Transient Increase in Calcium Efflux Accompanies Fertilization in *Chlamydomonas*

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ABSTRACT Mating in *Chlamydomonas* is a complex process initiated by contact of gametic flagellar surfaces, resulting in transmission of a signal from the flagella to the cell bodies. This signal triggers later events of cell wall loss, mating structure activation, and cell-cell fusion. Little is known about the nature of the signal or the role of Ca in these events. It was found that extracellular Ca is not necessary for successful mating in *Chlamydomonas*. However, cells will take up Ca from the medium in a linear manner for many hours and will accumulate micromolar concentrations, presumably by sequestering Ca within intracellular storage sites. If gametic cells of one mating type (preloaded with ^{45}Ca) are mated with gametes of the opposite mating type (preloaded with unlabeled calcium), there is a rapid, transient increase in calcium efflux rate (20 times that of the control) that lasts ~6 min. This effect is not associated with cell-cell fusion, since the same observation is made if (+) gametes preloaded with 45-Ca are agglutinated by isolated flagella from (−) gametes preloaded with unlabeled Ca. Other experiments have shown that the increased efflux rate is not a simple consequence of cell wall release. Ca efflux in unmated gametes is greatly reduced in deflagellated cells, suggesting that much of the Ca movement is associated with the flagellar membrane. Although signaling itself may involve Ca fluxes across the flagellar membrane, it is also possible that a consequence of signaling is release of Ca from intracellular storage sites (perhaps functional equivalents of the sarcoplasmic reticulum). The observed transient increase in Ca efflux rate may reflect a transient increase in the cytoplasmic free-Ca concentration. This increase in cytoplasmic Ca may regulate the later events in mating (such as cell wall release and mating structure activation).

Ca has been frequently implicated in the early events of fertilization, particularly in marine eggs (see references 13, 25, 58, 68 for reviews). In these systems, external Ca is generally necessary for sperm activation, but is not necessary for fertilization of eggs by previously acrosome-reacted sperm (55, 56). Fertilization results in a transient increase in free cytoplasmic Ca, presumably released from some intracellular membrane-bounded compartment. The increase in cytoplasmic free-Ca concentration induces the cortical granule reaction, which results in formation of the fertilization membrane from the vitelline envelope.

Mating events in *Chlamydomonas* have been the subject of intense study (17, 19, 22). *Chlamydomonas* is an isogamous green alga in which the gametes of the two different mating types are of equal size and of almost identical structure. Gametes of opposite mating types interact by their flagellar surfaces by means of species-, gamete-, and mating type (mt)-specific agglutinin molecules (1, 39); this reaction has been intensively studied as a model system for specific cell-cell interactions. Flagellar membrane contact is followed by reorientation of the flagella (6, 32, 36), flagellar tip activation (38), transmission of a signal from the flagella to the cell bodies (14, 64), loss of cell walls due to release of a specific lysin (53, 60, 65), activation of a specific mating structure on each partner (20, 21), cell-cell fusion (67), and de-adhesion of the flagellar surfaces (61, 62). This carefully programmed sequence of events can be completed in under 5 min, resulting in a heterokaryon that will later become a zygote after flagellar resorption and nuclear fusion.

Little is known about the role of Ca during fertilization in *Chlamydomonas*. Flagellar surface motility is known to be Ca-dependent (7). Flagellar surface motility, cell-wall release, and cell fusion in *Chlamydomonas* are all inhibited by similar concentrations of lidocaine (xylocaine) (63) and trifluoperazine (11). Since lidocaine is known to affect the interaction of calcium with membranes (33, 57) and to affect capping in

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lymphocytes and neutrophils (8, 49), the observations of Snell et al. (63) suggest that calcium may be involved in flagellar surface motility and in signaling events in *Chlamydomonas* mating. Since calmodulin, a Ca-binding regulatory protein, is known to be present in the flagella and cell bodies of *Chlamydomonas* (16) and since trifluoperazine is known to inhibit calmodulin function in at least some systems, the observations of Detmers and Condeelis (11) suggest that Ca and calmodulin may regulate both flagellar surface motility and the early events of mating. Both of these sets of observations suggest that flagellar surface motility is intimately involved in the early events of mating, something that has been previously suggested by several laboratories (6, 7, 22, 23, 32). One of the immediate consequences of signaling is cell-wall release. Claes (9) reported that treatment of vegetative or gametic cells with the divalent cation ionophore A23187 in the presence of external Ca brought about cell wall loss, but only in flagellated cells.

For our study we used gametic cells preloaded with ^{45}Ca to determine whether there is an adhesion-dependent change in the rate of efflux of Ca.

