Two Distinct, Calcium-mediated, Signal Transduction Pathways Can Trigger Deflagellation in *Chlamydomonas reinhardtii*

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Abstract. The molecular machinery of deflagellation can be activated in detergent permeabilized *Chlamydomonas reinhardtii* by the addition of Ca^{2+} (Sanders, M. A., and J. L. Salisbury, 1989. *J. Cell Biol.* 108:1751–1760). This suggests that stimuli which induce deflagellation in living cells cause an increase in the intracellular concentration of Ca^{2+}, but this has never been demonstrated. In this paper we report that the wasp venom peptide, mastoparan, and the permeant organic acid, benzoate, activate two different signalling pathways to trigger deflagellation. We have characterized each pathway with respect to: (*a*) the requirement for extracellular Ca^{2+}; (*b*) sensitivity to Ca^{2+} channel blockers; and (*c*) ^{45}Ca influx. We also report that a new mutant strain of *C. reinhardtii*, *adf-1*, is specifically defective in the acid-activated signalling pathway. Both signalling pathways appear normal in another mutant, *fa-1*, that is defective in the machinery of deflagellation (Lewin, R. and C. Burrascano. 1983. *Experientia*. 39:1397–1398; Sanders, M. A., and J. L. Salisbury. 1989. *J. Cell Biol.* 108:1751–1760). We conclude that mastoparan induces the release of an intracellular pool of Ca^{2+} whereas acid induces an influx of extracellular Ca^{2+} to activate the machinery of deflagellation.

Deflagellation is a specific event whereby the flagella are precisely excised from the cell body (Rosenbaum and Carlson, 1969; Satir et al., 1976; Lewin and Lee, 1985; Sanders and Salisbury, 1989; Jarvik and Suhan, 1991). The physical mechanism of flagellar excision appears to involve both a microtubule severing activity (Vale, 1991; Shiina et al., 1992; McNally and Vale, 1993) and a mechanical force generated by centrin (for references see Hartzell et al., 1993). A stellate array of centrin-containing transition zone fibers contract during deflagellation (Sanders and Salisbury, 1989). *Chlamydomonas* cells permeabilized with the non-ionic detergent, NP-40, deflagellate when Ca^{2+} is added in μM concentrations (Sanders and Salisbury, 1989). Because Ca^{2+} is necessary and sufficient for deflagellation in detergent-permeabilized cells (Sanders and Salisbury, 1994), agents which induce deflagellation in vivo may act via increases in intracellular [Ca^{2+}]. Deflagellation can be produced in living cells by a variety of stimuli (Minz and Lewin, 1954; Thompson et al., 1974; Lewin et al., 1980; Witman, 1986). We have previously shown that acid flux into the cell triggers deflagellation in vivo (Hartzell et al., 1993) as does external application of the wasp venom peptide, mastoparan (Quarmby et al., 1992). We now pose the question: How do these agents generate an intracellular Ca^{2+} signal in vivo?

In the only published report to examine the requirement for extracellular Ca^{2+} during acid-induced deflagellation, the authors state that a 30-min pretreatment in [Ca^{2+}] below 0.1 μM inhibited acid-induced deflagellation, but this observation is difficult to interpret because the experiment was done at pH 4.3 where EGTA is a very poor Ca^{2+} buffer (Sanders and Salisbury, 1989; and J. Salisbury, personal communication; see Discussion). We now report that acid and mastoparan activate distinct signalling pathways to induce deflagellation. The pathways are distinguished by their requirements for extracellular Ca^{2+}, patterns of Ca^{2+} influx, and sensitivity to Cd^{2+} and La^{3+}. We report that a recently isolated mutant strain of *Chlamydomonas reinhardtii*, *adf*-1, is specifically defective in acid-activated ^{45}Ca influx and deflagellation.

Materials and Methods

Cells and Culture Conditions

C. reinhardtii wild-type cells (137c; mt+) and the *fa-1* mutant strain (cc1370; mt+) were obtained from Dr. E. Harris (*Chlamydomonas* Genetics Center, Botany Department, Duke University, Durham, NC). The *Adf-1* strain was a gift from Dr. U. Goodenough (Washington University, St. Louis, MO).

Cells were inoculated from TAP plates into 75 ml of TAP medium (Harris, 1989). Cultures were bubbled with 5% CO_2 in air and grown for 42–46 h with continuous light (cool white) at room temperature. All experiments and solutions were made at room temperature.

