The Role of Calcium in the *Chlamydomonas reinhardtii* Mating Reaction

Ursula W. Goodenough,* Brian Shames,* Linda Small,* Tatsuaki Saito,* Richard C. Crain,‡ Mark A. Sanders,§ and Jeffrey L. Salisbury§

*Department of Biology, Washington University, St. Louis, Missouri 63130; ‡Department of Molecular and Cell Biology, University of Connecticut, Storrs, Connecticut 06269; §Department of Biochemistry and Molecular Biology, Mayo Clinic Foundation, Rochester, Minnesota 55905

Abstract. The mating reaction of *Chlamydomonas reinhardtii* entails a rapid series of cell–cell interactions leading to cell fusion. We have demonstrated (Pasquale, S. M., and U. Goodenough. 1987. *J. Cell Biol.* 105:2279–2293) that cAMP plays a key role in this process: gametic flagellar adhesion elicits a sharp increase in intracellular cAMP, and presentation of dibutyryl-cAMP to unmated gametes elicits all known mating responses. The present study evaluates the role of Ca^{2+} in this system. We document that the mating-induced increase in cAMP, and hence the mating responses themselves, are blocked by a variety of drugs known to interfere with Ca^{2+}-sensitive processes. These data suggest that Ca^{2+}-mediated events may couple adhesion to the generation of cAMP. Such events, however, appear to be localized to the flagellar membrane; we find no evidence for the mating-related increase in cytosolic free Ca^{2+} that has been postulated by others. Indeed, by monitoring the length of the Ca^{2+}-sensitive centrin-containing nucleus-basal body connector, we show that cytosolic free Ca^{2+} levels, if anything, decrease in response to cAMP signaling. We confirm a previous report that Ca^{2+} levels increase in the mating medium, but document that this represents a response to augmented cAMP levels and not a prelude. Finally, we show that IP_3 levels remain constant throughout the mating reaction. These results are discussed in terms of the various signal transduction systems that have now been identified in *Chlamydomonas*.

RECENT studies make a strong case for the role of cAMP as a primary signaling agent during the *Chlamydomonas* mating reaction: sexual adhesion between plus and minus gametes generates an immediate rise in intracellular cAMP levels (Pijst et al., 1984; Pasquale and Goodenough, 1987; Kooijman et al., 1990; Schuring et al., 1991), and exogenous presentation of cAMP elicits all known mating responses from gametes of a single mating type (Pasquale and Goodenough, 1987; Goodenough, 1989; Hunnicutt et al., 1990; Kooijman et al., 1990). Such data do not, however, rule out a role for other signaling agents, directly generated as a consequence of adhesion, which might go on to stimulate cAMP formation. Alternatively, the major role of cAMP might be to elicit an increase in additional second messenger(s) which would go on to elicit the mating responses.

An obvious additional candidate is calcium, which is widely used by eukaryotic cells as a rapid-acting signaling agent (Evered and Whelan, 1986; Berridge and Irvine, 1989; Cheek, 1991). The existing literature on the behavior of calcium during *Chlamydomonas* mating includes the following observations. Mating can occur normally in Ca-free medium (Bloodgood and Levin, 1983), and attempts to elicit mating responses using calcium ionophores alone have been negative (Bloodgood and Levin, 1983; Kaska et al., 1985; Pasquale and Goodenough, 1987). On the other hand, Bloodgood and Levin (1983) have detected increased ^{45}Ca levels in the medium during mating, and Kaska and coworkers (Kaska et al., 1985) have used x-ray microanalysis to argue that Ca^{2+} stored in discrete granules diffuses throughout the cytosol during mating. These observations have been interpreted to reflect the occurrence of a (transient) increase in cytoplasmic free Ca^{2+} levels, presumably caused by the release of Ca^{2+} from internal stores, which might signal mating responses. More recently, studies on the related species *C. eugametos* have suggested that an IP_3-induced Ca^{2+} increase elicits mating-structure activation and that IP_3 levels increase 10-fold at the time of gametic cell fusion (Shuring et al., 1990; Musgrave et al., 1992; van den Ende, 1992).

In the present study, we present data that are consistent with a role for Ca^{2+} in stimulating the rise in cAMP that activates mating responses, but inconsistent with a role for Ca^{2+} as a bona fide second messenger in the fashion postulated by the above-cited investigators.

Address correspondence to Dr. Goodenough at Department of Biology, Washington University, Campus Box 1229, St. Louis, MO 63120-4899.

Materials and Methods

Strains, Culture Conditions, and Reagents

Wild-type *C. reinhardtti* 137c (strains CC-620 mt^+ and CC-621 mt^-), available from the *Chlamydomonas* Genetics Center, Duke University (Durham, NC), were prepared as plate gametes (Martin and Goodenough, 1975). Plates were flooded with 10 mM K-Pipes, pH 7.4 for 1–2 h; a flask containing the suspended gametes was placed on an illuminated rotary shaker for 1 h; the gametes were pelleted and suspended in fresh Pipes; and the cells were shaken another 15–30 min before use. The achievement of full sexual-signaling capacity by the washed cells was ascertained by the occurrence of immediate wall release when mt^+ and mt^- gametes were mixed. Mating efficiency was calculated by counting biflagellate (BFC) and quadriflagellate cells (QFC) in glutaraldehyde-fixed samples and applying the formula: % cell fusion $\approx 2 \times QFC \times 100/(2\ QFC + BFC)$.

Reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except for H-8[1] (Seikagaku Inc., St. Petersburg, FL). Amiprophos-methyl (APM) (Morejohn and Fosket, 1984) was a gift from Dr. Donald Weeks, University of Nebraska. When drugs were used to block the mating reaction, gametes were carefully monitored to ascertain that the drugs were not compromising their adhesion or motility or inducing deflagellation, responses that indicate that excessive concentrations are being employed.

cAMP Determinations

Gametes at 2×10^7 cells/ml (hemacytometer counted) were incubated 5 min in the various inhibitors (concentrations given in Table I). They were then mixed and aliquots taken at the time points indicated in Fig. 1 for % cell fusion determinations and for cAMP assay. The latter was performed as described by Pasquale and Goodenough (1987).

