

Release of Ca^{2+} from the Endoplasmic Reticulum Contributes to Ca^{2+} Signaling in *Dictyostelium discoideum*

Zofia Wilczynska,¹ Kathrin Happle,² Annette Müller-Taubenberger,³ Christina Schlatterer,² Dieter Malchow,² and Paul R. Fisher^{1*}

Department of Microbiology, La Trobe University, Victoria 3086, Australia,¹ and Universität Konstanz, 78464 Konstanz,² and Max-Planck-Institut für Biochemie, 82152 Martinsried bei München,³ Federal Republic of Germany

Received 19 April 2005/Accepted 17 June 2005

Ca^{2+} responses to two chemoattractants, folate and cyclic AMP (cAMP), were assayed in *Dictyostelium D. discoideum* mutants deficient in one or both of two abundant Ca^{2+} -binding proteins of the endoplasmic reticulum (ER), calreticulin and calnexin. Mutants deficient in either or both proteins exhibited enhanced cytosolic Ca^{2+} responses to both attractants. Not only were the mutant responses greater in amplitude, but they also exhibited earlier onsets, faster rise rates, earlier peaks, and faster fall rates. Correlations among these kinetic parameters and the response amplitudes suggested that key events in the Ca^{2+} response are autoregulated by the magnitude of the response itself, i.e., by cytosolic Ca^{2+} levels. This autoregulation was sufficient to explain the altered kinetics of the mutant responses: larger responses are faster in both mutant and wild-type cells in response to both folate (vegetative cells) and cAMP (differentiated cells). Searches of the predicted *D. discoideum* proteome revealed three putative Ca^{2+} pumps and four putative Ca^{2+} channels. All but one contained sequence motifs for Ca^{2+} - or calmodulin-binding sites, consistent with Ca^{2+} signals being autoregulatory. Although cytosolic Ca^{2+} responses in the calnexin and calreticulin mutants are enhanced, the influx of Ca^{2+} from the extracellular medium into the mutant cells was smaller. Compared to wild-type cells, Ca^{2+} release from the ER in the mutants thus contributes more to the total cytosolic Ca^{2+} response while influx from the extracellular medium contributes less. These results provide the first molecular genetic evidence that release of Ca^{2+} from the ER contributes to cytosolic Ca^{2+} responses in *D. discoideum*.

Dictyostelium discoideum cells are responsive to various chemoattractants at different stages of their life cycle. In the growth phase *Dictyostelium* amoebae respond chemotactically to folate and other pterins and are believed to use this response to actively hunt bacteria that secrete these compounds. Upon starvation, the amoebae differentiate with the result that they begin to secrete and respond chemotactically to another chemoattractant, cyclic AMP (cAMP). Chemotactic aggregation in response to extracellular cAMP signals mediates the transition to the multicellular stages of the *Dictyostelium* life cycle.

Chemotactic responses to cAMP in *Dictyostelium* amoebae have been intensively studied: many of the molecular events in the signal transduction pathways involved have been identified so that this is perhaps the best understood example of chemotactic motility by amoeboid cells (for recent reviews see references 7, 40, and 55). The major receptor involved in cAMP chemotaxis is cAR1, one of four closely related seven-transmembrane domain cAMP receptors in *D. discoideum*, all of which couple to the heterotrimeric G-protein $\text{G}\alpha 2\beta\gamma$. Downstream signaling molecules involved in chemotaxis include guanylyl cyclases that produce cGMP from GTP, the small GTP-binding protein RasC, phosphatidylinositol-3 kinases that phosphorylate phosphatidylinositol bisphosphate, converting it to phosphatidylinositol trisphosphate phosphatase PTEN, Pleckstrin Homology (PH) domain-containing proteins such as

the docking protein PhdA, and the protein kinase Akt/PKB and other protein kinases such as PAKa and several myosin heavy- and light-chain kinases. Some of these molecules act at the leading edge of the cell where they activate pseudopod extension, while others act in the rear cortex of the cell to mediate retraction.

Not all of the intracellular signaling events activated by cAMP binding to cAR1 are G protein-dependent. The cAR1 receptor was the first G-protein-coupled receptor shown to elicit some downstream events in a G-protein-independent manner including an influx of extracellular Ca^{2+} (32, 33, 39, 45) and activation of the mitogen-activated protein kinase ERK2 (25). During chemotaxis in a spatiotemporal attractant gradient, the cytosolic Ca^{2+} elevation in response to an attractant occurs primarily in the rear cortex of the cell (59) where it may contribute to retraction of the rear and inhibition of inappropriate pseudopod extension in incorrect directions. The Ca^{2+} response is not essential for chemotaxis (53), but may play a role in its fine tuning, especially in shallow gradients.

Molecular genetic evidence indicates that there are two signaling pathways coupling the cAMP receptor to the Ca^{2+} channel responsible for Ca^{2+} influx, both of which are present in the early stages of differentiation (4 h differentiation) (39). One of these is G-protein-dependent and down regulated during early development, while a second is G-protein-independent and dominates responses in cells that are fully differentiated to aggregation competence (8 h differentiation).

Aside from the roles of the heterotrimeric G-proteins $\text{G}\alpha 4\beta\gamma$ (for responses to folate) and $\text{G}\alpha 2\beta\gamma$ (for responses to cAMP), little is known of the signaling events that connect the

* Corresponding author. Mailing address: Department of Microbiology, La Trobe University, Victoria 3086, Australia. Phone: 61 3 9479 2229. Fax: 61 3 9479 1222. E-mail: P.Fisher@latrobe.edu.au.

chemoattractant receptors to the Ca^{2+} influx channels. In the early, G-protein-dependent pathway, the unknown product of the *stmF* gene plays a role, since mutant alleles of this gene cause prolongation and enhancement of both cGMP and Ca^{2+} elevations in response to chemoattractant (23, 28, 39). Originally identified by chemical mutagenesis and classical genetics, *stmF* had been thought to encode the phosphodiesterase responsible for attenuating the cGMP responses to folate and cAMP. The prolonged G-protein-dependent Ca^{2+} responses of *stmF* mutants were therefore suggested to be caused by the prolonged cGMP responses. However this has recently been shown not to be the case.

A *pdeD* null mutant lacking the major cGMP-specific phosphodiesterase exhibits enhanced and prolonged cGMP responses like those of the *stmF* mutant (27). Yet in the early stages of differentiation (3 h) its Ca^{2+} responses are reduced rather than enhanced, consistent with the early suggestion that cGMP acts to inhibit Ca^{2+} influx (24). At later stages of development Ca^{2+} responses are unaltered in this (24) and another independent *pdeD* null strain as well as in several other mutants whose cGMP responses are dramatically altered by disruption of the guanylyl cyclase and cGMP phosphodiesterase genes (57). The *stmF* gene product thus appears to represent an upstream regulatory element in the G-protein-dependent pathway that restricts the duration of both the cGMP and Ca^{2+} responses.

Several observations led to the suggestion that, by analogy with hormone-stimulated mammalian cells, cAMP stimulation would elicit a rapid release of Ca^{2+} from the endoplasmic reticulum. These were reports that inositol (1, 4, 5)triphosphate (IP_3) elicits release of Ca^{2+} from intracellular stores in permeabilized cells (12, 16), cAMP stimulates a rapid, transient elevation of IP_3 in intact cells (peak within 5 seconds) (13, 56), and GTP γ S stimulates IP_3 synthesis in isolated membranes (14, 56).

However the predicted rapid Ca^{2+} response was not observed when measurements of cytoplasmic Ca^{2+} responses to chemoattractant were made in intact cells expressing the Ca^{2+} -sensitive luminescent protein, aequorin (38). This assay has a temporal resolution of 20 ms and can detect changes in cytosolic Ca^{2+} levels of as little as 2 to 3 nM. The only observable intracellular Ca^{2+} response in wild-type cells occurs after the reported IP_3 response has already subsided and it coincides with the influx of extracellular Ca^{2+} . No intracellular Ca^{2+} transient is observed in the absence of extracellular calcium or in the presence of known Ca^{2+} channel blockers (38), while Ca^{2+} responses to chemoattractants are close to normal (39, 46) in a mutant in which the phospholipase C gene has been disrupted (10). This mutant remains able to synthesize IP_3 from higher-order inositol polyphosphates through the action of a Ca^{2+} -dependent phosphatase, but it exhibits IP_3 responses only to cAMP concentrations several orders of magnitude higher than those required to elicit cytosolic Ca^{2+} responses (54).