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Quantification of Deflagellation

For deflagellation experiments, 5 × 10^5 cells were harvested from TAP medium by centrifugation (30 s, 12,000 *g*, room temperature) and resuspended by gentle trituration into 0.5 ml of either 50 mM Na benzoate (pH 6.0), 1 mM $MgCl_2$ (Hartzell et al., 1993) or 10 μM mastoparan in 10 mM Hepes, 1 mM $MgCl_2$ (Quarmby et al., 1992). Deflagellation-inducing solutions also contained $CaCl_2$ and/or BAPTA, as described below and in the figure legends. We estimate that TAP medium in the cell pellet contributed <0.5 μM total Ca^{2+} to the final solutions. Cells were treated with the deflagellation-inducing solution for 30 s and then fixed by the addition of an equal volume of 4% glutaraldehyde. Cells were scored for the loss of flagella by phase-contrast microscopy. The effect of Ca^{2+} channel blockers was tested by pre-incubating cells (at 10^6 cells/ml) with the blocker for 1 min before the addition of the deflagellation-inducing agent, except where noted.

Preparation of Ca^{2+} Buffers

A calcium electrode (Orion, Cambridge, MA) was used to titrate the BAPTA (Molecular Probes, Eugene, OR) stock solution using a calcium standard (Fisher, Pittsburgh, PA). Working Ca^{2+} solutions were calibrated by titration with an EGTA solution previously calibrated against the standard. For deflagellation experiments, solutions contained 1 mM BAPTA and an appropriate amount of $CaCl_2$ to produce the desired [Ca^{2+}]. A computer program (Fabiato, 1988) that takes into account the binding of Ca^{2+}, Mg^{2+}, and H^+ to the BAPTA was used for the necessary calculations. For some experiments, solutions were treated with Chelex-100 resin (Bio-rad Labs, Melville, NY) to remove divalent cations. 1 g of resin was added to 50 ml of the solution. The resin was resuspended and allowed to settle three times. After the resin was removed, the pH of the Chelex-treated solutions was adjusted to either pH 7.0 (for the Hepes solution) or pH 6.0 (for the Na benzoate solution) by addition of 1 N NaOH. The Chelex-treated solutions, used on the same day as Chelex treatment, were presumed to be Ca^{2+}-free; additions of a calibrated $CaCl_2$ stock solution were used to produce a range of final [Ca^{2+}].

^{45}Ca Flux

For ^{45}Ca influx experiments, cells were harvested by centrifugation (10 min, 2,000 *g*; 4°C) and resuspended in 10 mM Na-Hepes (pH 7.0), 1 mM $MgCl_2$ and $CaCl_2$ (5 or 50 μM). Cell concentration was adjusted to 2 × 10^7 cells/ml and the cells were stored in this buffered solution for 1 h. 250 μl of a solution containing ^{45}Ca (5 or 50 μM; ~0.4 mCi/μmol), 1 mM $MgCl_2$, and either 100 mM Na-Benzoate (pH 6.0) or 10 μM mastoparan in 10 mM Hepes (pH 7.0) was aliquoted into test tubes. 250 μl of the cell suspension in the same [Ca^{2+}] was pipetted at intervals into the ^{45}Ca solution. Influx was terminated 3 s after the final addition of cells by the simultaneous addition of 1.5 ml of ice-cold wash buffer (25 mM $CaCl_2$; 1 mM $MgCl_2$; 10 mM Na-Hepes, pH 7.0) to all of the tubes. To obtain an estimate of "time-zero" binding of ^{45}Ca to the cells, an aliquot of cells was added to ^{45}Ca immediately after the addition of wash buffer. The cells were then immediately (within 1 s) separated from the solution by filtration (using a Cell Harvester, Brandel, Gaithersburg, MD) onto glass fiber filters (#32; Schleicher & Schuell, Inc., Keene, NH). Test tubes and filters were washed twice with 1.5 ml of ice-cold wash buffer. Filters were placed in 3 ml of Bio-Safe II counting cocktail (Research Products Int., Mt. Prospect, IL) and radioactivity counted in a Beckman liquid scintillation counter.

The cell wall of *Chlamydomonas* has a high capacity for binding Ca^{2+}. Cells treated with 1% of the non-ionic detergent, NP-40 (Sigma Immunochemicals, St. Louis, MO), were used to control for cell wall binding of ^{45}Ca. We determined that the wash protocol described above reduced the amount of wall-bound ^{45}Ca to a low and reproducible level. This level was the same as the "time zero" controls described above, therefore, we report only "time-zero" values in this paper.

Mastoparan and the mastoparan analogue, Mas-17, were obtained from Peninsula Laboratories (Belmont, CA). ^{45}Ca (21.0 mCi/mg of Ca) was from DuPont NEN (Boston, MA).