External Ca Measurements

Equal volumes of mt^+ and mt^- gametes were mixed together at 2×10^7 cells per ml; unmated gametes at this concentration served as controls. At various times, 1-ml samples were withdrawn, transferred to a 1.5-ml plastic tube, and spun in a microfuge for 1 min (Beckman Instruments, Inc., Fullerton, CA). The supernatants (0.2–0.8 ml, depending on the experiment) were immediately transferred to fresh tubes. At the conclusion of the mating, each supernatant was mixed with a 100-μM stock solution of arsenezo III, a calcium indicator dye (Koch and Kaupp, 1985; Koch et al., 1987), to yield a final dye concentration between 10 and 40 μM. (Koch and Kaupp [1985] note that while the reliability of arsenezo III as an intracellular calcium indicator has been correctly questioned, it is very reliable, when used at low concentrations, to determine free Ca^{2+} levels in solution). The absorbance of the samples was then measured using the wavelength pair A_{650}–A_{730}, where the 730-nm reading detects light scattering due, for example, to wall particles and the 650 nm reading reflects the peak absorbance of the arsenezo-Ca^{2+} complex. (Arsenezo III is at least 50 × more sensitive to Ca^{2+} than Mg^{++} [Koch and Kaupp, 1987] and in controls in which 1 mM $MgCl_2$ was added to the supernatants, no changes were seen in the absorbance readings.) To calibrate the measurements, known amounts of $CaCl_2$ were added to supernatants from control (unmated) cell samples; absorbance readings were then recalculated as $\mu M\ Ca^{2+}$.

Unmated and mated supernatant samples were also spun at 100,000 *g* × 1 h to pellet organic particulates, diluted 1:10 and subjected to inductively coupled plasma-atomic emission spectroscopy (TCP-AES) (Jarrell-Ash) at the Monsanto Company, St. Louis, MO.

Determination of Nucleus-basal Body Connector Length

Gametes obtained by flooding plates with N-free medium (Martin and Goodenough, 1975) were shaken for 1 h, pelleted, and resuspended in 10 mM Hepes pH 7. Vegetative cells were grown in liquid TAP medium (Gorman and Levine, 1965) to a density of $\sim 5 \times 10^6$ cells/ml; they were then pelleted and resuspended in 10 mM Hepes pH 7. Sample aliquots were mixed with an equal volume of 8% paraformaldehyde and stored at 4°C until subjected to microscopic analysis.

They were first washed several times in 10 mM Hepes, then treated with two 5 min washes of 2 mg/ml sodium borohydride (Sigma Chemical Co.) to reduce free aldehyde groups. Cell suspensions were then allowed to adhere to eight-welled slides (Carlson Scientific, Peotone, IL) that previously had been coated with 0.1% polyethyleneimine (Sigma Chemical Co.). Cells were permeabilized in −20°C methanol for 10 min followed by −20°C acetone for 5 min, then air dried. The cells were blocked for nonspecific binding by a 30-min incubation at 37°C with 5% goat serum (Gibco Laboratories, Grand Island, NY), 5% glycerol, and 5% DMSO in PBS. They were then incubated with 17E10 anti-centrin monoclonal primary antibody (Salisbury et al., 1988) diluted 1:500 in blocking buffer for 3 h at 37°C, washed in several changes of PBS, and reacted with FITC-conjugated goat anti-mouse IgG secondary (Organon Teckníca-Cappel Laboratories, Malvern, PA), diluted 1:400 in blocking buffer, for 1 h at 37°C. The preparations were washed several times in PBS and mounted in Gevatol mountant (Rodríquez and Deinhardt, 1960) containing 2% *n*-propyl galate and 1 μg/ml DAPI (both from Sigma Chemical Co.), pH 8.5. The samples were observed using a Nikon FXA microscope (Fryer Co., Inc., Carpentersville, Il) equipped for epifluorescence and photographed using a 60× plan apo, 1.4 n.a. objective and recorded on Hypertech film (Microfluor, Ltd., Stony Brook, NY) at an ASA setting of 1600–3200. Fields of labeled cells were recorded and measurements of 30 cells were made after transfer of the negative images to an Image 1™ image processing system (Universal Imaging Corporation, Media, PA).

IP_3 Measurements

Samples of gametes, mated or deflagellated at the indicated times (Table III), were immediately mixed with TCA and the extracts subjected to IP_3 radioreceptor assay, with detailed protocols given in Quarmby and coworkers (1992).

Results

Inhibitors that Block Both Mating and cAMP Increases

The mating reaction between mt^+ (plus) and mt^- (minus) gametes in *C. reinhardtii* displays the following features that are pertinent to this report (details and references are given in Goodenough, 1991). The initial adhesion reaction occurs as soon as plus and minus gametes are mixed, and involves complementary agglutinin glycoproteins displayed on the flagellar surfaces. Adhesion elicits the primary sexual signal which, we argue, is a rise in intracellular cAMP, and there follows a cAMP-dependent phosphorylation of target proteins. Five mating responses to cAMP elevation can be monitored by microscopy: (*a*) agglutinin proteins move to the flagellar tips (tipping); (*b*) new agglutinins are added to replace those inactivated by the adhesion reaction (recruitment); (*c*) cell walls are lysed and shed into the medium; (*d*) mating structures are activated which, in plus gametes, entails actin polymerization into an extended fertilization tubule; and (*e*) activated mating structures fuse, allowing total cell fusion and the creation of quadriflagellated zygotes, which rapidly lose their adhesiveness by unknown mechanisms (Hunnicutt and Snell, 1991).

Table I lists inhibitors that we and others have found to block all five mating responses but to be without effect on the initial flagellar adhesion reaction (see also Goodenough, 1993). To dissipate the notion that the mating reaction is sensitive to any pharmacological manipulation, a partial list of inhibitors that fail to block mating includes: sodium vanadate (1 mM); $ZnCl_2$ (5 mM); NaF (100 mM); $CsCl_2$ (10 mM); tetraethylammonium (TEA), (10 mM); and 4-aminopyridine (5 mM).

Gametes pre-incubated in each inhibitor (5 min) and then

1. *Abbreviations used in this paper*: APM, amiprophos-methyl; db-cAMP, dibutyryl cAMP; GLE, gametic lytic enzyme; H-8, N-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; IBMX, isobutylmethylxanthine; NBBC, nucleus-basal body connector; TFP, trifluoperazine.