MATERIALS AND METHODS

Cell Strains and Culture Conditions: *Chlamydomonas reinhardii* wild-type strains NO+ (mt+) and NO− (mt−) were used for all experiments. Vegetative cells were grown synchronously at 22°C in Medium I of Sager and Granick (50) using an alternating cycle of 14 h light and 10 h dark. For induction of gametogenesis, vegetatively grown cells were washed into nitrogen-free growth medium and exposed to continuous light for 15 h. Mating was induced by mixing equal numbers of mating type plus (mt+) and mating type minus (mt−) cells together in nitrogen-free growth medium (51), in 10 mM Tris-HCl, pH 7.4, or one of the other buffers described in Table I. To quantitate mating efficiency, an aliquot of cells was mixed with an equal volume of 2% glutaraldehyde in 10 mM phosphate buffer, pH 7.0, at various times after mixing of the mt+ and mt− gametes. The relative number of biflagellate (BF) and quadriflagellate (QF) cells was determined with a phase-contrast microscope and the mating efficiency (%) was calculated as: % of gametes forming zygotes = 2 QFs/2 QFs + BFs × 100.

Ca Uptake Measurements: Gametes (at a concentration of 10^6–10^7 cells/ml) were incubated at room temperature in 10 mM Tris-HCl, pH 7.4, with 10 μCi/ml of ^{45}Ca chloride (New England Nuclear, Boston, MA). This represented a concentration of 2.1–5.6 μM Ca, depending on the specific activity of the particular batch of ^{45}Ca (varied from 12.9–35.6 mCi/mg). At various time intervals, duplicate samples of cells (0.4 ml) were layered on 1 ml of 12.5% sucrose in 10 mM Tris-HCl, pH 7.4, in 1.5-ml plastic centrifuge tubes and centrifuged for 2 min at 15,600 *g* in the Eppendorf Model 5412 centrifuge. The tubes were removed and frozen in lipid nitrogen. The tips of the plastic tubes, with the cell pellets, were cut off with a Foredom Model 73B dental drill (Foredom Electric Co., Bethel, CT) equipped with a cutting wheel, and the tips were incubated overnight at room temperature in 1.0 ml of Protosol (New England Nuclear). 10 ml of Aquasol 2 (New England Nuclear) were then added to each vial and the vials were incubated in the dark for several hours before counting in a Beckman LS8000 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, CA). In some cases, 100 μl of glacial acetic acid were added to each vial to eliminate chemiluminescence. The amount of uptake was calculated using cell number, estimated cell volume and specific activity of the particular batch of ^{45}Ca. Cell volume was based on average measurements of the long and short axes of 100 cell bodies along with flagellar lengths determined with an ocular micrometer and a phase contrast microscope; the flagellar diameter is known from electron microscopy. Average cell volume was estimated at 110 μm^3 while average cell surface area was 140 μm^2. The flagella on each cell contribute ~1.5% of the total cell volume and 17% of the total surface area.

Ca Efflux Measurements: Gametes of one mating type were preloaded with 10 μCi/ml ^{45}Ca (2.1–5.6 μM total Ca concentration) in 10 mM Tris-HCl, pH 7.4, for 120 min as described above while the opposite mating type was incubated for the same period of time in 10 mM Tris-HCl, pH 7.4, containing 1 mM unlabeled calcium chloride. Both mating types were then washed with 10 mM Tris-HCl and the cell densities adjusted to be the same (~4–5 × 10^6/ml). A baseline of ^{45}Ca efflux was measured for 30 min using only the labeled gametic cells; then an equal volume of the opposite mating type cells, which had been preincubated in unlabeled Ca, was added. The control involved addition of an equal volume (and equal number) of unlabeled gametes of the same mating type as the labeled gametes. The cells were kept in intense fluorescent light and constantly stirred on a magnetic mixer. Cells were monitored at 15-min intervals for cell health and mating efficiency; no results were used from any experiment that achieved <80% mating efficiency. At various intervals before and after the initiation of mating, duplicate 0.5-ml samples were put in 1.5-ml plastic centrifuge tubes and centrifuged for 1 min at 15,600 *g* in the Eppendorf Model 5412 centrifuge. 0.2 ml of each supernatant was carefully removed and added to 10 ml of Aquasol 2, and the samples were counted in a Beckman LS8000 liquid scintillation counter (Beckman Instruments, Inc.)

For the efflux studies involving agglutination of gametes of one mating type by isolated gametic flagella from the opposite mating type, one 8-liter culture of each mating type was grown and the cells were induced to become gametes as described above. Flagella were isolated from the mt+ and mt− gametes by the pH stock method of Witman et al. (70). Intact mt+ gametes were incubated in ^{45}Ca for 2 h in 10 mM Tris-HCl, pH 7.4, washed in the same buffer without calcium, and then incubated with either mt+ or mt− flagella that had been incubated in 10 mM Tris-HCl, pH 7.4, containing 1 mM unlabeled calcium. The number ratio of isolated flagella to whole gametes was 9:1. Agglutination of mt+ gametic cells occurred only in the presence of the mt− flagella. Bovine serum albumin (0.25%) was included in the reaction mixture to minimize sticking of the isolated flagella to the walls of the glass beaker. At various time points before and after addition of the gametic flagella, ^{45}Ca efflux from the whole gametic cells was measured as described above.

For studies of efflux in deflagellated, unmated gametes, mt+ gametes were preloaded with ^{45}Ca as described above and then washed into 10 mM Tris-HCl, pH 7.4, containing 4 mM colchicine (Sigma Chemical Co., St. Louis, MO). Half of these cells were deflagellated by the pH shock method of Witman et al. (70). ^{45}Ca efflux was then followed in both populations of cells as described above.