Although chemoattractants do not stimulate an observable intracellular release of Ca^{2+} into the cytoplasm that is independent of the influx of Ca^{2+} from the medium, it remains possible that the influx is accompanied by and coupled to a release of Ca^{2+} from intracellular stores (38). By analogy with Ca^{2+} -signaling systems in other organisms (3), there are two

possible scenarios that are not mutually exclusive. One is that the Ca^{2+} influx causes and is accompanied by a Ca^{2+} -induced release of Ca^{2+} from the endoplasmic reticulum. A second is that the Ca^{2+} influx after chemoattractant stimulation is activated by depletion of intracellular stores which elicits the opening of store-operated Ca^{2+} channels in the plasma membrane. This is referred to as capacitative Ca^{2+} entry. The store depletion that elicits it would result from the opening of Ca^{2+} channels in the endoplasmic reticulum (ER) by IP_3 as described above or by other second messengers such as long-chain fatty acids. It has been shown that arachidonic acid elicits both an influx of Ca^{2+} into intact *D. discoideum* cells and a Ca^{2+} release from intracellular vesicles of permeabilized cells (46). This accords with the suggestion that chemoattractants such as cAMP may activate phospholipase A2, resulting in the production of long chain fatty acids and the release of Ca^{2+} from intracellular stores, depleting them and thereby activating influx (45).

To investigate these various possibilities, we have studied Ca^{2+} responses to chemoattractants by mutants lacking either or both of calreticulin and calnexin, two of the major Ca^{2+} -binding proteins in the endoplasmic reticulum (30, 31, 35). All three knockout mutants exhibit qualitatively normal chemotactic responses and almost normal growth in liquid medium, while the double mutant (but not the single mutants) grows much more slowly on bacterial lawns because of a severe defect in phagocytosis (35). We report here that all three mutants exhibit enhanced cytosolic Ca^{2+} responses to chemoattractant. This is the first molecular genetic evidence that, in *Dictyostelium* cells, Ca^{2+} release from the endoplasmic reticulum contributes to the intracellular Ca^{2+} responses to chemoattractant stimuli.

MATERIALS AND METHODS

***Dictyostelium* strains and culture conditions.** All work was done with *Dictyostelium discoideum* strain AX2 (2), with the mutant strains derived from it, HG1774 (calreticulin deficient), HG1770 (calnexin deficient), HG1772 (calreticulin and calnexin deficient) (35), and with aequorin-expressing transformants derived from them (HPF401 from AX2, HPF608 from HG1774, and HPF609 and HPF610 from HG1770). For axenic growth the amoebae were incubated as previously in shaken culture (150 rpm) at 23°C in axenic medium (45) or at 21°C in HL5 medium (38).

Measurement of intracellular Ca^{2+} responses using the aequorin method. Intracellular Ca^{2+} levels were measured in transformants of wild-type and mutant cells expressing recombinant aequorin as previously described (38). Cells were harvested from a growing axenic culture at a density of 1×10^6 to 2×10^6 cells/ml. For cAMP responses they were washed by centrifugation and suspended in morpholineethanesulfonic acid (MES)-DB buffer containing 0.416 $\mu\text{g/ml}$ coelenterazine (the cofactor for aequorin) for development at 21°C with shaking for 6 h at a density of 2×10^7 cells/ml. For folate responses the washed cells were resuspended at a density of 2×10^7 cells/ml in fresh HL5 medium containing 0.416 $\mu\text{g/ml}$ coelenterazine and incubated for 4 h at 21°C with shaking at 150 rpm. Prior to assay the cells were washed free of unbound coelenterazine by centrifugation and suspended at a density of 2×10^7 cells/ml in MES-DB for assay.

Aequorin light emission was measured with a New Brunswick Lumitran photometer whose output was digitized by a data logging card (PCI-20428W-1 Multifunction Board) from Intelligent Instrumentation Inc. installed in a personal computer. To assay intracellular Ca^{2+} concentrations in real time, a 500 μl aliquot of a 10^{-3} dilution of the cells (i.e., 10^4 cells) was first injected into 5 ml of lysis buffer containing an excess of Ca^{2+} . This results in complete discharge of all active aequorin in the cells and the total light emission therefrom was measured for subsequent use in the recording software to calculate the fractional rate of light emission and Ca^{2+} concentration in real time. The Ca^{2+} responses were assayed in a gently stirred 5-ml suspension (i.e., 10^8 cells) in a plastic scintillation

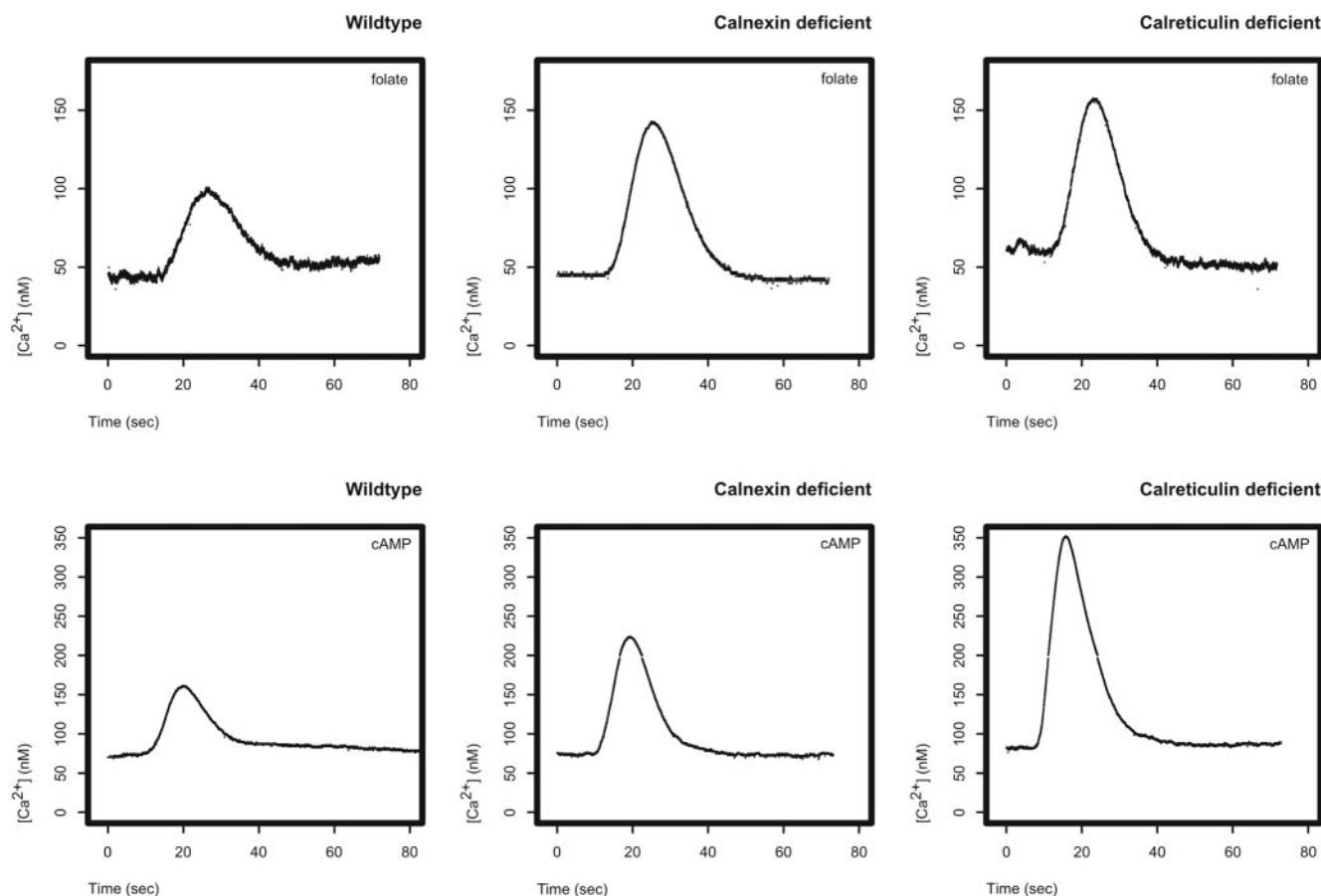


FIG. 1. Cytosolic Ca^{2+} responses by calnexin- and calreticulin-deficient mutants derived from wild-type strain AX2. Cytosolic Ca^{2+} levels were monitored in real time by the aequorin method in a gently stirred suspension of 10^8 cells during responses to $1 \mu\text{M}$ folate (vegetative cells) or $1 \mu\text{M}$ cAMP (aggregation competent cells after 6 h starvation). Recording began at 0 s and stimuli were delivered 2 s later.

vial to which a $50\text{-}\mu\text{l}$ volume of attractant was added at the time of stimulation. Intracellular Ca^{2+} concentrations were saved to file and output graphically to the personal computer monitor in real time by the recording software. Data analysis was conducted on a SUN workstation in the S statistical and graphical programming environment.