Table I. Inhibitors Used in Present Study

Reagent	Concentration blocking mating	Concentration blocking phototaxis	Concentration blocking vegetative cell tipping
$LaCl_3$	50 μM	1 mM‡	No effect**
$CdCl_2$	50 μM	10 μM	No effect **
Diltiazem	100 μM	50 μM§	100 μM‖
Lidocaine	1.25 mM*	No effect	14 mM‖
Trifluoperizine (TFP)	10 μM	10 μM (partial)	16 μM¶
Colchicine	20 mM	Not tested	No effect¶
Amiprophos-methyl (APM)	50 μM	No effect	Not tested

Superscripts indicate prior publications from other laboratories utilizing these reagents: * Snell et al., 1982; ‡ Nultsch, 1979; § Nultsch et al., 1986; ‖ Bloodgood and Salomonsky, 1990; ¶ Bloodgood, 1990. The unreferenced phototaxis results were obtained by U. W. Goodenough, monitoring gametic swimming towards blue light. ** The results represent personal communications from Dr. R. A. Bloodgood, University of Virginia, Charlottesville, VA.

mixed show an efficient early agglutination reaction but start to disadhere after 10–15 min. Addition of 10 mM db-cAMP/1 mM IBMX to such disadhered cells restores their ability to agglutinate by recruiting agglutinin pools (Goodenough, 1989, 1993), and they proceed to activate the other mating responses and fuse normally (except diltiazem and colchicine, as discussed below). Alternatively, gametes of a single mating type can be incubated with inhibitor (5 min) and then with db-cAMP/IBMX; except as discussed below, mating responses are also elicited normally. Thus, db-cAMP/IBMX can rescue the inhibited state, suggesting that each inhibitor acts upstream of adhesion-induced cAMP generation.

To evaluate this interpretation, the effects of each inhibitor on cAMP generation were directly tested using radioimmunoassay. Fig. 1 shows that normal cAMP dynamics during a mating reaction: adhesion elicits a transient 10-fold increase in cAMP, followed by cell fusion, disadhesion, and an attendant drop in cAMP levels. Also shown are the data for five of the inhibitors (Cd^{2+}, La^{3+}, APM, colchicine, and lidocaine) listed in Table I. All tend to elevate somewhat the basal cAMP levels, but none show the further dramatic elevation in cAMP normally elicited by sexual adhesion. Data documenting that TFP inhibits the cAMP increase have been previously published (Pasquale and Goodenough, 1987).

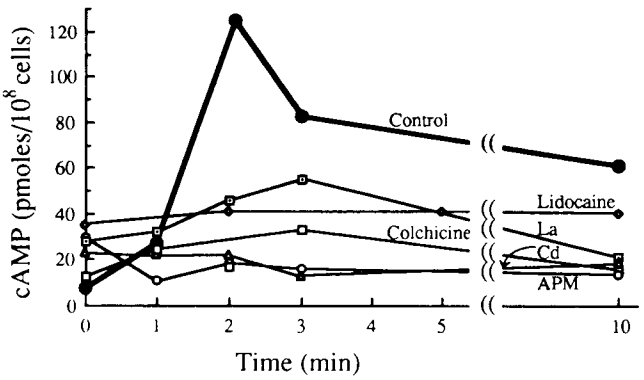

Figure 1. Levels of cAMP during mating in controls (●—●) and in gametes preincubated (5 min) and then mated in $CdCl_2$ (50 μm) (△—△); $LaCl_3$ (50 μM) (⊡—⊡); APM (50 μM) (○—○); colchicine (20 mM) (□—□); lidocaine 1.25 mM) (◇—◇). Controls mated at 82% at 10 min; inhibitors blocked mating completely.

Two of the inhibitors give puzzling results. Diltiazem (100 μM) does not block the adhesion-induced rise in cAMP (data not shown); db-cAMP/IBMX can nonetheless rescue tipping, agglutinin recruitment, and cell-wall loss in diltiazem-treated gametes, but cell fusion is not rescued. Colchicine (20 mM) blocks the adhesion-induced rise in cAMP (Fig. 1) and db-cAMP/IBMX treatment rescues tipping and agglutinin recruitment, but it fails to rescue cell-wall loss or cell fusion. Apparently, therefore, these inhibitors each affect the mating reaction at more than one point.

Most of the reagents listed in Table I have been implicated as having effects on Ca^{2+} transport and/or utilization. APM and colchicine are apparent exceptions: both are best known for their effects on tubulin polymerization (Lefebvre et al., 1978; Morejohn and Foskett, 1984). In addition, however, APM has been reported to affect Ca^{2+} transport (Hertel et al., 1980), and we find that the colchicine inhibition of mating is abolished if 0.5 mM Ca^{2+} is added along with the drug; hence a calcium connection pertains here as well. As published previously (Snell et al., 1982), the lidocaine inhibition is also reversed by 1 mM Ca^{2+}. The La^{3+}-blocked cells are partially rescued (30% mating) by adding 1 mM $CaCl_2$ directly, whereas the Cd^{2+}-blocked cells can be partially rescued by 1 mM $CaCl_2$ (56% mating) only if the cells are first washed; washing alone fails to release the block.

Additional evidence that these reagents affect Ca^{2+} transport/utilization is given by their effects on the calcium-sensitive processes of phototaxis and flagellar tipping in vegetative cells (Stavis and Hirschberg, 1973; Nultsch, 1979; Laurens, 1987; Bloodgood and Salomonsky, 1990; Harz and Hegemann, 1991). As summarized in Table I, most of the drugs that have been tested are found to affect one or both behaviors. Obvious discrepancies (e.g., the failure of La^{3+} and Cd^{2+} to inhibit vegetative-cell tipping, the failure of APM to inhibit phototaxis) suggest that distinctive Ca^{2+} entry/utilization mechanisms are employed for each behavior.