To look at the effect of wall release on ^{45}Ca efflux from unmated mt+ gametes, the specific *C. reinhardii* lysin was obtained by the method of Snell (60). The supernatant (containing lysin) obtained from a mating mixture of highly concentrated mt+ and mt− gametes in 10 mM Tris-HCl, pH 7.4, was used without dilution or concentration. Mt+ gametes were incubated with ^{45}Ca in Tris-HCl, pH 7.4, washed in the same buffer without Ca, and then divided into two aliquots. One was pelleted and resuspended in 10 mM Tris-HCl while the other was pelleted and resuspended in the full strength lysin preparation. At various times, samples were taken for measurement of ^{45}Ca efflux. Cell-wall loss was quantitated by the spectrophotometric method of Snell (60).

General Data Analysis: All data points in Figs. 1–6 represent the average of two determinations and all of the straight lines shown are fitted by the method of least squares (linear regression); correlation coefficients were in all cases in excess of 0.95.

RESULTS

The presence of free Ca ions in the external medium is not necessary for successful completion of mating in *C. reinhardii*. Table I shows that the mating efficiency in a variety of media is only slightly decreased by the absence of external Ca. Therefore, if Ca is obligatorily involved in any of the steps in mating through the quadriflagellate state (postcell fusion), the source of such Ca must be within the gametic cells themselves.

TABLE I

Effect of Extracellular Calcium on Mating Efficiency

Medium	Mating efficiency*
	%
Gametogenesis medium, pH 6.7‡	99.5
Gametogenesis medium, pH 6.7, + 5 mM EGTA‡	96.4
10 mM Tris-HCl, pH 7.4, + 1 mM $CaCl_2$	99.0
10 mM Tris-HCl, pH 7.4	89.8
10 mM Tris-HCl, pH 7.4, + 5 mM EGTA	75.4
10 mM HEPES, pH 7.8, + 1 mM $CaCl_2$	98.7
10 mM HEPES, pH 7.8, + 5 mM EGTA	89.9

* Mating efficiency determined 15 min after mixing mt+ and mt− gametes.
‡ Nitrogen-free medium developed by Sager and Granick (51) for inducing gametogenesis. Contains 0.36 mM $CaCl_2$.

When mt+ gametic cells are incubated in 10 μCi/ml of ^{45}Ca (giving a concentration of 2.1–5.6 μM Ca, depending on the specific activity of the particular batch of ^{45}Ca used) in 10 mM Tris-HCl, pH 7.4, in the absence of unlabeled Ca, the cells take up ^{45}Ca from the medium in a linear manner for a minimum of 9 h (Fig. 1). For all subsequent experiments, gametic cells were used that had been preloaded with ^{45}Ca for 2 h. Using the data shown in Fig. 1, it is calculated that the intracellular concentration of labeled Ca (if it were uniformly distributed through the entire volume of the cell) is 0.66 mM at 2 h and 2.0 mM at 9 h in addition to whatever unlabeled Ca was already in the cell. This indicates that the gametes have the ability to concentrate Ca as much as 1,000 times over the concentration in the medium. Since these concentrations of Ca are far higher than the typical physiological concentrations of cytoplasmic free Ca (usually reported in the range of 10^{-8}–10^{-5} M), it is likely that the gametes are sequestering much of the Ca that is being taken up. Results similar to those shown in Fig. 1 for mt+ gametes have been observed for uptake of ^{45}Ca by mt− gametes.

Fig. 2 shows the results from a typical experiment in which the mt− gametes preloaded with ^{45}Ca were mixed with either mt+ or mt− gametes that were preloaded with unlabeled Ca chloride. In the case where successful mating occurred (the mt+/mt− mixture), there was observed a transient, rapid increase in the rate of efflux of ^{45}Ca; the efflux rate returned to control level within 6 min after the initiation of mating. The Ca efflux rate under the mating conditions (mt+ gametes/mt− gametes) reached a level that was at least 20 times the rate observed in the control, nonmating situation (mt+ gametes/mt+ gametes). Had it been possible to obtain data points sooner than 6 min after mixing of the mt+ and mt− gametes, the maximum rate of Ca efflux might have been observed to be much greater. In this experiment, the mating efficiency reached 82% by 35 min. Subjectively, there was a correlation between the efficiency of mating in a particular batch of cells and the extent of the transient increase in efflux rate observed. Similar results were obtained when the experiment was performed using mt− gametes that were preloaded for 90 min with ^{45}Ca and mixed with mt+ gametes that were preincubated with unlabeled Ca.