Results

Requirement for Extracellular Ca^{2+}

We first determined the extracellular Ca^{2+} requirement for deflagellation induced in vivo by either acid or mastoparan. We made solutions of defined [Ca^{2+}] using BAPTA, which is an effective Ca^{2+} buffer at pH 6 as well as at neutral pH (Tsien, 1980), and examined the ability of Na benzoate (pH 6) or mastoparan in 10 mM Hepes (pH 7) to induce deflagellation as a function of extracellular [Ca^{2+}] (Fig. 1). We have previously shown that although many organic acids induce deflagellation, benzoate induces deflagellation with greater potency than the more commonly used acetate (see Hartzell et al., 1993). Benzoate (50 mM, pH 6) triggered deflagellation with an EC_{50} for [Ca^{2+}] of ~100 μM. In contrast, mastoparan (10 μM) caused a significant proportion of the cells (60%) to deflagellate at [Ca^{2+}] as low as ~1 nM. Deflagellation was efficient in response to either benzoate or mastoparan at high [Ca^{2+}] (1 mM); however, when [Ca^{2+}] was buffered at 1 μM mastoparan induced deflagellation, but benzoate did not (Fig. 1). Thus, the [Ca^{2+}] requirement is greater for benzoate induced than for mastoparan-induced deflagellation. To control for the presence of BAPTA, we repeated the experiments using solutions of 1 μM and 1 mM $CaCl_2$ in Hepes or benzoate solutions previously treated with Chelex resin to remove divalent cations (Fig. 2). As we found with the BAPTA solutions, mastoparan induced deflagellation in both high and low [Ca^{2+}], but benzoate was only effective at high concentrations (1 mM).

In the experiments of Figs. 1 and 2, the deflagellation stimulus was provided at the same time cells were placed in

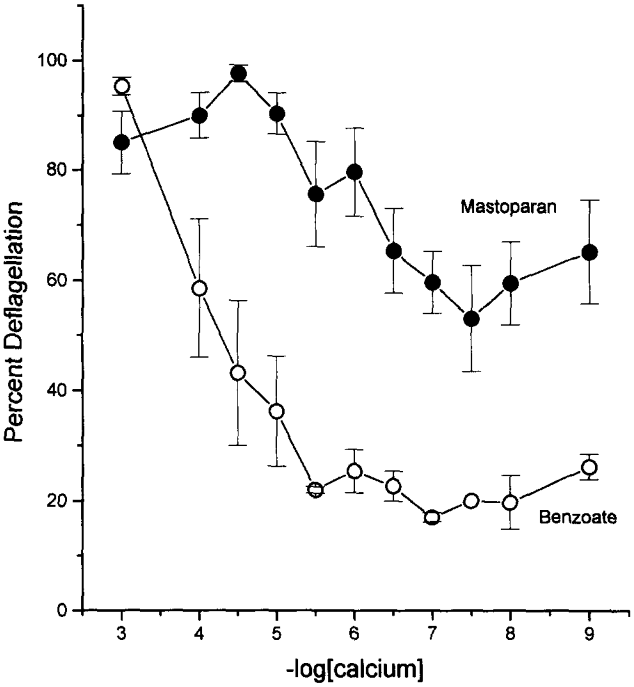


Figure 1. The dependence of deflagellation on extracellular Ca^{2+}. For each sample, 5 × 10^5 cells were pelleted, the media aspirated, and the cells resuspended in 0.5 ml of deflagellation-inducing solution for 30 s before fixation with 2% glutaraldehyde. Solutions were buffered for Ca^{2+} with 1 mM BAPTA as described in Materials and Methods. Deflagellation was induced by 50 mM Na Benzoate, pH 6.0 (○) or 10 μM mastoparan, pH 7.0 (●). Each data point is the mean of seven samples from a total of three independent experiments. At least 100 cells were scored for each sample. Error bars in this and subsequent figures are the standard error.

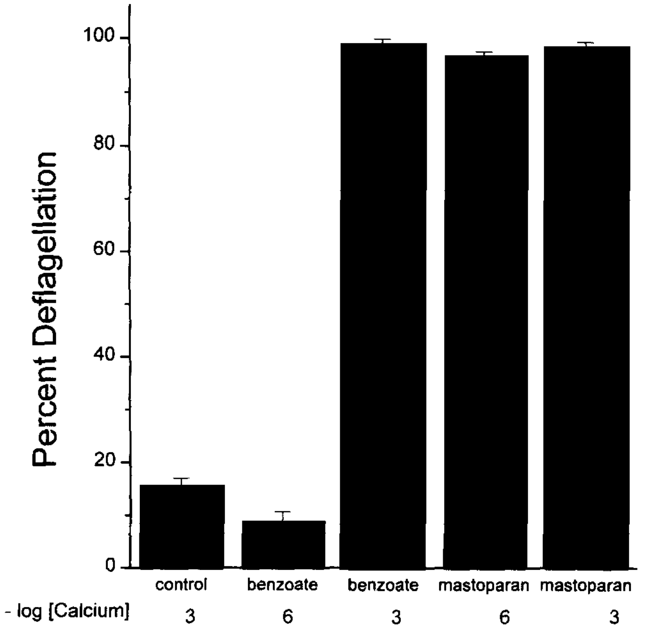


Figure 2. Chelex-treated solutions give the same results as BAPTA-buffered solutions. Solutions were made cation-free by Chelex treatment as described in Materials and Methods. Either 1 μM [Ca^{2+}] or 1 mM [Ca^{2+}] was added to the solutions and deflagellation experiments were done as described in Fig. 1. Data are the mean of two independent experiments each done in duplicate.

the appropriate [Ca^{2+}] solution. To investigate the effect of Ca^{2+} applied at different times relative to the deflagellation stimulus, cells were pelleted and resuspended at 10^6 cells/ml in 0.25 ml of 10 mM Hepes (pH 7.0). When 0.25 ml of 100 mM benzoate (pH 6.0) was added to cells held in low [Ca^{2+}], they did not deflagellate; however, they did deflagellate when 1 mM Ca^{2+} was subsequently added (Fig. 3 *a*).