Any conclusion about the mode of action of these inhibitors need to take into account the data shown in Fig. 2. In the course of experiments evaluating the effects of elevated cAMP on protein phosphorylation, phosphate-starved gametes were pre-incubated (10 min) with the inhibitors shown and then pulsed (6 min) with $^{32}P_i$. In contrast to the controls, and to cells treated with the protein kinase inhibitors

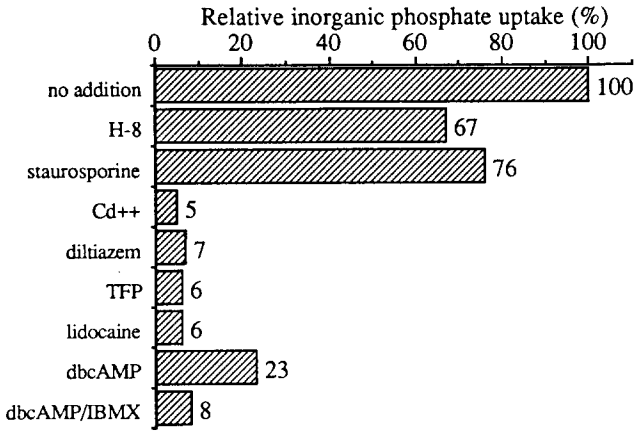

Figure 2. Inhibition of inorganic phosphate uptake. Phosphate-starved gametes (1×10^8 cells/ml) were dewalled with GLE and pre-incubated with a series of drugs for 10 min. 1.5 ml of cells were mixed with inorganic $^{32}P_i$ (10 μCi/ml) for 6 min, cooled on ice, quickly harvested and resuspended in 100 μl of nitrogen-free medium, mixed with a liquid scintillation cocktail and counted. Drug concentrations were 2 mM H-8, 1 μM staurosporine, 100 μM Cd^{++}, 125 μM diltiazem, 30 μM TFP, 1.25 mM lidocaine, 10 mM db-cAMP and 10 mM db-cAMP/1 mM IBMX.

staurosporine and H-8, virtually no $^{32}P_i$ uptake was observed, even though cell motility and flagellar integrity, two parameters of cellular viability in *Chlamydomonas*, were unimpaired. Curiously, db-cAMP/IBMX also blocks phosphate uptake, possibly because cAMP and phosphate compete for the same carrier.

External Calcium and the Mating Reaction

Reagents such as heavy metal cations, diltiazem, and the amphipathic amines are capable of blocking Ca^{2+} entry into cells, which would suggest a mating-reaction requirement for external calcium. Cells washed and resuspended in 10 mM K-Pipes pH 7.3 mate with near-100% efficiency if they are maintained in the Pipes buffer at least 30 min before mixing. When the [Ca^{2+}] of the buffer is analyzed spectrophotometrically before and then after the 30-min incubation, however, it is found to increase from 0.04 to ~25 μM. Thus, the cells release substantial amounts of Ca^{2+} into the medium under these circumstances and hence have available to them substantial Ca^{2+} levels at the time of mating. While the source of this released Ca^{2+} is not known, it is likely to derive from the cell walls.

The obvious experiment, the chelation of this released Ca^{2+} with EGTA, was performed by Bloodgood and Levin (1983), who report at most a 25% inhibition of mating in 10 mM Tris, 5 mM EGTA. In our hands, such EGTA concentrations strip agglutinins from the flagellar surface, compromising adhesion and mating efficiency, and inhibit the activity of GLE (gametic lytic enzyme), the metalloprotease that catalyzes cell-wall lysis (Matsuda et al., 1985). We therefore analyzed the effect of repeatedly washing the gametes in Ca-free Pipes buffer. As summarized in Table II, each washing reduces mating efficiency, and in each case it can be restored with extracellular Ca^{2+}. We conclude, therefore, that external Ca^{2+} is important for mating and is likely to be required, but since gametes continuously add

Table II. Effect of Ca^{2+} Depletion and Addition on Mating Efficiency

	Mating
Experiment 1	%
A. Wash 2 × in Pipes + 0.1 mM $CaCl_2$	97
B. Wash 2 × in Pipes alone	69
C. Add 0.1 mM $CaCl_2$ to sample B	97
Experiment 2	
A. Wash 1 × in Pipes alone	92
B. Wash 2 × in Pipes alone	73
C. Wash 3 × in Pipes alone	67
D. Add 0.1 mM $CaCl_2$ to sample B	88
E. Add 0.1 mM $CaCl_2$ to sample C	91

Ca^{2+} to the medium and may well bind Ca^{2+} to their membrane surfaces for use in mating, it is not possible to document the levels required, nor the Ca^{2+} source. It is worth noting that after two washes in Ca-free Pipes, gametes are rendered completely blind to phototaxis, and are incapable of undergoing deflagellation in response to pH shock; both behaviors are fully restored if 0.1 mM Ca^{2+} is subsequently added to the buffer (data not shown). Therefore, the requirement for external Ca^{2+} during mating is clearly different than for these other responses.

Calcium Release During Mating

The rapid release of Ca^{2+} into the medium when cells are suspended in Ca-free buffer equilibrates after ~30 min, with equilibration levels ranging from 20–40 μM in different preparations. When such equilibrated cells are then mated, an additional rapid release of ~5 μM Ca^{2+} occurs; in Fig. 3, for example, the medium changes from 25 → 30 μM Ca^{2+}. A slow rise in Ca^{2+} levels then continues over the next 2 h, but there are no further discontinuities. This 5 μM mating-related increase in extracellular Ca^{2+} levels presumably corresponds to the mating-induced $^{45}Ca^{2+}$ efflux observed in the experiments of Bloodgood and Levin (1983).

To confirm that the change in arsenazo III absorbance ac-

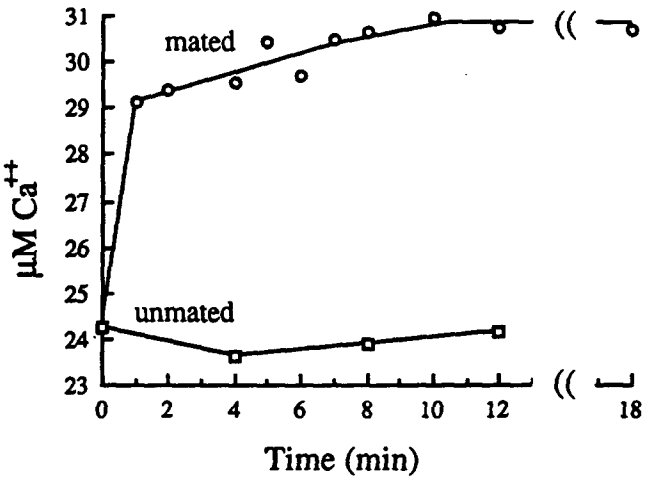

Figure 3. Levels of external Ca^{2+} during mating (first 20 min) (o—o) compared with unmated controls (□—□).

curately reflects Ca^{2+} levels, the equilibrated medium from unmated mt^+ and mt^- gametes, and the medium after these gametes had mated for 10 min, was subjected to atomic emission spectroscopy (courtesy Dr. Cliss Ling, Monsanto Company, St. Louis, MO). The unmated samples each yielded 0.5 parts per million (ppm) Ca^{2+}, corresponding to 13 μM; the mated sample yielded 0.7 ppm, corresponding to 18 μM. These values, close to those obtained with arsenazo III (Fig. 3), independently document that Ca^{2+} levels change by 5 μM. In the same samples there was no change in levels of Mg^{2+} (0.4 ppm), Na^+ (11 ppm), K^+ (680 ppm) and Zn^{++} (0.2 ppm); most of the K^+ and Na^+ was presumably contributed by the K^+-Pipes buffer.