During the interval of increased efflux of Ca shown in Fig. 2, a number of mating-associated events are occurring: flagella-flagella adhesion, reorientation of flagella, flagellar tip activation, flagellar signaling, cell-wall release mediated by a specific lysin, mating structure activation, and cell-cell fusion (17). With the limited temporal resolution of the assay used to measure Ca efflux and the rapidity with which this sequence of events occurs, it is not possible at the present time to relate the increased Ca flux to one or another of these phenomena. However, since the process of cell-cell fusion can be uncoupled from the earlier events of mating by interacting isolated flagella from gametes of one mating type with whole gametes of the opposite mating type (27, 37, 59, 62), it is possible to ask whether cell-cell fusion is necessary to observe the transient increase in Ca efflux. Fig. 3 shows an experiment in which Ca efflux was measured after mixing mt+ whole gametes (preloaded with ^{45}Ca) with flagella isolated from either mt+ or mt− gametes (preincubated with unlabeled Ca). The mt+ gametic cell/mt− gametic flagella mixture (with a flagella: gamete number ratio of 9:1) resulted in a transient increase in the rate of efflux of Ca relative to the control situation (mt+ gametic cells/mt+ gametic flagella mixture) that was very similar to the result obtained using gamete-gamete mating (Fig. 2). Phase-contrast microscopic observations indicated that the mt− gametic flagella agglutinated the mt+ gametic cells by their flagellar surfaces; no agglutination was observed in the mixture of mt+ gametic cells with mt+ gametic flagella. The maximum rate of Ca efflux in the experimental case (mt+ gametic cells/mt− gametic flagella)

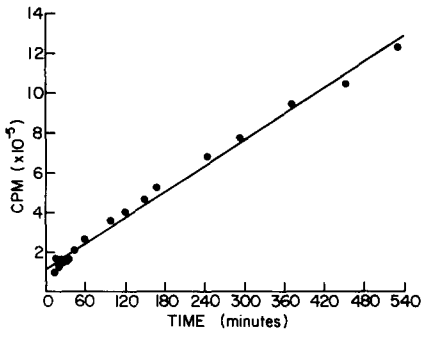

FIGURE 1 Uptake of ^{45}Ca by mt+ gametes. 6 × 10^6 cells/ml were incubated in 10 mM Tris-HCl, pH 7.4, containing 10 μCi/ml ^{45}Ca (sp act of 12.9 mCi/mg) giving a Ca concentration of 2.05 μM. At various times, duplicate 0.4-ml samples (each containing 2.4 × 10^6 cells) were layered on 12.5% sucrose, centrifuged, and the amount of radioactivity in the pellets was measured. At 2 h the intracellular concentration of ^{45}Ca (assuming uniform distribution throughout the entire volume of the cell) was 0.66 mM and at 9 h it was 2.0 mM. The latter figure represents a 1,000-fold concentration of ^{45}Ca over the concentration in the medium.

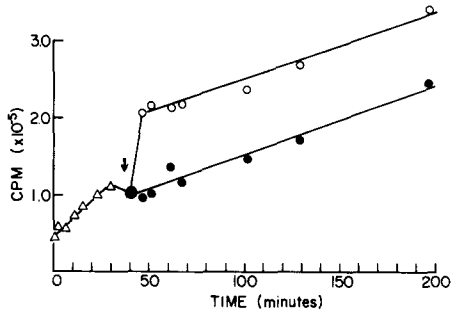

FIGURE 2 Efflux of ^{45}Ca from gametes under mating and nonmating conditions. Mt+ gametes were incubated in 10 μCi/ml ^{45}Ca for 2 h and then washed with 10 mM Tris-HCl, pH 7.4. At time zero, the labeled cells were resuspended in this same buffer at a concentration of 5 × 10^6 cells/ml. At various times after that, duplicate 0.5-ml aliquots of cells were centrifuged and 0.2 ml of each supernatant was removed and the radioactivity measured. The ordinate represents CPM/0.2 ml. Efflux from labeled mt+ gametes alone is shown by triangles. The arrow indicates the time (37.5 min) at which an equal number of unlabeled mt− gametes were added to half of the labeled mt+ gametes (open circles) while an equal number of unlabeled mt+ gametes were added to the other half of the labeled mt+ gametes (closed circles). The slope (efflux rate) for the labeled mt+ alone is 11,500 CPM/ml/min. During the first 6 min after addition of unlabeled gametes, the mt+/mt− mixture has an efflux rate (92,300 CPM/ml/min) 20 times that of the mt+/mt+ mixture (4,500 CPM/ml/min). After the first 6 min, the rate of efflux of mt+/mt− mixture returns to a value (4,300 CPM/ml/min) similar to that of the mt+/mt+ mixture (4,500 CPM/ml/min). The 2.5-fold reduction in efflux rate after the labeled mt+ cells were mixed with the unlabeled mt+ cells is due primarily to dilution. At 35 min after mixing, the mating efficiency of the mt+/mt− mixture was 82% and the mating efficiency of the mt+/mt+ mixture was 0%. At the time of mixing (37.5 min), the extracellular Ca concentration was 0.2 μM; in the mt+/mt− mixture, this concentration rose to 0.4 μm within 6 min after mixing.