Measurement of intracellular Ca^{2+} responses by fluorescence microscopy using fura2-dextran. Intracellular Ca^{2+} concentrations in individual cells were measured using the Ca^{2+} -sensitive fluorescent indicator fura2 coupled to dextran as previously described (46, 50). Cells were harvested after 4 to 6 h of development, washed by centrifugation and resuspended in cold Sørensen phosphate buffer (17 mM Na^+/K^+ -phosphate, pH 6.0). Amoebae were shaken at 2×10^7 cells/ml, 150 rpm, 23°C until use. The use of fura2-dextran prevents the rapid sequestration and extrusion of the dye that occurs when either fura2 is loaded or cells are incubated with the membrane-permeant dye fura2-AM (49). Cells were loaded with fura2-dextran by electroporation, after which nonviable cells (typically 20 to 30% of the cells) and extracellular fura2-dextran were removed by repeated washing in an Eppendorf centrifuge (50). Aliquots of washed amoebae in H5-buffer (5 mM HEPES, 5 mM KCl, pH 7.0; 2 to $5 \mu\text{l}$) on glass coverslips were placed in a humid chamber until use. Under control conditions, 85 to $88 \mu\text{l}$ of H5 buffer containing 1 mM CaCl_2 was added 15 min prior to the internal Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) imaging experiment.

Measurement of the influx of Ca^{2+} during Ca^{2+} responses. Calcium influxes were measured using a Ca^{2+} -sensitive electrode to monitor extracellular Ca^{2+} concentrations as previously described (5, 45). Cells were harvested and washed in Tricine buffer (5 mM Tricine/5 mM KCl, pH 7.0) and resuspended at a density of 5×10^7 cells/ml in the presence of 5 or $10 \mu\text{M}$ extracellular Ca^{2+} . Extracellular Ca^{2+} levels were assayed in a gently stirred, aerated 2 ml suspension with a Ca^{2+} -sensitive electrode (ETH 1001; Möller, Zürich, Switzerland). The minimum extracellular Ca^{2+} concentration occurs 30 to 40 seconds following cAMP-

stimulation. The maximal rate of decrease occurs at around 20 seconds (28), close to the time of maximal intracellular $[\text{Ca}^{2+}]_i$ elevation.

RESULTS

Ca^{2+} responses to chemoattractants are larger and faster in calnexin/calreticulin-deficient mutants. To investigate a possible contribution by the ER to cytosolic Ca^{2+} responses, we studied responses in mutants deficient in calnexin or calreticulin, both of which are abundant Ca^{2+} -binding proteins resident in the endoplasmic reticulum (ER), calreticulin in the lumen and calnexin anchored in the membrane by a single transmembrane domain near its C-terminal end (19, 30, 31, 35). Both proteins are believed to play roles in Ca^{2+} buffering in the ER, suggesting that their absence should impair Ca^{2+} homeostasis in the ER lumen and potentially alter any ER contribution to cytosolic Ca^{2+} signals.

Figure 1 shows an example of real-time recordings of cytosolic Ca^{2+} levels in aequorin-expressing transformants of either the calnexin-deficient or the calreticulin-deficient mutant stimulated with either folate or cAMP. For both attractants the magnitude of the ensuing Ca^{2+} responses was noticeably larger. To verify these findings and to determine if the calnexin/calreticulin-deficient double mutant showed similarly en-

TABLE 1. Calcium responses in individual cells of the wild-type strain and calreticulin- and calnexin-deficient mutants^a

Parameter	Wild type ^b	Calreticulin deficient ^b	Calnexin deficient ^b	Calreticulin and calnexin deficient ^b
% Responding cells (no. positive/total)	39.5 (167/423)	40.7 (122/300)	36.4 (124/341)	35.6 (119/334)
$\Delta [Ca]_{rise}$ (nM)	117 \pm 9	195 \pm 16	154 \pm 15	147 \pm 15
Basal Ca (nM)	49 \pm 1.6	94 \pm 4	77 \pm 2.5	89 \pm 4
No. of experiments	14	10	11	10

^a Responses were assayed by fluorescence microscopy of cells loaded with fura2-dextran.

^b Errors are standard errors of the mean.

hanced responses, we used cells loaded with fura2-dextran to assay the cytosolic Ca²⁺ responses in individual cells microscopically. The results in Table 1 showed that the percentage of responding cells was similar in the mutants and the wild type, but in the cells that did respond, the amplitude of the Ca²⁺ transient was greater in all three mutants. The basal calcium levels also appeared slightly elevated in the mutants. There was no evidence in the double mutant for an additive effect of the absence of the two proteins.

Table 2 summarizes the responses to folate and cAMP observed in the single mutants using the aequorin method to measure Ca²⁺ responses in populations of cells in suspension in a large number of independent experiments. The resting cytosolic Ca²⁺ concentrations were found not to be significantly altered in the mutants and to fall within the range normally observed with wild-type cells (40 to 100 nM). However, when the cells were stimulated with either of the attractants, cytosolic Ca²⁺ levels began to rise sooner and peaked earlier at higher concentrations in both mutants than in the parental strain. This is shown graphically in Fig. 2 where the prestimulus Ca²⁺ levels 2 seconds before stimulation have been normalized to 70 nM, and the mean Ca²⁺ concentrations are plotted at the mean time of onset of the response, the mean time of the response peak, as well as at 60 seconds and 70 seconds after the onset of recording. We conclude that deficiencies in either calreticulin or calnexin result in larger, faster cytosolic Ca²⁺ responses to chemoattractant, indicating that the endoplasmic reticulum makes a contribution to the responses.

Magnitude and timing of Ca²⁺ responses to chemoattractants are coupled. Not only were there clear differences between the mutants and the wild-type strain, but all three strains exhibited smaller, slower responses to folate than to cAMP. Folate receptors are coupled to the Ca²⁺ channels by a G-protein-dependent pathway while cAMP receptors in the fully aggregation competent cells used here are coupled to the Ca²⁺ responses by a pathway that is almost completely independent of heterotrimeric G-proteins (38). That the Ca²⁺ responses to cAMP are faster than the responses to folate could therefore have been due to the fact that the receptors for these two attractants are coupled to the Ca²⁺ channels by different signaling pathways. However this hypothesis fails to explain why the larger responses by the mutants should also be faster for both attractants. The results suggest instead that the timing of the Ca²⁺ response is coupled in some way to the magnitude of the response itself.

To test this idea we plotted the magnitude of the response against the time of its onset and the time at which Ca²⁺ levels peaked. This was done for a large number of individual exper-

iments with cells of all three strains stimulated with folate (vegetative cells) or cAMP (aggregation competent cells). For both the onset and the peak of the response, the timing and magnitude of individual responses to the different attractants by the different strains were scattered around the same quasi-hyperbolic line of best fit (Fig. 3). Since larger responses begin earlier, they would be expected to peak earlier even if the Ca²⁺ levels rise for the same length of time during the response. In this case the rise time would be constant regardless of the onset time.

Figure 4 shows that this is not so, but that there is instead a strong positive correlation between the rise time and onset time. Thus, the earlier peak associated with larger responses is due not only to an earlier onset time but also a shorter rise time involving a faster net rate of entry of Ca²⁺ into the cytosol from intracellular stores and/or the extracellular medium. Furthermore the measurements from individual experiments for the different strains and attractants are again scattered around the same line of best fit. Clearly the differences in response kinetics among the strains and between the two attractants can be accounted for by the differences in the magnitudes of the responses. This indicates that the timing of both the onset of the Ca²⁺ response and its peak are coupled to the magnitude of the response and are controlled not by upstream signaling elements but by rate-limiting events common to the cAMP and folate signal transduction pathways after they converge.