From these data, we hypothesize that acid-induced deflagellation requires Ca^{2+}-influx. Mastoparan-induced deflagellation requires <1 nM extracellular [Ca^{2+}]. If mastoparan triggers deflagellation by releasing internal Ca^{2+} stores, then deflagellation would not occur if the intracellular pools were depleted. Consistent with this idea, fewer cells deflagellated (20%) when placed at very low [Ca^{2+}] for 30 s before the addition of mastoparan than if the cells were placed in very low [Ca^{2+}] at the same time as the mastoparan was added (60%; Fig. 3 *b*). In further support of the idea that the status of intracellular Ca^{2+} stores is important for mastoparan-induced deflagellation, we observed that ~80% of cells deflagellate if 1 mM Ca^{2+} is added 30 s after the cells are resuspended in low [Ca^{2+}] regardless of whether mastoparan is added at the time of resuspension or 60 s later (Fig. 3 *b*).

Effects of Ca^{2+} Channel Blockers

We found that several Ca^{2+} channel blockers known to inhibit other Ca^{2+}-mediated behaviors in *Chlamydomonas* (Hegemann et al., 1990; Goodenough et al., 1993) were ineffective at blocking acid- or mastoparan-induced deflagellation. These included omega-conotoxin (up to 5 μM with a 3-h preincubation), diltiazem (up to 100 μM), D-600 (10 μM), Ni^{2+} (up to 1 mM), and Co^{2+} (up to 1 mM) (data not shown). However, two inorganic Ca^{2+} channel blockers, Cd^{2+} and La^{3+}, which interfere with other flagellar signalling pathways in *Chlamydomonas* (Goodenough, 1993; Saito et al., 1993) did inhibit deflagellation, as described below.

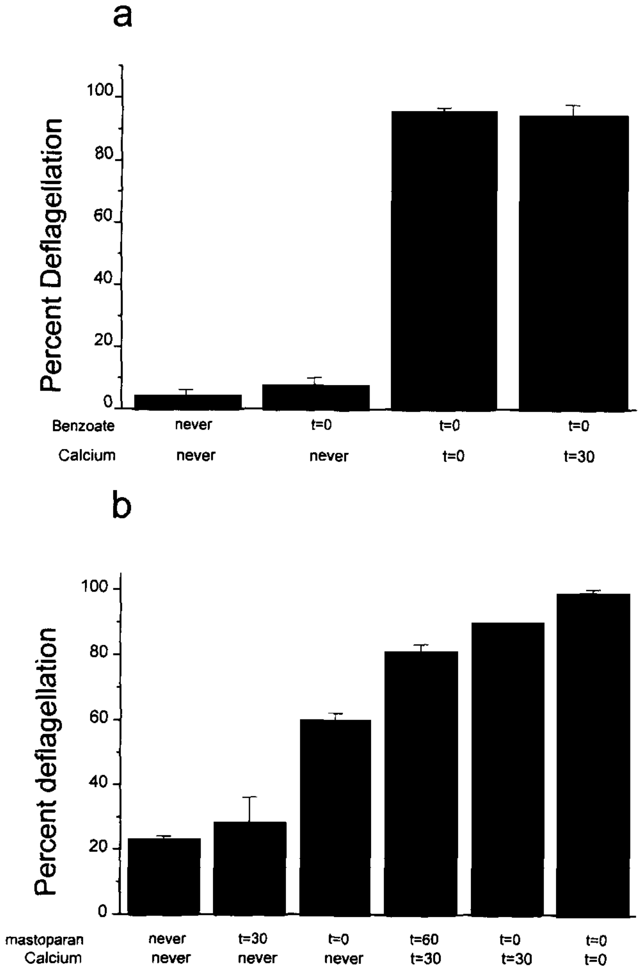
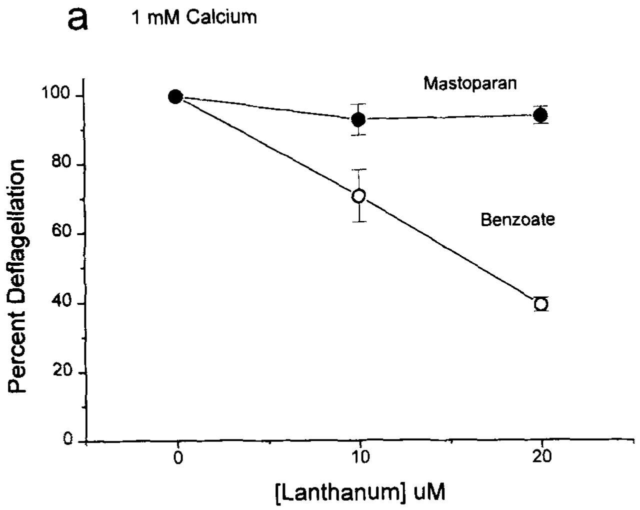