When mt^+ gametes are isoagglutinated with an antiserum raised against gametic flagella, the full panoply of mating responses is elicited (Goodenough and Jurivich, 1978), including cAMP elevation (Saito et al., 1993). As shown in Fig. 4, this isoagglutination also elicits 5-μM increase in extracellular Ca^{2+} levels.

To ask whether the Ca^{2+} release is a prelude to cAMP signaling or a response, equilibrated mt^+ gametes were treated with 10 mM db-cAMP/1 mM IBMX and monitored relative to untreated controls. After a 5-min lag, Ca^{2+} levels began to rise, attaining a 5-μM increase by ~30 min (Fig. 5). Similar kinetics are observed for the stimulation of other mating traits by db-cAMP/IBMX (Pasquale and Goodenough, 1987); we attribute the lag and the asynchronous response to the uptake of the reagents. These data indicate that Ca^{2+} release is a response to cAMP elevation and not the reverse.

We also assessed the effects of several inhibitors on Ca^{2+} release, in each case adjusting for any absorbance changes caused by the direct interaction of the inhibitor with arsenazo III. In an experiment wherein the $[Ca^{2+}]$ of the medium increased by 13 μM in a control mating, there was a 1-μm increase in the presence of Cd^{2+} (100 μM) and no increase in the presence of TFP (10 μM) or diltiazem (50 μM). Again, therefore, cAMP signaling appears necessary to elicit Ca^{2+} release.

To evaluate whether the Ca^{2+} derives from the cell walls shed at the time of mating, mt^+ and mt^- gametes were

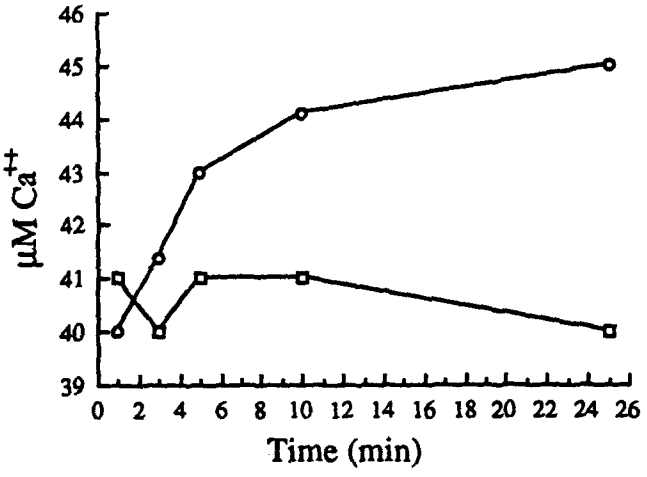

Figure 4. Levels of external Ca^{2+} when mt^+ gametes are isoagglutinated with antisera directed against flagellar surface carbohydrate epitopes (o—o) vs nonagglutinated controls (□—□).

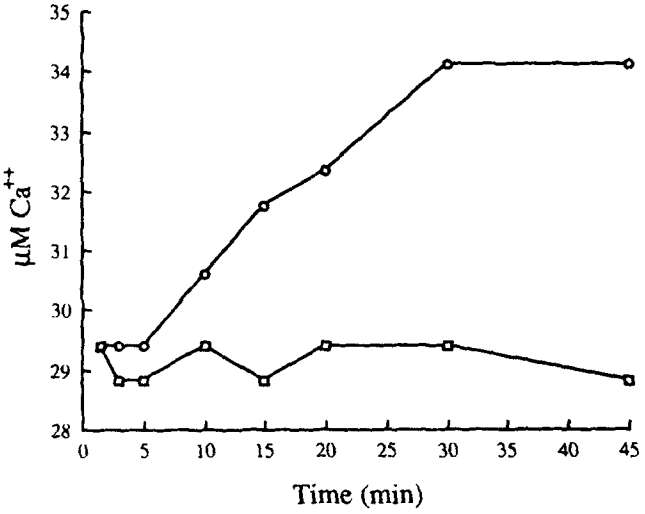

Figure 5. Levels of external Ca^{2+} when mt^- gametes are treated with 10 mM db-cAMP/1 mM IBMX (o—o) vs untreated controls (□—□).

separately incubated in GLE, washed twice with Pipes buffer, equilibrated for 15 min, and then mated. Results are plotted in Fig. 6. Equilibration levels of Ca^{2+} tend to be lower in the GLE-treated gametes than in similarly washed and equilibrated controls, indicating that one source of this Ca^{2+} may be the cell walls. However, both sets of gametes released equivalent amounts of Ca^{2+} upon mating. It therefore seems unlikely that the mating-released Ca^{2+} derives from disassembling cell walls at the time of mating, a conclusion reached in a somewhat different fashion by Bloodgood and Levin (1983).

Levels of Cytosolic Free Ca^{2+} during the Mating Reaction

An alternate source of the released Ca^{2+}, suggested by

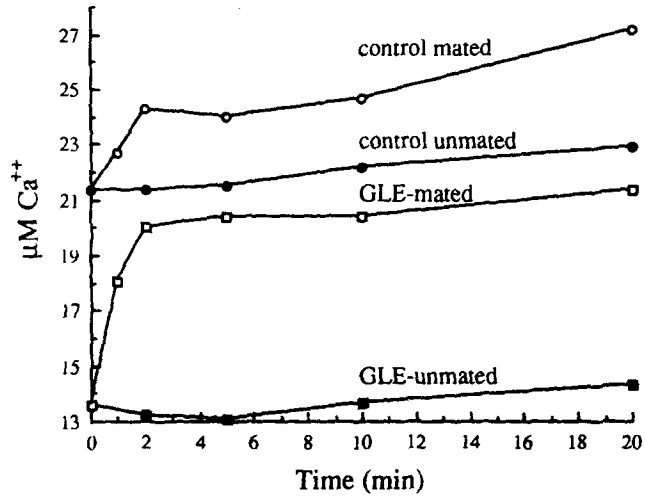

Figure 6. Two experiments following levels of external Ca^{2+} during the mating of walled gametes (o—o) and gametes pre-treated with GLE to remove walls (□—□). Controls are the mean values of the mt^+ and mt^- walled (●—●) or wall-free (■—■) gametes determined separately.