Uptake of extracellular Ca²⁺ in response to chemoattractants is reduced in calnexin/calreticulin-deficient mutants. The simplest explanation for the observed coupling between response magnitudes and kinetics is that key events in the response are Ca²⁺-regulated. At the onset of the response, such events could include Ca²⁺-induced calcium release from the ER and the opening of store-operated channels in the plasma membrane. At the peak of the response when Ca²⁺ levels begin to decline back to prestimulus levels, they could include Ca²⁺-induced closure of plasma membrane Ca²⁺ channels and Ca²⁺-mediated activation of Ca²⁺ pumps in the plasma membrane or endoplasmic reticulum. If the mechanisms for terminating Ca²⁺ responses are indeed Ca²⁺ induced (either directly or indirectly), then larger responses resulting from earlier, more rapid rates of increase in cytosolic Ca²⁺ levels would also exhibit more rapid rates of decline in Ca²⁺ levels after the peak.

We therefore measured the rate of change of cytosolic Ca²⁺ levels during the calcium responses to both chemoattractants by the wild-type and mutant strains. Figure 5a illustrates this measurement for a typical response to cAMP. Using data from all three strains for both attractants, we found a strong correlation between the maximum rates of increase in the Ca²⁺

TABLE 2. Calcium responses to chemoattractants by wild-type cells and calreticulin- and calnexin-deficient mutants^a

Attractant	Strain (no.)	Prestimulus [Ca ²⁺] _i (nM)	Time of onset ^b (s)	Onset [Ca ²⁺] _i (nM)	Time of peak (s)	Peak [Ca ²⁺] _i (nM)	60 s [Ca ²⁺] _i (nM)	Poststimulus [Ca ²⁺] _i (nM)	Δt _{rise} (s)	Δ[Ca ²⁺] _{rise} (nM)
Folate	AX2 wild type (13)	76.7 ± 5.6	11.9 ± 0.6	76.6 ± 5.5	26.9 ± 1.1	128.9 ± 9.3	85.4 ± 5.5	83.6 ± 5.0	15.0 ± 0.9	52.3 ± 7.1
	Calnexin deficient (11)	71.9 ± 5.5	10.1 ± 0.5	72.8 ± 5.5	22.8 ± 0.6	155.2 ± 10.6	74.0 ± 5.5	73.4 ± 5.6	12.7 ± 0.7	82.4 ± 9.2
	Calreticulin deficient (10)	60.2 ± 4.3	10.1 ± 0.6	60.3 ± 4.1	22.2 ± 0.8	151.1 ± 9.5	62.9 ± 5.1	63.7 ± 5.1	12.1 ± 0.6	90.7 ± 10.4
cAMP	AX2 wild type (18)	70.8 ± 4.3	8.6 ± 0.4	71.0 ± 4.5	20.8 ± 0.6	197.9 ± 18.7	77.2 ± 4.9	76.6 ± 4.8	12.2 ± 0.5	126.9 ± 16.5
	Calnexin deficient (17)	66.6 ± 4.2	7.8 ± 0.3	68.0 ± 4.0	18.9 ± 0.6	234.3 ± 14.9	69.7 ± 4.2	68.4 ± 4.0	11.1 ± 0.4	166.4 ± 13.7
	Calreticulin deficient (17)	75.5 ± 3.9	7.1 ± 0.2	78.5 ± 3.4	17.1 ± 0.5	271.5 ± 14.3	84.9 ± 3.3	83.6 ± 3.1	10.0 ± 0.4	193.0 ± 15.3

^a Responses were measured in a stirred suspension of 10⁸ cells using the aequorin method. Errors are standard errors of the mean.

^b Times are measured from the time of delivery of the stimulus. Recording began at ca. -2 s before stimuli were given.

concentration in the rising phase of the response and the maximum rates of decrease in the falling phase of the response (Fig. 5b). The maximal increase rates ranged from about 2 nM/second to just over 60 nM/second (ca. 0.01 to 0.30 pmol/10⁷ cells/second), while the maximal decrease rates were smaller in magnitude so that the return to resting levels of cytosolic Ca²⁺ took longer than the rise time. The observed correlation between the rise and fall rates is consistent with the Ca²⁺ response being self-limiting because of Ca²⁺-induced mechanisms for response termination and Ca²⁺ removal.

One of the mechanisms that may contribute to Ca²⁺-induced termination of Ca²⁺ responses is closure of the Ca²⁺ uptake channels. Earlier closure of the channels in the mutants could even result in smaller Ca²⁺ influxes from the extracellular medium, with a greater proportion of the cytosolic Ca²⁺ response being contributed by calcium release from intracellular stores, particularly the endoplasmic reticulum. We therefore compared the influx of Ca²⁺ in response to cAMP in the parental strain and the mutants, including the double mutant lacking both calnexin and calreticulin.

The results in Fig. 6 show that at two different extracellular calcium concentrations (5 and 10 μM), stimulating the cells with any of three different cAMP concentrations produced a smaller uptake of calcium by the mutants than the wild-type strain. Indeed the Ca²⁺ influx after a 1 μM cAMP stimulus was reduced by more than 50%. Clearly the additional cytosolic Ca²⁺ observed in the mutant responses comes from intracellular sources, not from the medium. This is consistent with enhanced intracellular release of Ca²⁺ from the endoplasmic reticulum producing larger cytosolic responses and either earlier closure of the influx channels or earlier activation of Ca²⁺ pumps in the plasma membrane.

Dictyostelium genome encodes at least three putative Ca²⁺ pumps and four putative Ca²⁺ channels that may be regulated by calcium. Because the *Dictyostelium* genome has been completely sequenced (11), we were able to search the predicted proteome for homologs to examples of each of the subunit families of known Ca²⁺ channels (6, 7, 20, 22, 41, 42) and pumps (51, 52, 61). The results (Table 3) revealed that *D. discoideum* has genes encoding at least four putative Ca²⁺ channels and at least three putative Ca²⁺ ATPases. The subcellular locations of these pumps and channels are unknown, except for the PatA ATPase, which resides in the membrane of the contractile vacuole (34).

Based on the known subcellular location of their closest homologs (20, 37, 41–43, 52, 61), it is possible to predict the organelles to which most of the others are likely to be targeted. With three identifiable P-type Ca²⁺ ATPases, it seems likely that *D. discoideum* is provisioned with a single Ca²⁺ pump in each of the ER and vacuolar and plasma membranes. Of the Ca²⁺ channels, one could reside in each of the plasma membrane (polycystin-2 homolog), late endosomes/lysosomes (mucolipin homolog), the contractile vacuole (TPC, the two-pore Ca²⁺ channel homolog), and the ER (IPRL).

Which of these Ca²⁺ channels and pumps are likely to be regulated by calcium signals? Based on recognizable Ca²⁺- or calmodulin-binding sequence motifs and by analogy with their regulatory properties in other organisms, most if not all of them. We found putative calmodulin-binding domains in all three Ca²⁺ pumps and in IPRL, while the mucolipin and TPC