Figure 3. The effect of Ca^{2+} applied at different times relative to the deflagellation stimulus. (*a*) Benzoate-induced deflagellation was done as described in Fig. 1 except that 1 mM Ca^{2+} was either not added (*never*), added with the acid ($t = 0$), or added 30 s later ($t = 30$). (*b*) 10 μM mastoparan and 1 mM Ca^{2+} were either present when the cells were resuspended in 10 mM Hepes (pH 7.0), or added at the times indicated (0, 30 or 60 s). Cells were fixed 30 s after all additions had been made. Data are the mean of duplicates.

Consistent with our hypothesis that benzoate induces deflagellation via influx of extracellular Ca^{2+} whereas mastoparan does not, La^{3+} had little effect on mastoparan-induced deflagellation, but inhibited benzoate-induced deflagellation in a dose-dependent manner up to 20 μM [La^{3+}] (Fig. 4 *a*). Higher [La^{3+}] (e.g., 50 μM) sometimes inhibited both mastoparan and benzoate-induced deflagellation, but we believe these effects were nonspecific, because cells treated with ≥50 μM La^{3+} clumped and the effects on deflagellation were variable. The IC_{50} for [La^{3+}] could not be determined at 1 mM [Ca^{2+}]. In 50 μM [Ca^{2+}] (Fig. 4 *b*), the IC_{50} for [La^{3+}] was 2 μM for benzoate-induced deflagellation. For ^{45}Ca experiments (below) we used 20 μM [La^{3+}], which concentration substantially blocks benzoate-induced deflagellation and inhibits mastoparan-induced deflagellation less than 25%.



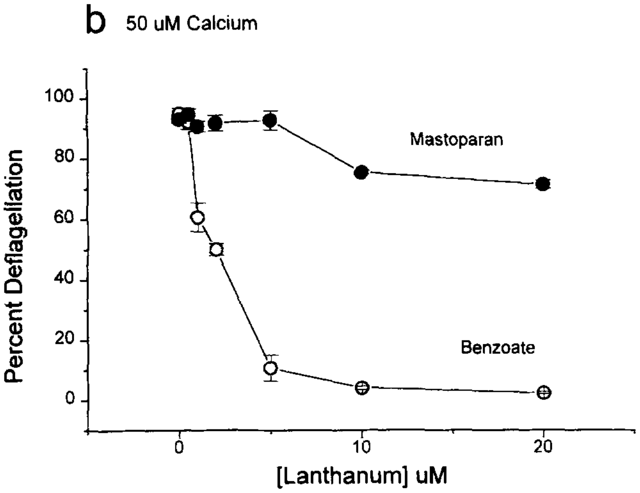
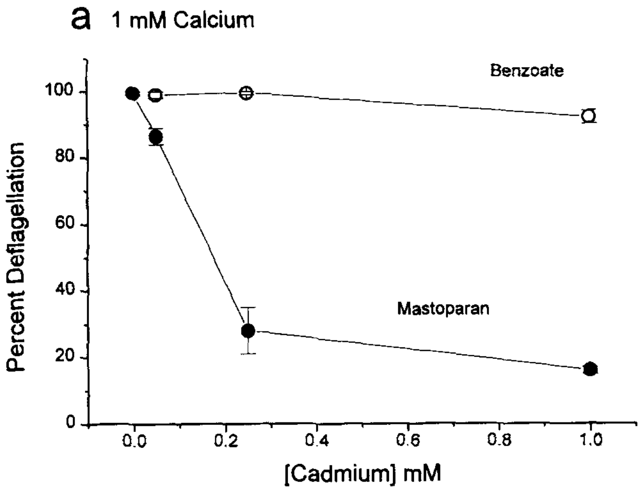


Figure 4. Inhibition of deflagellation by La^{3+}. (*a*) Cells were resuspended in 10 mM Hepes with 1 mM [Ca^{2+}] and the specified [La^{3+}] and then incubated for 1 min before the addition of an equal volume of 100 mM Na benzoate, pH 6.0 (○) or 20 μM mastoparan, pH 7.0 (●). The final pH value for the acid-treated cells was 6.3. Cells were fixed 30 s after induction of deflagellation. (*b*) Cells were resuspended in 10 mM Hepes with 50 μM [Ca^{2+}] and incubated for 1 h before the addition of the specified [La^{3+}]. Cells were incubated for 1 min in La^{3+}, then an equal volume of 100 mM Na benzoate, pH 6.0 (○) or 20 μM mastoparan, pH 7.0 (●) was added. Data are the mean of two independent experiments each done in triplicate.