Bloodgood and Levin (1983) and Kaska and coworkers (1985), is that there occurs during mating a global increase in cytosolic free Ca^{2+} which spills over into the medium. Evaluation of cytosolic free Ca^{2+} is traditionally made with fluorescent Ca^{2+}-indicator dyes such as fura-2, but we and others have thus far been unsuccessful at inducing *C. reinhardtii* to take up these dyes.

Fortunately, it is possible to monitor calcium-sensitive structural elements as indicators of free Ca^{2+} levels in the *Chlamydomonas* cytosol. A system of contractile fibers known as the nucleus-basal body connector (NBBC) (Wright et al., 1985) has been shown to contain the calcium-binding protein centrin (caltractin) (Schulze et al., 1987; Salisbury et al., 1988; Huang et al., 1988). Using detergent-permeabilized cell models, Salisbury and co-workers (1987) have demonstrated that the NBBC shortens upon increase in prevailing free Ca^{2+} levels, and have shown that a similar shortening occurs in vivo when cells are deflagellated by pH shock, an operation that elevates cytosolic Ca^{2+} levels (Salisbury et al., 1987; Quarmby et al., 1992).

Fig. 7 documents that the mean resting length of NBBCs is 4.3 μm in the gamete and 5.6 μm in the vegetative cell. This difference does not appear to be due to contraction but rather to the smaller size of gametes (1/2–2/3 the size of vegetative cells), and a concomitant reduction in size of the NBBC: the descending fiber between the basal body and the nucleus is shorter, whereas the fiber system that embraces the nuclear envelope is equivalent in size to its vegetative counterpart. Fig. 7 also documents that NBBC lengths remain constant when external [Ca^{2+}] is changed by 10^3, indicating that the cytosolic free Ca^{2+} concentrations are well buffered.

Within 1 min of mating, the mean NBBC length increases to 6.1 μm (Figs. 8 and 9), an increase of ~29% over unmated gametes. The fiber system remains extended over the next hour until the time of nuclear fusion (as monitored by DAPI staining) which occurs 90–135 min after cell fusion. Upon completion of nuclear fusion, the NBBC has contracted to a length of 3.9 μm (Fig. 8), displacing the nucleus to the anterior end of the cell.

The lengthening of the NBBC during the mating reaction may in fact indicate that the centrin has been subjected to phosphorylation because, in permeabilized cell models, NBBCs have been shown to relax when Ca^{2+} is chelated and ATP is added (Salisbury et al., 1987). It is not, on the other hand, the expected outcome if cytosolic Ca^{2+} levels increase during the mating reaction; instead, one would expect a shortening.

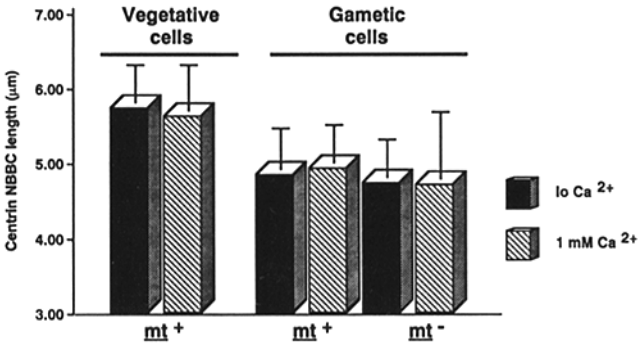

Figure 7. Mean lengths of NBBCs for vegetative mt^+ cells and for mt^+ and mt^- gametes in 10 mM Hepes pH 7 (lo Ca) and with the addition of 1 mM $CaCl_2$ (hi Ca).

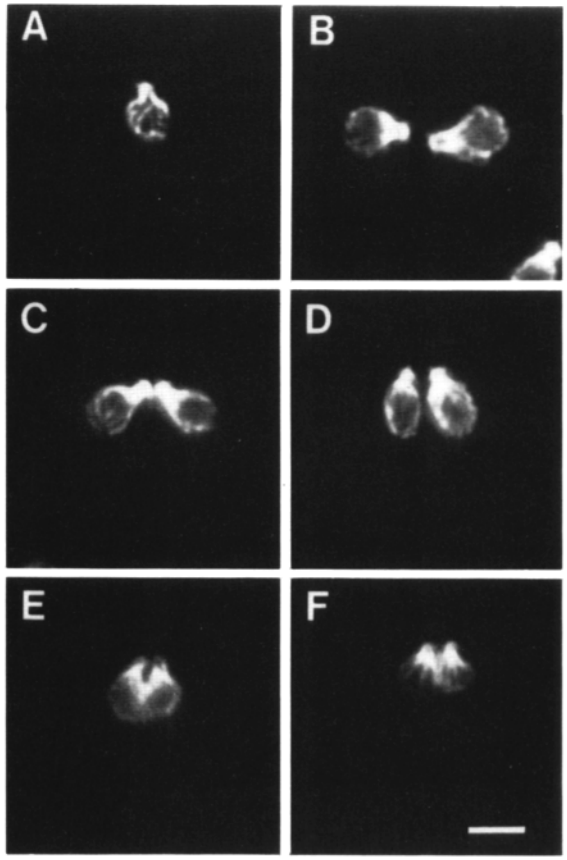

Figure 8. Fluorescence micrographs of gametic centrin localization (NBBCs) before (*A*) and following (*B–F*) the onset of the mating reaction. A, mt^- gamete; B-F, mated pairs at 2, 3, 5, 90, and 165 min following mating. Scale bar, 5 μM.