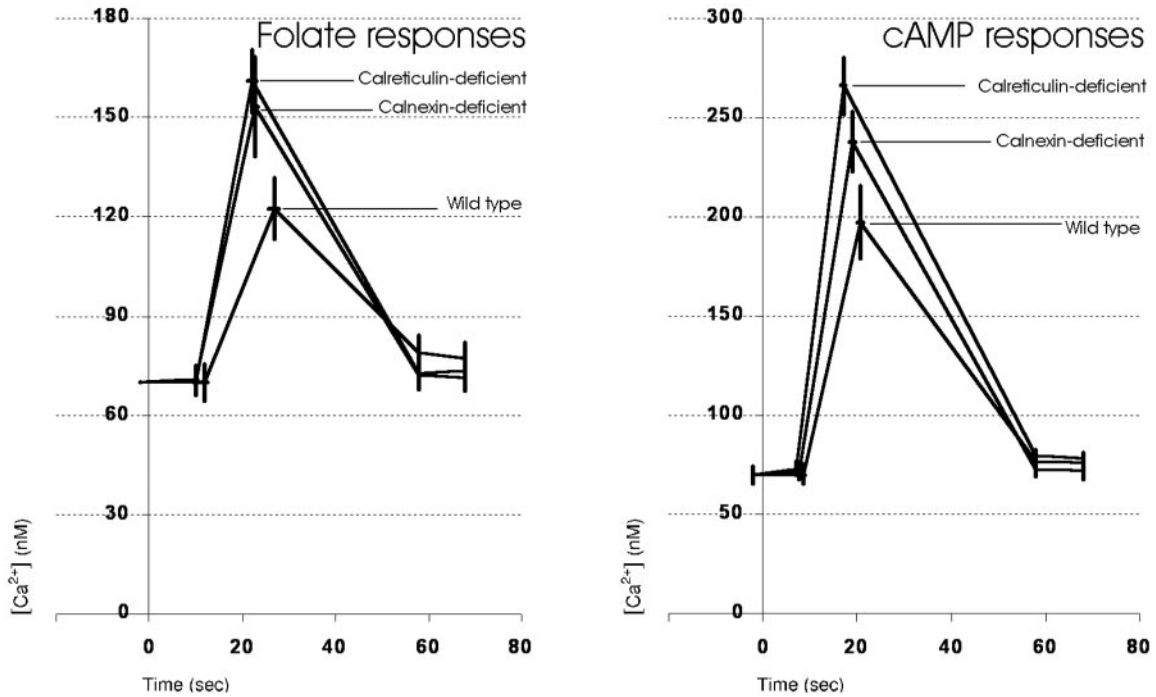


FIG. 2. Normalized mean Ca^{2+} responses by calnexin- and calreticulin-deficient mutants compared to the wild-type strain AX2. The left panel shows response to folate and the right panel shows responses to cAMP. Points indicate the mean cytosolic Ca^{2+} concentrations at the mean time of onset of the response, the mean time of the response peak, and at the set times of 60 and 70 s after recording started. Stimuli were delivered 2 s after recording began. Mean responses were normalized to a prestimulus resting Ca^{2+} concentration of 70 nM. Vertical and horizontal bars indicate standard errors of the mean for $[Ca^{2+}]$ and for the onset and peak response times. In some cases the horizontal bars at the onset and peak times are too short (very small standard errors) to be clearly visible in the plot.

homologs both contained putative EF-hand Ca^{2+} -binding sites (Table 3). Polycystin-2 and the mucolipins are Ca^{2+} -activatable channels belonging to the TRP (Transient Receptor Potential) family, various members of which are involved in

vision, olfaction, osmoregulation, thermo- and mechanoreception (8). We were unable to detect a putative Ca^{2+} -binding site in the sequence of the *Dictyostelium* polycystin-2 homolog, even though all other known examples of this little studied

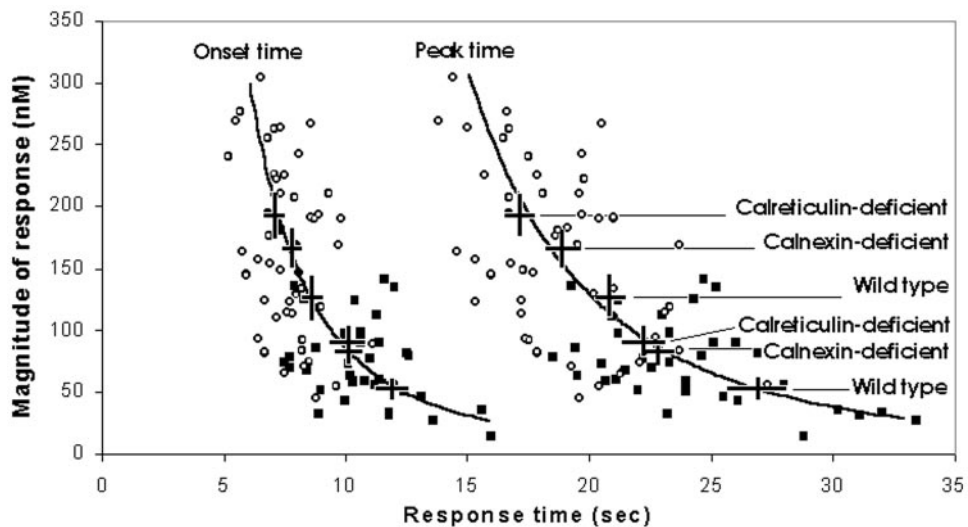


FIG. 3. Negative correlation between response magnitudes and the times of the onset and peak of cytosolic Ca^{2+} responses. Open circles represent individual experiments measuring the responses of aggregation competent cells to 1 μ M cAMP. Squares represent individual experiments measuring the responses of vegetative cells to 1 μ M folate. Vertical error bars show means and standard errors of response amplitudes for the calnexin- and calreticulin-deficient mutants and the wild-type cells. Horizontal bars show means and standard errors for the onset and peak response times. Response magnitudes were the difference between the cytosolic $[Ca^{2+}]$ at the onset and the peak of the response.

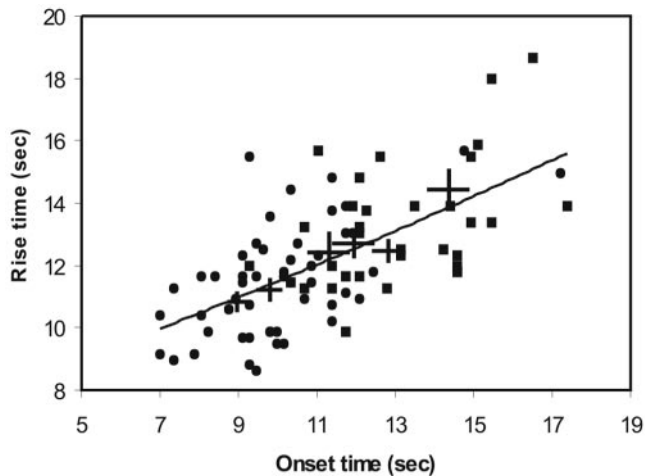


FIG. 4. Positive correlation between onset times and rise times for cytosolic Ca^{2+} responses. Circles represent individual experiments measuring the response of aggregation competent cells to $1 \mu\text{M}$ cAMP. Squares represent individual experiments measuring the responses of vegetative cells to $1 \mu\text{M}$ folate. Error bars indicate means and standard errors. From left to right, the error bars indicate in order the responses to cAMP by the calreticulin-deficient, calnexin-deficient, and wild-type cells and then the slower responses to folate by the calreticulin-deficient, calnexin-deficient, and wild-type cells. Cells were stimulated at 2 s.

channel contain an EF-hand Ca^{2+} -binding motif. We conclude that *D. discoideum* possesses at least six putative Ca^{2+} pumps and channels whose sequences contain Ca^{2+} - or calmodulin-binding motifs. This is consistent with our observation that Ca^{2+} responses appear to be autoregulated by the magnitude of the cytosolic Ca^{2+} signal.

DISCUSSION

Calreticulin and calnexin reside in the ER, where they play roles both as Ca^{2+} -sequestering proteins and as Ca^{2+} -dependent chaperones of N-linked glycoproteins imported into the ER and destined for subsequent targeting to various cellular destinations (19, 30, 31, 35). Whereas calreticulin is found in the lumen of the ER, calnexin is anchored to the ER membrane by a single transmembrane domain and has a regulatory cytosolic domain as well as an ER-luminal domain related to calreticulin. In this paper we have shown that cytosolic Ca^{2+} responses to chemoattractants are larger in mutants deficient in calreticulin and/or calnexin. Our results thus reveal that Ca^{2+} release from the endoplasmic reticulum contributes to the measured cytosolic Ca^{2+} responses in *D. discoideum*. This release from the ER coincides with and is coupled to the well-characterized influx of Ca^{2+} from the extracellular medium.