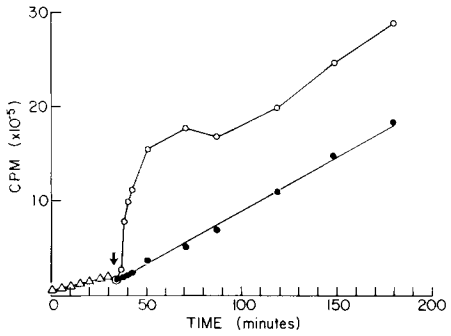

FIGURE 3 Efflux of ^{45}Ca from mt+ gametes incubated with isolated flagella obtained from either mt+ or mt− gametes. Mt+ gametes were incubated in 10 μCi/ml of ^{45}calcium for 2 h and then washed into 10 mM Tris-HCl, pH 7.4, at time zero. At various times after that, duplicate 0.5-ml aliquots of the mixture were centrifuged and 0.2 ml of the supernatants was removed and the radioactivity measured. Efflux from labeled mt+ gametes alone is shown by the triangles. The arrow indicates the time (33.8 min) at which mt+ (solid circles) or mt− (open circles) gametic flagella were added to the mt+ gametes in a number ratio of 9.2 and 9.7 flagella/cell, respectively. The flagella were preincubated for 2 h in 1 mM unlabeled Ca chloride in 10 mM Tris-HCl, pH 7.4, and 0.25% bovine serum albumin was included in the cell-flagella mixture in order to minimize sticking of the isolated flagella to the glass surfaces of the beaker. The ordinate represents CPM/0.2 ml. The slope (efflux rate) for the labeled mt+ gametes alone is 25,000 CPM/ml/min. During the first 5 min after addition of isolated flagella, the mt+ gametes with the mt− flagella exhibited an efflux rate (1,440,000 CPM/ml/min) 25 times that of the mt+ gametes mixed with the mt+ flagella (56,600 CPM/ml/min). At ~18 min after addition of the flagella, the rate of efflux of ^{45}Ca from the mt+ gametes mixed with mt+ flagella returned to a value (50,850 CPM/ml/min) very similar to that of the mt+ gametes mixed with mt+ flagella (56,600 CPM/ml/min). Significant amounts of clumping of the mt+ gametes by the mt− flagella was observed at all time points after the addition of the flagella (maximum time observed was 160 min after addition of flagella). No agglutination was observed at any time in the mixture of mt+ gametes and mt+ flagella.

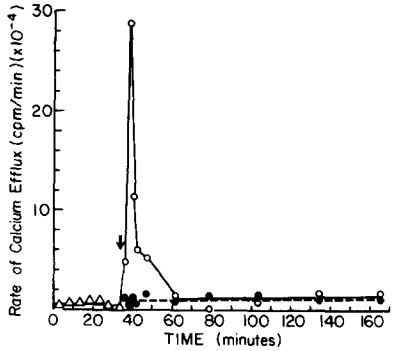

FIGURE 4 A replotting of the same data shown in Fig. 3 as the first derivative in order to show the rate of calcium efflux (CPM/0.2 ml/min) as a function of time. The symbols are the same as those used in Fig. 3.

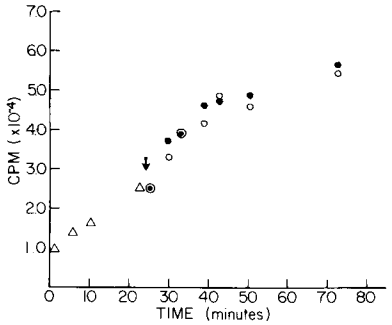

FIGURE 5 Effect of cell wall removal on the efflux of ^{45}Ca from mt+ gametic cells. Cells were preincubated in 10 μCi/ml of ^{45}Ca for 2 h and washed into buffer at time zero. At various times afterwards, 0.5-ml aliquots were centrifuged and 0.2 ml of each supernatant was removed and the radioactivity measured. Triangles indicate initial of ^{45}Ca efflux. The arrow indicates the time (24 min) at which the cells were divided in half and centrifuged. One-half was resuspended in 10 mM Tris-HCl, pH 7.4, (closed circles) while the other half was resuspended in an equal volume of the same buffer containing the specific lysin that removes the cell walls (open circles).

during the first 5 min after mixing was 25 times that of the control (nonmating) situation (mt+ gametic cells/mt+ gametic flagella). Using the same data as the experiment in Fig. 3, the change in the rate of efflux with time is graphically presented in Fig. 4. This figure clearly shows that most of the transient rise in Ca efflux occurs during the first 8 min after mixing the gametic cells of one mating type (mt+) with the isolated flagella from the opposite mating type cells (mt−), although the efflux rate does not completely return to the control level until ~30 min after mixing. This probably reflects, at least in part, the asynchrony of the cell-flagella interaction system, since the Ca efflux rate returned to control level within 6 min after initiation of normal gamete-gamete mating (Fig. 2). The results shown in Figs. 3 and 4 clearly demonstrate that the transient increase in Ca efflux rate is not related to cell-cell fusion.