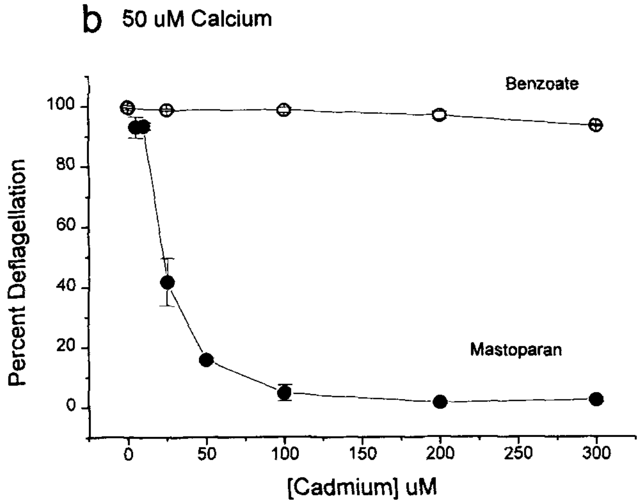


Figure 5. Inhibition of deflagellation by Cd^{2+}. (*a*) and (*b*) Cells were treated as described in the legend to Fig. 4, except that Cd^{2+} was used instead of La^{3+}. Data are the mean of triplicates. Similar results were obtained in two independent experiments.

Because Cd^{2+} also blocks many plasma membrane Ca^{2+} channels, we predicted that Cd^{2+} would behave like La^{3+} and inhibit benzoate-induced deflagellation but not mastoparan-induced deflagellation. Surprisingly, Cd^{2+} did not inhibit benzoate-induced deflagellation, but did inhibit mastoparan-induced deflagellation (Fig. 5). This was true both in the presence of 1 mM Ca^{2+} (Fig. 5 *a*) or 50 μM Ca^{2+} (Fig. 5 *b*). The IC$_{50}$ for Cd^{2+} inhibition of mastoparan-induced deflagellation was 180 μM in the presence of 1 mM Ca^{2+} and 25 μM in the presence of 50 μM Ca^{2+}. Because mastoparan-induced deflagellation is relatively insensitive to La^{3+} (Fig. 4) and Co^{2+} (not shown), and is relatively insensitive to extracellular [Ca^{2+}] (Figs. 1 and 2) we hypothesize that Cd^{2+} is not inhibiting the mastoparan pathway by blockade of a plasma membrane Ca^{2+} channel, but rather is inhibiting some other step in the mastoparan pathway.

^{45}Ca Influx

To test the hypothesis that acid was activating a La^{3+}-sensitive, Cd^{2+}-insensitive Ca^{2+} channel, we measured Ca^{2+} influx using ^{45}Ca. ^{45}Ca (50 μM, 0.4 mCi/μmol) was mixed with the deflagellation-inducing agent (benzoate or mastoparan), and an equal volume of cells (in 50 μM [Ca^{2+}]) was added at $t = 0$. Influx was terminated by the addition of ice-cold, 25 mM [Ca^{2+}] buffer (see Materials and Methods). In these experiments we are measuring the accumulation of ^{45}Ca. Because the ^{45}Ca is added at the same time as the stimulus, accumulation is a minimal estimate of influx. Benzoate produced a dramatic stimulation ^{45}Ca accumulation (compare circles and triangles, Fig. 6). In cells pre-treated with 100 μM [Cd^{2+}] for 1 min, benzoate-stimulated accumulation was unaffected for the first 3 s, and then was abruptly inhibited (compare circles and squares, Fig. 6). Because Cd^{2+} did not inhibit either benzoate-induced deflagel-