In other organisms, two kinds of coupling between intracellular Ca^{2+} release and Ca^{2+} influx have been observed, capacitative Ca^{2+} entry in which release of Ca^{2+} depletes intracellular stores and thereby opens store-operated channels in the plasma membrane, and Ca^{2+} -induced calcium release in which influxes of Ca^{2+} cause Ca^{2+} -sensitive Ca^{2+} channels in the ER to open (3). Enhanced responses in mutants deficient in cal-

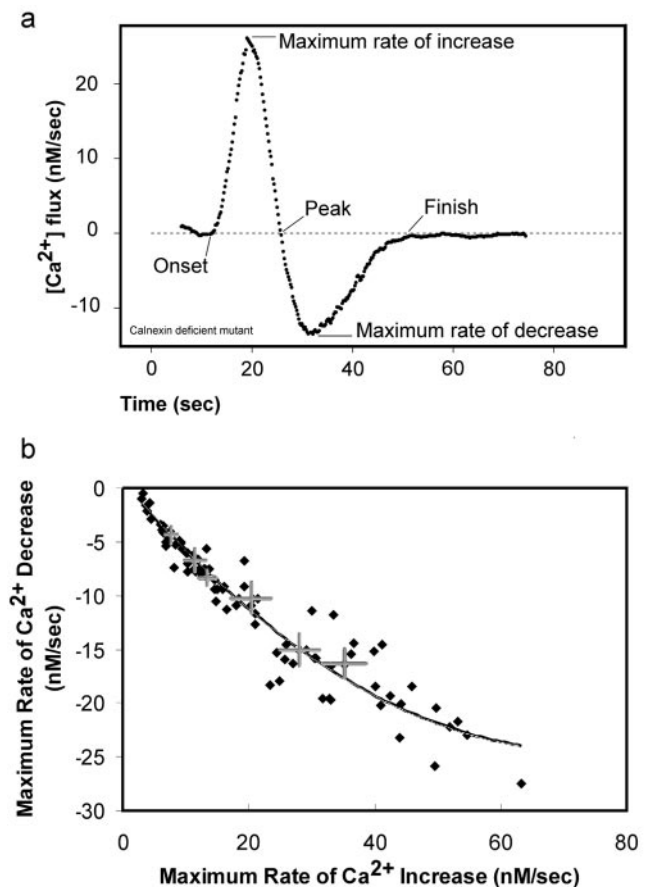


FIG. 5. Rates of change of cytosolic Ca^{2+} concentrations during responses to chemoattractant. Panel a shows an example of measurement of the rates of change of $[\text{Ca}^{2+}]$ during a response to an attractant stimulus (in this case, $1 \mu\text{M}$ cAMP delivered to aggregation competent cells of the calnexin-deficient mutant 2 s after recording started). The maximum rates of $[\text{Ca}^{2+}]$ increase during the rising phase and of $[\text{Ca}^{2+}]$ decrease during the falling phase of the response were measured graphically as indicated using features of the S statistics and graphics package. Panel b shows the correlation between the maximum rise and fall rates during individual experiments measuring Ca^{2+} responses by vegetative (to $1 \mu\text{M}$ folate) and aggregation competent (to $1 \mu\text{M}$ cAMP) cells of the calreticulin- and calnexin-deficient mutants and wild-type cells. The error bars indicate the means and standard errors, in order from left to right, for the responses to folate by the wild-type, calnexin-deficient, and calreticulin-deficient cells and then the larger, faster responses to cAMP by the wild-type, calnexin-deficient, and calreticulin-deficient cells.

nexin or calreticulin could be consistent with both kinds of coupling.

In the case of capacitative Ca^{2+} entry, it is known that overexpression of calreticulin in fibroblasts (29, 36) and *Xenopus laevis* oocytes (58) increases total Ca^{2+} storage capacity and attenuates calcium influx through store-operated channels. Conversely, the absence of calreticulin reduces the total calcium storage capacity of the ER (36). Thus, in the *D. discoideum* mutants, the ER Ca^{2+} stores are expected to be depleted more rapidly and extensively after an attractant stimulus. This could trigger earlier and more extensive Ca^{2+} entry from the extracellular medium through store-operated channels so that cytosolic Ca^{2+} levels begin to increase earlier and more rap-

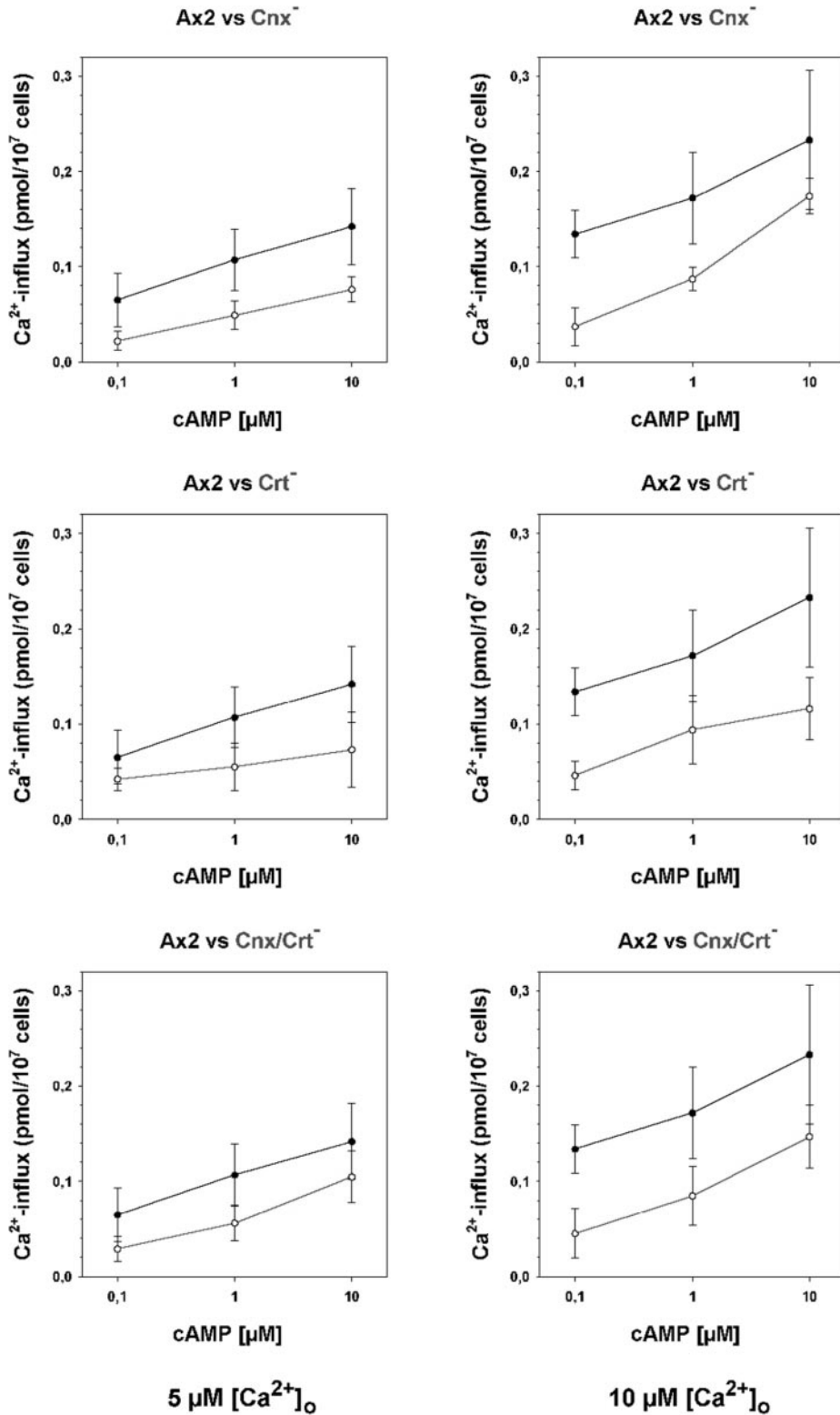


FIG. 6. Magnitude of the Ca^{2+} influxes elicited by cAMP stimuli in wild-type (AX2) cells and calreticulin-deficient (Crt^{-}) and calnexin-deficient (Cnx^{-}) single and double (Cnx/Crt^{-}) mutants. Response amplitudes at the time of the minimum extracellular $[Ca^{2+}]$ (40 s after stimulation with 0.1, 1.0, and 10 μ M cAMP) are shown for cells bathed in buffer containing either 5 μ M or 10 μ M Ca^{2+} . Wild-type cells (eight experiments) were starved for 5 to 7 h, the single mutants Cnx^{-} (five experiments) and Crt^{-} (five experiments) for 6 to 8 h and the double mutant for 7 to 10 h (four experiments). In each panel the solid circles show wild-type responses and the open circles show responses by the indicated mutant. The wild-type responses are redrawn in each row to facilitate direct pairwise comparisons with each mutant. Responses were measured using a Ca^{2+} -sensitive electrode. Error bars represent standard deviations.