One possible explanation of the mating-dependent increase in Ca efflux is that the release of the cell walls from both mating types that occurs during normal mating removes a permeability barrier to Ca efflux. This explanation is, of course, not consistent with the observation that the increased rate of efflux of Ca is transient and rapidly returns to the control rate (Figs. 2, 3, and 4). However, a direct test of this possibility is shown in Fig. 5, where the rate of Ca efflux from unmated mt+ gametes was measured in the presence and the absence of the cell walls. A single batch of mt+ gametes was preloaded with ^{45}Ca, then the cells were pelleted; one half of the cells was resuspended in 10 mM Tris-HCl, pH 7.4, and the other half was resuspended in the same buffer containing the specific lysin that removes the cell walls (53, 60). Cell-wall release was verified using the technique of Snell (60). Since there was no significant difference in the Ca efflux rate for cells with or without cell walls (Fig. 5), it is unlikely that the cell wall serves as a barrier to the movement of Ca or that the transient increase in Ca efflux rate during mating is a result of cell-wall release. A related possibility is that a large pool of ^{45}Ca is associated with the cell-wall and/or the periplasmic space between the cell wall and the plasma membrane; the act of cell-wall release during mating would then dump this ^{45}Ca into the medium resulting in an observed transient increase in the rate of appearance of ^{45}Ca in the medium after mating. This possibility is also ruled out by the data shown in Fig. 5.

The method used in this study for measuring Ca efflux does not distinguish between movement of Ca across the flagellar membrane and the rest of the cell-surface plasma membrane. Calculations, based on measurements of cell diameter, flagellar diameter, and flagellar length, indicate that the two flagella represent 1.5% of the total cell volume and 16.7% of the total surface area of the cell. To get an indication of the relative

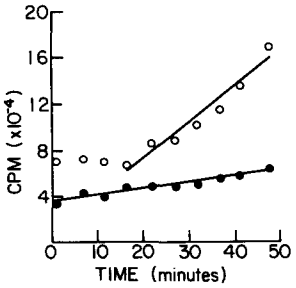

FIGURE 6 Rate of ^{45}Ca efflux from flagellated (open circles) and deflagellated (closed circles) mt+ gametic cells. Gametes were prelabeled for 2 h in 10 μCi/ml ^{45}Ca and washed into 10 mM Tris-HCl, pH 7.4, containing 4 mM colchicine. Half of the cells was deflagellated by pH shock at time zero. At various times after returning the pH to 7.4, duplicate 0.5-ml aliquots were centrifuged and the radioactivity measured in 0.2 ml of each supernatant; both the flagella and the cell bodies were pelleted during the one-min spin. The slope (rate of Ca efflux; 15,500 CPM/ml/min) for the flagellated cells (open circles) was 5.6 times greater than the slope (rate of Ca efflux; 2,770 CPM/ml/min) for the deflagellated cells (closed circles). The lag seen in the nondeflagellated cells before significant Ca efflux occurred was variable in length and not always present.

contribution of the flagellar membrane to total Ca efflux, mt+ cells were preloaded with ^{45}Ca and then half the cells was deflagellated by pH shock while the other half was not. Both populations of cells were treated with 4 mM colchicine; this was observed to totally prevent flagellar regeneration in the deflagellated population of cells while having no effects on flagellar length or whole cell motility in the nondeflagellated population of cells. Fig. 6 shows the results from one such experiment comparing efflux from flagellated versus deflagellated gametes under nonmating conditions. This type of experiment does not compare directly with the mating situation, but such a directly comparable experiment is not possible because the presence of the flagella is obligatory for the initial events in normal mating. The rate of efflux of ^{45}Ca from the flagellated cells in this experiment was 5.6 times that of the deflagellated cells. Although there was considerable variability noted in multiple repetitions of this experiment, the results generally showed the rate of efflux of the flagellated cells to be at least three times that of the deflagellated cells. Assuming that the pH shock procedure itself is not inhibiting Ca efflux across the general cell-surface membrane and considering the small percentage of the surface area of the cell contributed by the flagellar membrane, Fig. 6 suggests that the flagellar membrane is preferentially involved in the Ca efflux observed using gametic cells preloaded with ^{45}Ca.

Attempts have been made to use a commerical Ca-specific electrode (Orion Research Inc., Cambridge, MA) to confirm the observations on Ca fluxes made using ^{45}Ca. However, it turns out that, under the conditions used in these experiments, the extracellular Ca concentration does not rise above 0.5 μM and the Ca-specific electrode has very poor sensitivity at this Ca concentration.

DISCUSSION

Ca is involved in the regulation of many cellular events through its interaction with and transport across biological membranes. Such Ca fluxes (both passive and active) can produce Ca action potentials and can result in changes in the free-Ca concentration in cellular compartments. Ca movements have been shown to be associated with events of early development, particularly fertilization (13, 25, 58, 68). In the best studied systems (fertilization in sea urchins and other invertebrates), Ca has been demonstrated to be involved in both sperm activation (acrosomal reaction) and egg activation (particularly cortical granule release). Extracellular Ca is necessary for the sperm acrosome reaction in echinoderm (10) and mammalian (71) sperm, and Ca influx during the acrosome reaction has been documented (28, 52) using ^{45}Ca.