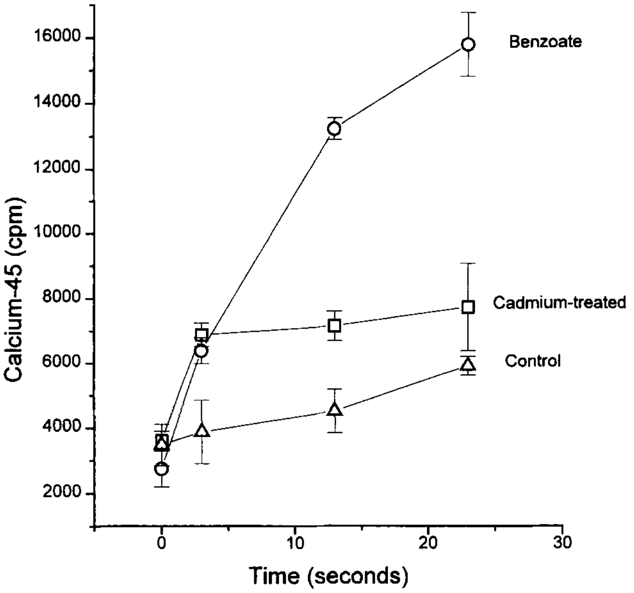


Figure 6. Induction of ^{45}Ca accumulation by benzoate. Cells were resuspended at 2 × 10^7 cells/ml in 10 mM Hepes (pH 7.0); 50 μM [Ca^{2+}]; 1 mM $MgCl_2$ and incubated for 1 h. 250 μl of cells were added at intervals to an equal volume of 50 μM [^{45}Ca] in 100 mM Na benzoate, pH 6.0 (○) and influx quenched as described in Materials and Methods. The control accumulation was obtained with ^{45}Ca in 100 mM Hepes, pH 7.0 (△). To test the effects of Cd^{2+}, cells were pre-treated with 100 μM Cd^{2+} for 1 min before the addition of Na benzoate/^{45}Ca (□). The data are the mean of duplicates in single experiment. Similar results were obtained in three independent experiments.

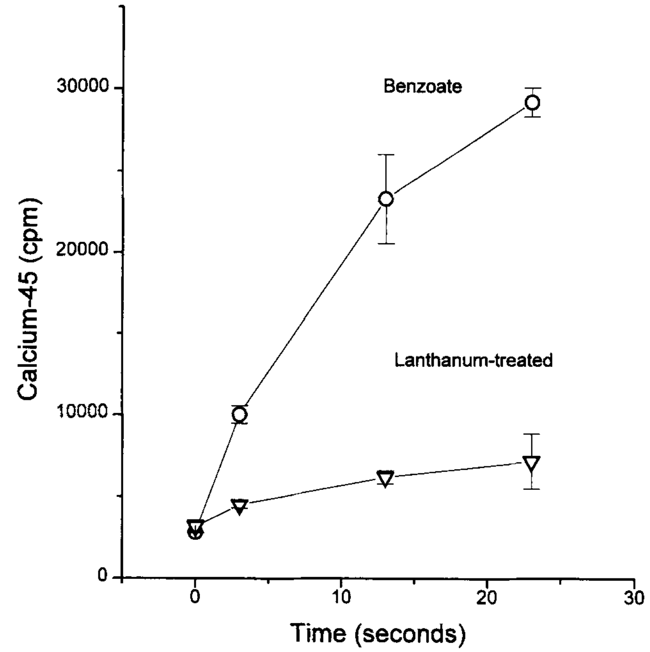


Figure 7. Effect of La^{3+} on benzoate-induced ^{45}Ca accumulation. The experiment was done as described in Materials and Methods and the legend to Fig. 6, except that 20 μM La^{3+} was used instead of Cd^{2+}. The data are the mean of duplicates in a single experiment. Similar results were obtained in two other experiments.

lation or the rapid initial accumulation of ^{45}Ca induced by benzoate, we hypothesized that a rapid influx of Ca^{2+} was involved in deflagellation, which occurs in <1 s (Quarmby et al., 1992; Yueh and Crain, 1993).

Because La^{3+} inhibits deflagellation produced by benzoate, we predicted that La^{3+} would inhibit the rapid phase of ^{45}Ca influx. Indeed, La^{3+} completely inhibited benzoate-induced ^{45}Ca accumulation (Fig. 7). The observation that La^{3+} inhibited both the fast and the slow components of benzoate-induced ^{45}Ca accumulation, whereas Cd^{2+} blocked only the slow phase is consistent with the idea that the rapid initial accumulation triggers deflagellation.

Mastoparan also triggered an accumulation of ^{45}Ca (Fig. 8 *a*). However, there are substantial differences between mastoparan- and benzoate-induced ^{45}Ca accumulation. First, in each of seven independent experiments, accumulation of ^{45}Ca in the mastoparan-treated cells after 30 s was about fivefold higher than into the benzoate-treated cells (Fig. 8 *a*, shows the results of a typical experiment). We hypothesize that the mastoparan-stimulated Ca^{2+} entry pathway has a high capacity whereas the Ca^{2+} influx activated by acid may be highly localized (perhaps to flagella or the flagellar transition zone). Second, the rate of benzoate-induced ^{45}Ca accumulation was maximal by 3 s, whereas the mastoparan-induced ^{45}Ca accumulation showed a lag of several seconds. The differences in kinetics are more apparent when the data is normalized to total flux at 23 s (Fig. 8 *b*). To facilitate comparison of the time courses, we sought experimental conditions where the total accumulation induced by the two agents was comparable. In 5 μM Ca^{2+}, mastoparan induced a smaller Ca^{2+} influx than it did at 50 μM Ca^{2+}, but the timecourse was comparable. Fig. 8 *c* compares the accumulation of ^{45}Ca in response to acid treatment at 50 μM [Ca^{2+}] with mastoparan-induced ^{45}Ca accumulation at 5 μM [Ca^{2+}]. Differences in the timecourses of stimulation of ^{45}Ca accumulation by mastoparan and by benzoate support the hypothesis that benzoate-induced deflagellation proceeds via an influx of extracellular Ca^{2+} whereas mastoparan-induced deflagellation is mediated by the mobilization of intracellular stores of Ca^{2+}, followed by an influx of Ca^{2+} which may serve to refill the depleted internal stores.

