single micropyle, which is the site of sperm entry (Gilkey, 1981), and the chorin may be impermeable to these compounds. However, injection of ryanodine (10 mM; tip concentration) was able to activate eggs – a result consistent with the presence in medaka eggs of RyRs that are the likely targets for the effects of cADPR reported here.

Whether medaka eggs employ both cADPR and $InsP_3$ as Ca^{2+}-mobilizing messengers at fertilisation, as sea urchin eggs do (Galione *et al.*, 1993*a*; Lee *et al.*, 1993), remains to be determined. An interesting correlation is that cGMP activates medaka (Iwamatsu *et al.*, 1988*b*; Iwamatsu, 1989) and sea urchin eggs (Whalley *et al.*, 1992; Galione *et al.*, 1993*b*) after a significant latency. In sea urchin eggs the mechanism of cGMP-induced Ca^{2+} release has been shown to involve the cGMP-stimulated synthesis of cADPR from β-NAD^+ by the synthetic enzyme ADP-ribosyl cyclase (Galione *et al.*, 1993*b*; Willmott *et al.*, 1996) and the subsequent activation of RyRs. Whether this is also the case in medaka is currently being investigated.

Acknowledgements

This work was supported by NSF DCB-9017210 and NSF-9316125 to R.A.F., NSF BIR9211855 to A.L.M. and Lionel Jaffe, and by grants from Franklin and Marshall College's Hackman Scholars Program and Harry W. and Mary B. Huffnagle Fund to V.C.A. We thank Tim Hesterberg for his help in statistical analysis and display of the data in Figs. 1–3. Fig. 1*B* was created with S-plus software (Becker *et al.*, 1988); Inc., 1991 #1018] and the sunflower function was authored by Andreas Ruckstuhl, Werner Stahel, Martin Maechler and Tim Hesterberg, from the StatLib archive (http://stat.cmu.edu/S/sunflower).

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