In invertebrate eggs, there have also been reports of increased uptake of Ca at fertilization, using ^{45}Ca as a tracer (2, 26, 40, 45). These observations are somewhat difficult to interpret since it has now been clearly shown that external free Ca is not necessary for fertilization or activation of most eggs (55, 56). There are also several reports of increased rate of Ca efflux during fertilization (2, 3, 26). These authors interpret this as a reflection of a fertilization-dependent transient rise in free intracellular cytoplasmic Ca. Intracellular release of Ca during fertilization was first reported by Mazia (35) and has since been carefully documented using the Ca-sensitive photoprotein, aequorin (15, 48, 66, 72). Numerous studies (see references 58, 68 for reviews) using isolated egg cortices, partially lysed eggs, injection of aequorin, injection of EGTA, or treatment with the ionophore A23187 have shown that cortical granule release, an early event in fertilization, is a direct consequence of a rise in cytoplasmic free-Ca concentration.

Ca has been implicated in a number of flagella-associated processes in *Chlamydomonas*. Flagellar elongation and shortening can be regulated by the free-Ca concentration in the medium (30, 46). Calcium has been demonstrated to affect the waveform of the flagellum (5, 24, 41) and the swimming speed of the cell (4, 44, 47). Phototactic swimming responses in *Chlamydomonas* are also dependent upon Ca (4, 42, 54).

Here we report the first study of Ca fluxes during fertilization of *Chlamydomonas* and one of the first results that suggests Ca involvement in the events of mating in this organism. Claes (9) reported that the divalent cation ionophore A23187 (only in the presence of extracellular Ca) induced release of lysin and cell-wall degradation in unmated gametic cells. This effect required the presence of the flagella, suggesting that the Ca was acting via the flagella (signal generation?) rather than directly on the processes of lysin release or wall release. These results were confirmed by Goodenough (18) in abstract form only, but other laboratories have been unable to obtain any wall release in the presence of A23187 and Ca (D. Kaska and A. Gibor, personal communication; M. Buchanan and W. Snell, personal communication). Snell et al. (63) showed that the anesthetic lidocaine (xylocaine) inhibited wall loss and zygote formation but not flagellar adhesion and flagellar tip activation. Since the lidocaine effect was antagonized by Ca and since lidocaine is known to interfere with divalent cation movement across membranes and to release membrane-bound Ca (33, 57), Snell et al. (63) interpreted their lidocaine observations as suggesting a role for Ca in the flagellar signaling mechanism. Detmers and Condeelis (11) reported that trifluoperazine allowed normal flagellar agglutination but interfered with flagellar tip activation, cell-wall release, and mating structure activation. Since trifluoperazine has been shown to bind specifically to calmodulin (31), these authors suggested that early events in *Chlamydomonas* mating may be regulated by the intracellular Ca concentration through the action of cal-