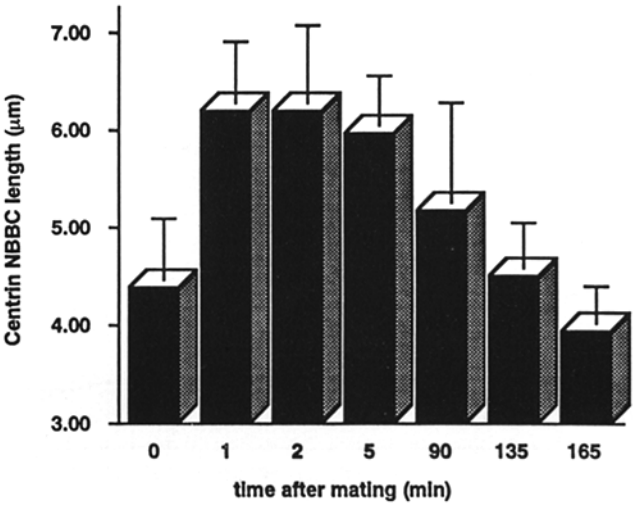

Figure 9. NBBC mean length determinations for gametes and mated cells at various times following the onset of the mating reaction. Each bar represents the mean NBBC length determined for 30 cells with standard error bars indicated.

Table III. IP_3 Levels During Mating and Deflagellation

	IP_3	Mating
	*(pmol/10_6 cells)**	%
A. Unmated mt^+ gametes	1.3	
B. Unmated mt^- gametes	0.8	
C. Mating, 0 time (A+B/2)	1.1	
D. Mating 1 min	1.4	15
E. Mating 2 min	1.1	60
F. Mating 5 min	1.1	82
G. Mating 10 min	0.9	92
H. mt^+ gametes (A), pH shock, 30 s	5.7	
I. mt^+ gametes (A), pH shock, 60 s	5.0	
J. mt^- gametes (B), pH shock, 30 s	4.9	
K. mt^- gametes (B), pH shock, 60 s	4.7	

* Data is representative of two separate experiments each assayed in duplicate. The differences in IP_3 levels in samples *A–G* are not statistically significant.

Levels of IP_3 during the Mating Reaction

A frequent, although not invariant, prelude to cytosolic Ca^{2+} increase in eukaryotic cells is an increase in IP_3 levels (Berridge and Irvine, 1989). Musgrave and colleagues (1992) report a 10-fold increase in IP_3 levels at the time of gametic cell fusion in *C. eugametos*, and predict that this generates a release of Ca^{2+} into the cytosol from membranous stores. The data in Table III document that IP_3 levels show no significant change throughout the mating reaction in *C. reinhardtii*. Samples of the same gametes were also subjected to pH shock, which causes deflagellation and dramatic increases in IP_3 levels in vegetative cells (Quarmby et al., 1992). As shown in Table III, fivefold increases in IP_3 levels were stimulated by pH shock. Therefore, *C. reinhardtii* gametes are capable of IP_3 responses, but they are not elicited during mating. Consistent with this observation, gametes (like vegetative cells) are incapable of deflagellation in 10 μM neomycin, which blocks PIP_2 hydrolysis and IP_3 generation in vegetative cells (Quarmby et al., 1992); they nonetheless mate at control levels (>90%) in 10 μM neomycin.

Discussion

The Upstream Block

We have shown (Saito et al., 1993) that the elevation of cAMP during the *C. reinhardtii* mating reaction is the consequence of an adhesion-induced activation of adenylyl cyclase (and not, for example, an inhibition of phosphodiesterase). The reagents listed in Table I, with the exception of diltiazem, block the adhesion-induced cAMP increase (Fig. 1) without affecting the initial adhesion reaction. We document elsewhere that they exert no direct inhibitory effect on adenylyl cyclase activity in vitro (Saito et al., 1993). We conclude, therefore, that adhesion stimulates upstream events that lead to the activation of the cyclase, and that each inhibitor blocks one or more of these activating events.

Most of the reagents listed in Table I are known to affect Ca^{2+}-related responses (Low et al., 1979; Miller, 1987; Weiss and Levin, 1978), and in previous studies where they have been used to block *Chlamydomonas* flagellar tipping (Bloodgood, 1990; Bloodgood and Salomonsky, 1990), phototaxis (Schmidt and Eckert 1976; Nultsche, 1979; Nultsche et al., 1986; Dolle and Nultsche, 1988), or mating (Snell et al., 1982; Detmers and Condeelis, 1986; Schuring et al., 1990), the authors have speculated that facets of Ca^{2+} transport/utilization were perturbed, although studies describing nonspecific effects were also cited. We in fact consider it likely that Ca-binding proteins couple gametic adhesion to cyclase activation, and that local flagellar Ca^{2+} fluxes participate in this transduction (Pasquale and Goodenough, 1987; Saito et al., 1993; see below). However, the collective drug data presented here are most conservatively interpreted as demonstrating that the successful completion of upstream events depends on the native state of the cell membrane, and that perturbation of this state is inhibitory.

The inhibitors that most persuasively argue for a role in Ca^{2+} uptake are Cd^{2+} and La^{3+} because recent studies indicate that they are not permeant and block vertebrate voltage-sensitive Ca^{2+} channels by binding to the external face of the pore (c.f., Lansman et al., 1986). However, they may also exert such effects as neutralization of negative external surface charge and/or inhibition of other time- and voltage-dependent ionic currents (Nathan et al., 1988; Sanguinetti and Jurkiewicz, 1990). The fact that all of the reagents employed affect phosphate uptake (Fig. 2), a poorly understood process that is thought to depend on membrane polarization, surface potential, and the co-transport of various cations (Borst-Pauwels, 1981), speaks to the potential complexity of their physiological effects (see also Shih et al., 1988; Browning and Nelson, 1976; Bonini et al., 1986). Interestingly, Segal and Luck (1985) note that $^{32}P_i$ uptake is inhibited by 80% in low (10^{-7} M) external Ca^{2+}, suggesting that the drugs may somehow mimic a low-Ca^{2+} state.

We wish instead to stress that because an unperturbed membrane is necessary for upstream events, adhesion is likely to generate change(s) in the properties of the flagellar membrane. Its conductances, transmembrane potential, and/or surface potential may be affected, the three parameters of course being intimately related, the important point being that the flagellar cyclase would be activated as a consequence of these changes. One possibility is that the cyclase itself is a channel, as suggested by the recent work of Schultz et al. (1992) for the adenylyl cyclase of *Paramecium*. These authors report an inhibition of the enzyme, and its conductance, by K^+-channel blockers such as $CsCl_2$ and TEA. The same (mM) concentrations of these inhibitors have no effect on the *Chlamydomonas* mating reaction; therefore, if we are dealing with a channel-cyclase, it is probably a different protein. A second possibility is that the flagellar cyclase is directly activated by changes in ion availability and/or voltages; a third possibility is that intermediary protein(s), akin to G proteins or calmodulin in other systems (Ross and Gilman, 1980), are activated by such changes and in turn stimulate the cyclase. Our in vitro studies of the flagellar enzyme, reported elsewhere (Saito et al., 1993), in fact indicate that the third possibility is the most applicable.