TABLE 3. *Dictyostelium discoideum* genes encoding putative Ca²⁺ channels and pumps

Channel or pump	DictyBase ^a accession number	DictyBase ^a gene name	Protein product ^b	No. of TM domains ^c	Subcellular location ^d	Putative calcium- or calmodulin-binding regulatory sites ^e	Functional roles ^f	Reference(s)
Channels	DDB0183788	BECSV2_0_00020	Mucolipin	6	Lysosomes (mucolipin-1), vesicles	Ca ²⁺ -binding EF-hand (614–626)	Ca ²⁺ -dependent lysosomal or vesicle biogenesis and trafficking	43
	DDB0217079	JC2V2_0_00823	Polycystin-2	6	Plasma membrane (mucolipin-3)		Mechanosensory Ca ²⁺ signaling in cilia	37
	DDB0201571	<i>ip1A</i>	IPRL (IP ₃ Receptor-Like protein)	6	ER membrane	Calmodulin-binding helical peptide IQ motif (148–167)	Cytosolic Ca ²⁺ signaling	53
	DDB0216059	BECSV2_0_00938	TPC (Two Pore Channel)	12	Vacuolar membrane	Ca ²⁺ -binding EF-hands (772–810 and 808–836)	Cytosolic Ca ²⁺ signaling	42
Pumps	DDB0188438	BC5V2_0_01117	PMCA or SERCA	10	Plasma or ER membrane	Calmodulin-binding helical peptide IQ motif (683–702)	Ca ²⁺ homeostasis	52, 61
	DDB0186093	BC4V2_0_00846	PMCA or SERCA	10	Plasma or ER membrane	Calmodulin-binding helical peptide IQ motif (127–146)	Ca ²⁺ homeostasis	52, 61
	DDB0214945	<i>patA</i>	PatA	10	Vacuolar membrane	Calmodulin-binding helical peptide IQ motif (642–661)	Ca ²⁺ homeostasis	34

^a DictyBase is an online informatics resource for *Dictyostelium* (15).

^b Indicates the closest homologs in other organisms, except for PatA (a P-type Ca²⁺-ATPase related to PMCA) and IPRL (an IP₃ receptor-like protein related to IP₃ and ryanodine receptors), both of which have been studied experimentally in *Dictyostelium*.

^c Based on the number of transmembrane domains in homologs in other organisms and on predictions from standard web-based programs for topology prediction, accessed through the mirror site at APAF Australia of the EXPASY Proteomics Server (<http://au.expasy.org>) at the Swiss Institute of Bioinformatics (17). Note that in many cases different programs produced slightly different predictions.

^d Known in the case of PatA, predicted from the subcellular location of known homologs for all others.

^e Detected using InterPro, MyHits, and ELM motif searches through the EXPASY Proteomics Tools page (<http://au.expasy.org/tools/>). Numbers in parentheses indicate positions in the protein sequence. In the case of polycystin-2, the automated DictyBase gene ontology annotation predicts Ca²⁺ ion binding because this has been linked to the polycystin-2 signatures. However, we have been unable to find a putative Ca²⁺-binding site in any sequence motif searches.

^f Observed function of IPRL in *Dictyostelium*, observed functions of known homologs in other organisms for all others.

idly. This hypothesis is attractive but is not readily reconciled with the findings that the null mutants exhibit reduced rather than enhanced influxes of Ca^{2+} from the extracellular medium.

In the case of Ca^{2+} -induced calcium release, the greater amplitudes of the mutant responses could result from higher resting levels of free Ca^{2+} in the lumen of the ER. Free calcium levels in the ER (40 to 600 μM when stores are filled) are substantially higher than in the cytoplasm (0.04 to 0.11 μM) in other organisms (31). However the concentrations of bound Ca^{2+} in the ER are an order of magnitude higher again (1 to 3 mM) because of the presence of major Ca^{2+} -binding proteins, including calreticulin and calnexin. The absence of one or both of these two Ca^{2+} -binding proteins could result in even higher free calcium levels in the ER, a steeper Ca^{2+} concentration gradient from the ER lumen to the cytosol and an increased flux of Ca^{2+} into the cytosol during responses to chemoattractant. Brini et al. (4) reported such a correlation between the resting $[\text{Ca}^{2+}]_{\text{ER}}$ and the extent of IP_3 -induced release of Ca^{2+} into the cytosol.

The enhancement of the Ca^{2+} release as $[\text{Ca}^{2+}]_{\text{ER}}$ increased was found to be saturable, so that beyond a certain threshold there was no further increase in the magnitude of the Ca^{2+} release. This was explained by the autoregulatory nature of Ca^{2+} responses; inhibition of the IP_3 channel by cytosolic Ca^{2+} appeared to limit the magnitude of the responses. A similar phenomenon could explain why we observed no synergistic effect of the calnexin/calreticulin double deficiency. Ca^{2+} pumps and channels in the endoplasmic reticulum are regulated by both cytosolic and ER Ca^{2+} and thereby contribute to homeostatic control of Ca^{2+} levels in both compartments. Such mechanisms could prevent the absence of both calnexin and calreticulin from having additive effects on the free Ca^{2+} levels in the ER. It is noteworthy that the double mutant is much more severely impaired than the single mutants with respect to other phenotypes such as phagocytosis (35), suggesting that these phenotypes may not be explicable in terms of altered Ca^{2+} responses alone.

Steeper transmembrane calcium gradients across cellular membranes can also result from lower cytosolic Ca^{2+} levels such as are produced rapidly by EGTA treatment of cells (Z. Wilczynska and P. R. Fisher, unpublished data). Under the foregoing hypothesis, brief EGTA pretreatment should enhance subsequent responses to chemoattractant and this was recently reported to be the case (48). However this finding may also be interpreted as resulting from an indirect EGTA-mediated depletion of intracellular calcium stores (48).

Resting calcium levels in the ER have been reported to be unaffected by calnexin deficiency in a T-lymphoblastoid leukemia cell line (60), by calreticulin deficiency in fibroblasts (36), and by overexpression of calreticulin in both fibroblasts and in *Xenopus* oocytes (36, 58). However Arnaudeau et al. (1) reported that ectopic overexpression of calreticulin caused the basal levels of free Ca^{2+} in the ER of HEK-293 (HeLa) cells to double, while John et al. (21) observed either no change or a decrease in $[\text{Ca}^{2+}]_{\text{ER}}$ as a result of calreticulin overexpression in *Xenopus* oocytes. The free Ca^{2+} levels in the ER are the outcome of a balance between sequestration and release from high capacity binding proteins and fluxes to and from the cytosol via Ca^{2+} pumps and channels. Depending on the relative rate constants for these processes and the autoregulatory

mechanisms controlling them, the absence of one or more major classes of Ca^{2+} binding protein in the ER could have different effects on Ca^{2+} homeostasis in different cell types. It would clearly be valuable to assay free Ca^{2+} levels in the ER of *Dictyostelium* cells to further our understanding of the roles of ER Ca^{2+} in *Dictyostelium* responses to chemoattractant. However such experiments must await the development for *D. discoideum* of sensitive and accurate assays for ER Ca^{2+} comparable to those deployed in other organisms, for example, by targeting recombinant aequorin to the ER (4).

Calcium homeostasis and ligand-induced Ca^{2+} responses in other cell types are influenced not only by the Ca^{2+} -binding capacities of calnexin and calreticulin, but also by their roles as Ca^{2+} -dependent chaperones assisting the folding and regulating the function of other proteins (19, 30). For example, the bradykinin receptors of fibroblasts are improperly folded in the absence of calreticulin, so that Ca^{2+} responses to bradykinin are dramatically reduced in calreticulin-deficient cells (18, 36). In *D. discoideum* the cAMP and folate receptors and downstream signaling proteins clearly do not require calreticulin or calnexin for their proper folding, since both attractants elicit normal chemotaxis and large calcium responses in the mutants.