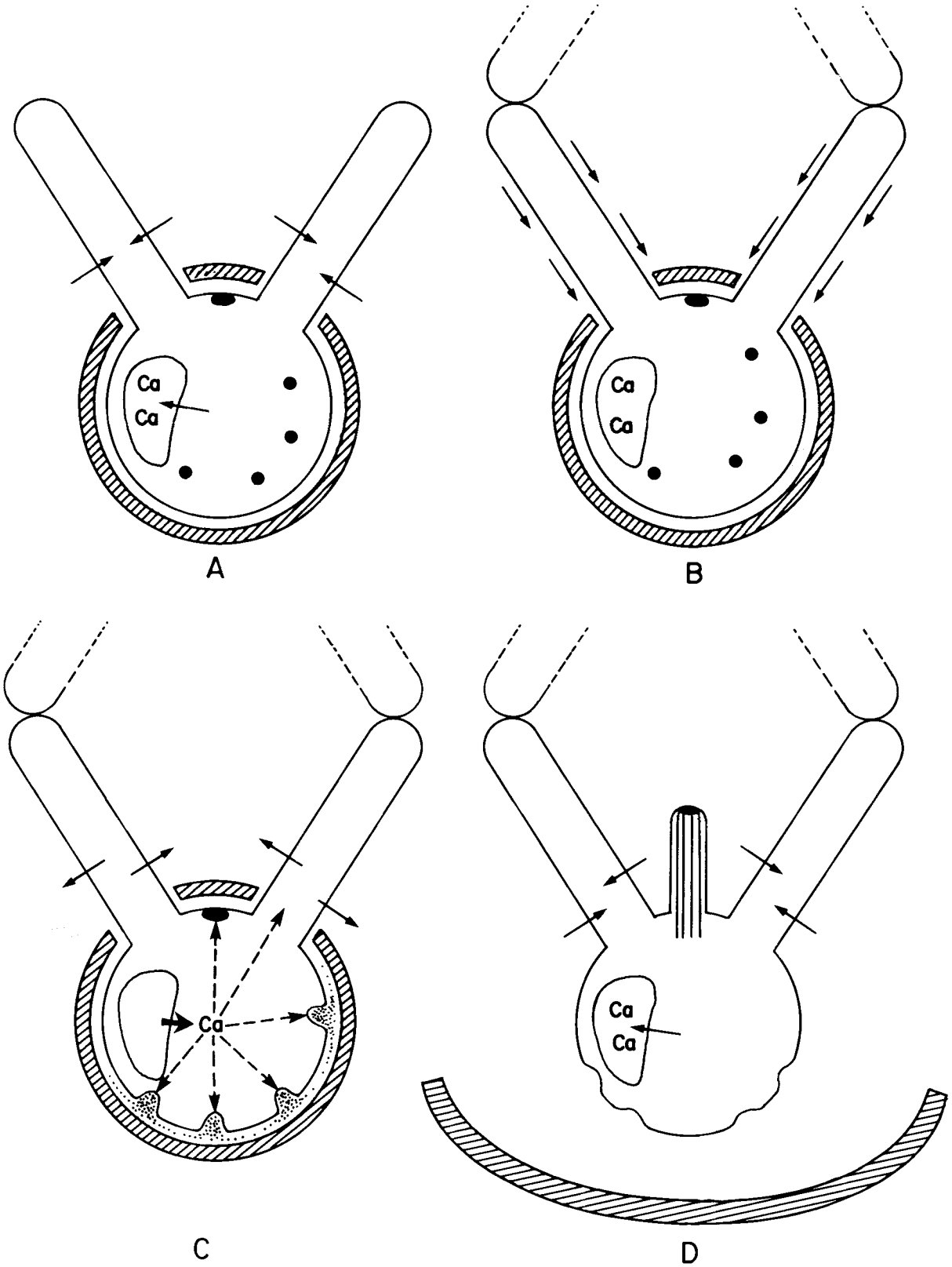

FIGURE 7 Model illustrating how an increase in cytoplasmic Ca concentration may be triggered by a flagellar signal and how this increase in Ca concentration may trigger later events in *Chlamydomonas* mating. (*A*) represents the situation in an unmated gamete which takes up and stores calcium in a cytoplasmic membrane-bounded compartment which may be considered equivalent to the sarcoplasmic reticulum. Upon flagellar adhesion occurring with a gamete of the opposite mating type (*B*), a signal is sent along the flagellar membranes triggering a transient increase in the Ca permeability of the membrane of the sarcoplasmic reticulum equivalent. This results (*C*) in a rapid flow of Ca out of the sarcoplasmic reticulum equivalent into the cytoplasm. The subsequent rise in cytoplasmic Ca concentration triggers later events of mating such as exocytosis of lysin storage granules and activation of the plasma-membrane associated mating structures. After these events have occurred (*D*), the Ca concentration of the cytoplasm is lowered by pumping Ca back into the sarcoplasmic reticulum equivalent.

modulin. Such an interpretation must be tempered with some caution, since trifluoperazine has also been shown to be a membrane-stabilizing agent that can thereby inhibit ionic fluxes (29). White and Raynor (69) have shown that trifluoperazine inhibits Ca transport across platelet microsomal membranes; they suggest but do not demonstrate that this effect is mediated through calmodulin.

Consistent with the literature on sea urchin fertilization (55, 56), the present study shows that extracellular free Ca is not necessary for any of the steps in *Chlamydomonas* mating (Table I), although *Chlamydomonas* can take up large amounts of Ca from the extracellular medium in a linear manner (Fig. 1). Recent experiments in this laboratory have looked for but found no evidence for a mating-dependent increase in the rate of influx of ^{45}Ca into *Chlamydomonas* gametes. The principal observation being reported here is the existence of a mating-dependent, transient increase in Ca efflux (Figs. 2–4). This transient Ca efflux is not associated with cell-cell fusion (Figs. 3 and 4) and is not a simple consequence of cell-wall release (Fig. 5). Deflagellation experiments suggest that much of this Ca efflux is associated with the flagellar membrane (Fig. 6). This is interesting since the voltage-sensitive Ca channels in *Paramecium* have been reported to reside entirely within ciliary membranes (12, 34, 43). The present study does not directly address the question of whether the transient increase in Ca efflux rate accompanying mating is due to changes in plasma membrane permeability or transport activity, to changes in free-Ca concentration in the cytoplasm or to a combination of these events. Our current working hypothesis is that the transient increase in Ca efflux rate results, at least in part, from a transient increase in the intracellular cytoplasmic concentration of free Ca resulting from release of Ca from an intracellular storage site, perhaps equivalent to the sarcoplasmic reticulum of striated muscle cells. This increased intracellular Ca concentration would then act to trigger one or more of the later events in the mating process (exocytosis of lysin-containing granules resulting in cell-wall release; mating structure activation; cell fusion). The previously described flagellar signal (14, 64) may be the trigger for release of Ca from the intracellular sarcoplasmic reticulum equivalent or it may be a consequence of increased Ca flux across the flagellar membrane. This scheme of events is diagrammatically portrayed in Fig. 7.

Results of the present study should serve as an impetus to explore the sites of Ca sequestration in *Chlamydomonas* gametes and to attempt to directly implicate Ca in the regulation of specific steps (such as signaling, lysin and wall release, mating structure activation, and cell fusion) in the mating process.

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