Regardless of mechanism, the effects of the upstream inhibitors strengthen our conclusion (Pasquale and Goodenough, 1987) that cAMP is a primary signaling molecule in the *C. reinhardtii* mating reaction: their ability to block the adhesion-induced rise in cAMP (Fig. 1) accounts for their inhibition of mating since the exogenous application of

db-cAMP/IBMX restores, in most cases, full mating ability to drug-treated gametes.

The Downstream Calcium Release

Three sets of published observations suggest the occurrence of increased cytosolic Ca^{2+} during the *Chlamydomonas* mating reaction and hence a role for Ca^{2+} as a second messenger sensu metazoan fertilization (Azarnia and Chambers, 1976). Kaska and coworkers (1985) report that Ca^{2+} stored in cytoplasmic granules is released into the cytosol of mating cells. We have concerns about these data, however. While it is stated that the unmated gametes were stripped of their walls with GLE, wall removal may have been incomplete, in which case the process of air drying the samples may have ruptured the granules in the wall-free mating cells but left them intact in the walled unmated cells. Certainly, if cytosolic Ca^{2+} were elevated in vivo to the levels indicated by the STEM images, the cells would have deflagellated, and this is not the case (Kaska et al., 1985; Figs. 2 and 3).

The van den Ende lab (Schuring et al., 1990; 1991; Musgrave et al., 1992; van den Ende, 1992) finds that when *C. eugametos* gametes are treated with reagents that induce deflagellation (and hence elevate cytosolic IP_3 and presumably Ca^{2+} levels), the cells often respond by rupturing their apical cell walls and protruding balloons interpreted to be activated mating structures. Such cells, however, are also osmotically compromised—their contractile vacuoles are greatly impaired by the treatment—and it is not clear that they are viable. The Amsterdam group also detects an elevation in IP_3 levels after 15 min of mating, when some of the cells are fusing, and interprets this as stimulating an increase in cytosolic Ca^{2+} necessary for actin polymerization (Musgrave et al., 1992). The *C. reinhardtii* mating reaction also entails actin polymerization (Detmers et al., 1983); however, we find no change in IP_3 levels (Table III), nor is there any evidence for an increase in cytosolic Ca^{2+} (Figs. 8 and 9). Therefore, *C. reinhardtii* and *C. eugametos* may differ in this regard.

Finally, Bloodgood and Levin (1983) report a transient efflux of $^{45}Ca^{2+}$ accompanying the *C. reinhardtii* mating reaction, and speculate that this efflux results, at least in part, from a transient increase in cytosolic free Ca^{2+} which might trigger such events as cell-wall lysis and mating-structure activation. We confirm, by monitoring the absorbance change of a Ca^{2+}-sensitive dye, that levels of extracellular Ca^{2+} indeed increase during mating, but our data do not support the interpretation of Bloodgood and Levin. Instead, we find that the increased levels of external Ca^{2+}, triggered by adhesion (Fig. 5) and fully elicited by db-cAMP/IBMX in the absence of adhesion (Fig. 6), occur without obvious change in cytosolic free Ca^{2+}.

That the Ca^{2+} does not derive from a cytosolic spillover can also be argued a priori. The increase in extracellular Ca^{2+} is typically ~4–5 μM, as measured independently by the indicator dye and by atomic emission spectroscopy. This is effected by gametes at 2×10^7 cells/ml, yielding a net contribution of 2×10^8 ions per cell. Given a cell diameter of 10 μm, a calculated cell volume of 5×10^{-13} l, and assuming a resting level of free cytosolic Ca^{2+} of 10^{-7} M, each unmated gamete would be expected to contain only 3×10^3 free Ca^{2+} ions. Assuming that this resting level is maintained during mating, the spillover hypothesis requires that the additional 2×10^8 Ca^{2+} ions would need to move through the cytoplasm of each cell from intracellular stores. One would imagine that this flux would induce deflagellation, or backwards swimming, neither of which occurs during the mating reaction or in response to exogenous cAMP.

Therefore, we propose that the Ca^{2+} released into the medium during the mating reaction derives either from the direct exocytosis of Ca^{2+}, perhaps from the Ca^{2+}-sequestering vesicles visualized by Kaska et al. (1985) and in the related alga *Tetraselmis subcordiformis* by Salisbury (1982), and/or from a release of Ca^{2+} bound to membrane surfaces (Ca^{2+} associated with the cell wall is not an important contributor, as shown in Fig. 6 and by Bloodgood and Levin, 1983). Whether or not this release has any purpose in the mating reaction (e.g., in the activation of GLE, c.f., Snell et al., 1989), or whether it is an indirect manifestation of a critical event (e.g., a change in net surface charge), remains to be determined.

Signal Transduction in Chlamydomonas

Chlamydomonas is both a cell and an organism. Its signal-transduction pathways must therefore be designed such that a variety of complex organismal behaviors can be carried out without ambiguity. In the case of an ion like Ca^{2+}, there are two ways to maximize its spectrum of effectiveness: (*a*) different behaviors can be activated/suppressed by different concentrations of Ca^{2+}; (*b*) a given activating/suppressing Ca^{2+} concentration can be achieved locally, in one cellular compartment, such that other Ca^{2+}-sensitive systems never detect the fluctuation (c.f., Lechleiter et al., 1991; Allbritton et al., 1992).

Applying this reasoning to the mating reaction, we imagine that a strong Ca^{2+}-buffering protein, such as calmodulin or centrin, sequesters a flagellar-localized Ca^{2+} flux that attends adhesion. Therefore, the rest of the cell never sees this Ca^{2+} and hence never responds by phototaxis, backwards swimming, or deflagellation. The phototactic and photophobic swimming responses are also likely to be localized (Kamiya and Witman, 1984; Schmidt and Eckert, 1976; Hyams and Borisy, 1978), while a far more global Ca^{2+} increase appears to attend the deflagellation event. Snell et al. (1982) have aptly pointed out that gametes do not display mating responses every time they are stimulated by light. To the extent that *Chlamydomonas* cells have both localized their Ca^{2+} fluxes and designed different thresholds of Ca^{2+} sensitivity, a large range of behaviors can be governed by this single ionic species.

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