In animal cells, both calnexin (44) and calreticulin (21) bind to and regulate SERCA2b (Sarco/Endoplasmic Reticulum Ca^{2+} ATPase 2b) which pumps cytosolic Ca^{2+} into the ER. This Ca^{2+} -dependent interaction provides an inhibitory feedback mechanism that contributes to Ca^{2+} homeostasis in several cell types and participates in regulating cytosolic Ca^{2+} oscillations in *Xenopus* oocytes. Overexpression of calreticulin or ectopic coexpression of calnexin with SERCA2b thus dampens the oscillations in *Xenopus laevis*, while the absence of either protein is expected to enhance them. If calnexin and calreticulin have similar regulatory roles in *D. discoideum*, their removal should enhance Ca^{2+} responses as observed.

Not only were the amplitudes of Ca^{2+} responses to chemoattractants greater in mutants lacking calnexin or calreticulin, but the responses were accelerated. Compared to the responses in control cells, those in the mutants began sooner, peaked earlier, and exhibited shorter rise times and faster rates of increase in the rising phase of the response as well as faster rates of decrease in the falling phase of the response. These kinetic changes in the mutants are explicable in terms of the observed relationship between the magnitudes and the kinetics of the responses; larger responses are faster, regardless of the chemoattractant used and the presence or absence of calnexin and calreticulin. This indicates that the timing of key events such as opening and closing of Ca^{2+} channels and activation of Ca^{2+} pumps is autoregulated by mechanisms common to the signaling pathways for both the folate and cAMP receptors. Furthermore these mechanisms serve to couple the kinetics and the amplitudes of the Ca^{2+} responses in such a way that deficiencies in calnexin or calreticulin can affect both.

How might this coupling between Ca^{2+} response amplitudes and kinetics occur? The larger responses in calnexin- or calreticulin deficient mutants occur in combination with an earlier onset and a greater net rate of Ca^{2+} entry into the cytosol during the rising phase of the response. This indicates that during the larger responses in the mutants, a higher proportion of the responsible Ca^{2+} channels in the ER and/or the plasma membrane are opened. Such would be the case if Ca^{2+} re-

sponses are autoactivatory, as occurs in Ca²⁺-induced calcium influx or release and in capacitative Ca²⁺ entry following calcium store depletion. The shorter rise times associated with larger responses indicate that the Ca²⁺ responses are also autoinhibitory, that the Ca²⁺ flux into the cytosol is terminated sooner by Ca²⁺-induced channel closure and/or balanced earlier by Ca²⁺ activation of calcium pumps.

Consistent with this apparent autoregulation of the Ca²⁺ signal in chemoattractant responses, we found that the *Dictyostelium* genome encodes at least four putative Ca²⁺ channels and at least three presumptive Ca²⁺ pumps, most or all of them likely to be regulated by Ca²⁺. The sequences for six of them included recognizable motifs for Ca²⁺- or calmodulin-binding sites. All three of the Ca²⁺ pumps were related to the highly conserved and heavily studied PMCA (Plasma Membrane Ca²⁺ ATPase) (61) and SERCA (Sarcoendoplasmic Reticulum Ca²⁺ ATPase) (52) families and contained a helical peptide, calmodulin-binding IQ motif. It is difficult to predict the subcellular localization of these three putative Ca²⁺ pumps from sequence similarities as they are all more closely related to the PMCA than to the SERCA. PatA is known to localize to the vacuolar membrane (34), so the other two are likely to reside in the ER and plasma membranes—one in each.

Of the four *Dictyostelium* Ca²⁺ channels, only IPRL belonged to a well-studied class, the ER-resident IP₃ (41) and ryanodine (20) receptors. Although its subcellular location is unknown, IPRL seems the most likely candidate for an ER channel, and has been shown to play a role in cytosolic Ca²⁺ signaling in response to chemoattractants (53). In our search of the predicted *Dictyostelium* proteome we found no putative voltage-gated (6) or cyclic nucleotide-gated (22) channels and no homologs of the better known families (TRPC, TRPM, and TRPV) within the TRP (Transient Receptor Potential) superfamily of channels (8). Instead we found, in addition to IPRL, three channels that were homologs of mucolipins (43), polycystin-2 (37) and TPC (42). The first two belong to recently recognized groups within the TRP superfamily (mucolipins or TRPML and polycystins or TRPP) while TPCs (Two Pore Channels) are distantly related to the alpha subunits of the voltage-gated channels.

The best studied of the mucolipins, mucolipin-1, releases Ca²⁺ from the late endosome to facilitate the Ca²⁺-dependent interactions between endosomes and lysosomes (43). Mucolipin-1 mutations in humans cause a lysosomal storage disease, mucopolipidosis type IV, which is associated with neurological and ophthalmological defects. Mucolipin-3 is found in vesicle membranes in cochlear hair cells and melanocytes—mutations in this protein in mice cause pigmentation defects, deafness and erratic circling behavior suggestive of inner ear pathology (9). By analogy with the animal mucolipins, the *Dictyostelium* homolog identified here is likely to be found associated with late endocytic vesicles and lysosomes.

Polycystin-2 (or PKD2) in animals is expressed in ciliated cells where it localizes to the plasma membrane of the ciliary shaft, interacts with the mechanoreceptor polycystin-1 and mediates Ca²⁺ signaling in response to mechanical stimuli (37). Mutant forms disrupt the mechanosensory function of ciliated epithelial cells—in the case of those lining the nephrons of the kidney this leads to polycystic kidney disease. *Dictyostelium* cells are not ciliated and we were unable to find a homolog of

the polycystin-1 mechanoreceptor in the *Dictyostelium* genome. It is possible that the *Dictyostelium* polycystin-2 homolog is coupled instead to chemoreceptors and mediates Ca²⁺ influx through the plasma membrane in response to chemoattractant instead of mechanical stimuli.

TPC, the two-pore Ca²⁺ channel, is found in animals and in plants, but has been studied functionally only in the latter, where it is located in the vacuolar membrane and mediates Ca²⁺ release into the cytoplasm in response to hormonal and other stimuli (42). If it plays analogous roles in *D. discoideum* it could also participate in Ca²⁺ responses to chemoattractants, in which case the contractile vacuole would also contribute Ca²⁺ to the cytosolic Ca²⁺ signal.

Clearly any or all of these putative Ca²⁺ channels and pumps could participate in Ca²⁺ homeostasis and signaling in *D. discoideum*. Their existence reinforces the fact that our present results reveal only the contribution from the ER and that other intracellular organelles are likely also to participate in controlling cytosolic Ca²⁺ responses to chemoattractant. Understanding their roles will require functional studies using appropriately constructed mutants and accurate *in vivo* assays of Ca²⁺ concentrations in the various subcellular compartments.

Before accurate assays of cytosolic Ca²⁺ levels in *D. discoideum* became available, it was predicted that there would be two intracellular Ca²⁺ responses to chemoattractant—a rapid intracellular release accompanying the reported synthesis of IP₃ and a slower influx which had been revealed by measuring the uptake of ⁴⁵Ca²⁺. This would have paralleled the findings in other cells in which a rapid IP₃-induced release of Ca²⁺ into the cytosol depleted the ER calcium stores and was followed by a slower store-operated influx of Ca²⁺ from the extracellular medium (see Fig. 4 of reference 58 for an example). Nebl and Fisher (38) showed that this prediction was not correct and that the only measurable cytosolic Ca²⁺ response coincided with the influx and was completely dependent on extracellular Ca²⁺. Nonetheless there is evidence that *D. discoideum* contains all of the elements necessary for intracellular Ca²⁺ release in response to chemoattractants (26, 46, 47).

In this paper we have provided the first molecular genetic evidence that the *Dictyostelium* ER does indeed contribute to the elevation of cytosolic Ca²⁺ after a chemoattractant stimulus. We cannot determine from our results whether this ER contribution is in the form of Ca²⁺-induced calcium release or of an attractant-stimulated depletion of intracellular Ca²⁺ stores or both. Positive autoregulatory mechanisms regulating the calcium responses are implicit in both mechanisms, i.e., Ca²⁺ influx induces release or *vice versa*. The coupling we observed between the kinetics of the calcium responses and their amplitudes indicates that both positive and negative autoregulatory elements participate in regulating the calcium responses. Further molecular genetic dissection is required to unravel these mechanisms that control chemoattractant-induced calcium responses in *Dictyostelium*.

ACKNOWLEDGMENTS

We are grateful to Frank Zucchetti for his expert technical assistance.

We are grateful to the Deutsche Forschungsgemeinschaft for financial support.

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