If the mastoparan-induced influx of Ca^{2+} is not the trigger for deflagellation, then it should be possible to block the Ca^{2+} influx without inhibiting deflagellation. Fig. 9 shows that 20 μM [La^{3+}], a concentration which has little effect on mastoparan-induced deflagellation (Fig. 4), completely inhibited mastoparan-induced ^{45}Ca accumulation. We conclude that mastoparan-induced Ca^{2+} influx is not the trigger for mastoparan-induced deflagellation.

Mastoparan-induced ^{45}Ca accumulation was inhibited ~85% when cells were pretreated for 1 min with Cd^{2+} (Fig. 10). Unlike the inhibition of acid-induced Ca^{2+} accumulation (Fig. 6), inhibition of mastoparan-induced ^{45}Ca accumulation by Cd^{2+} was apparent even at the earliest time points (Fig. 10). Because Cd^{2+} inhibited mastoparan-induced deflagellation, it is not possible to distinguish whether the lack of ^{45}Ca accumulation is attributable to a blockade of the relevant channel or to inhibition of the pathway responsible for generating a signal for the influx. Cd^{2+} may be exerting multiple effects.

The mastoparan analogue, mas-17, is similar in structure to mastoparan, but does not activate G proteins (Higashijima

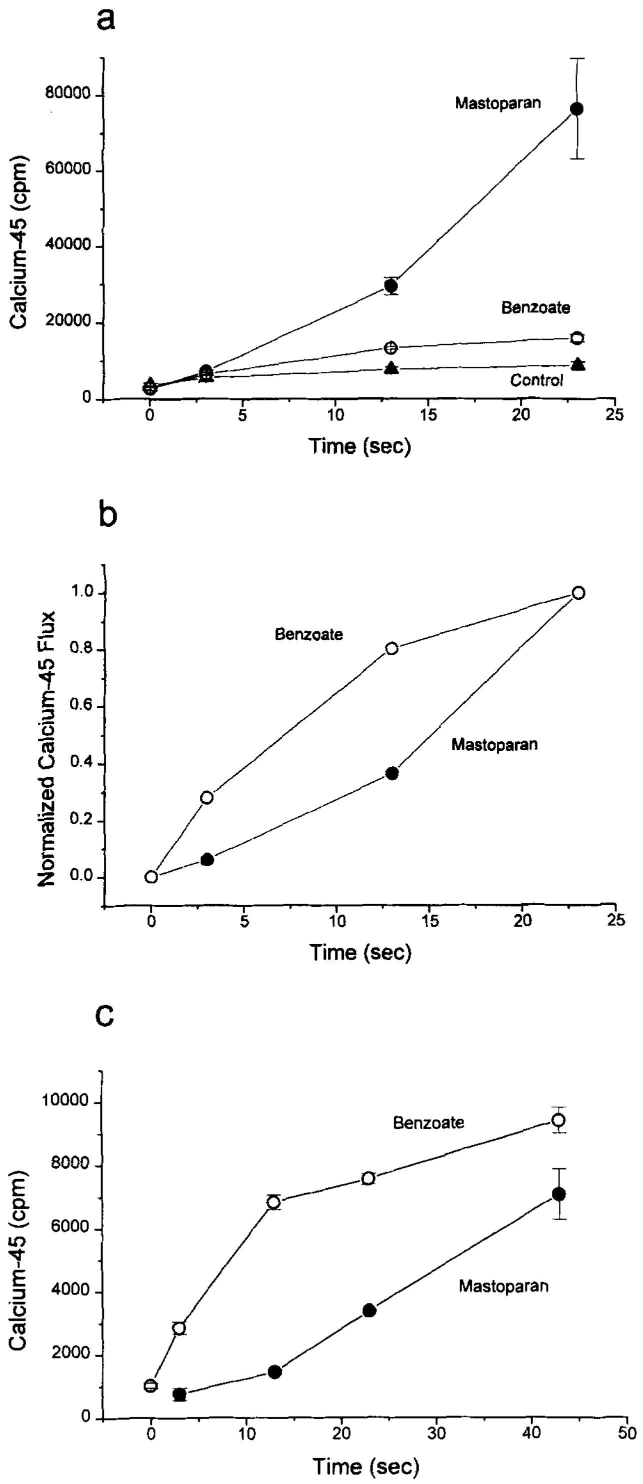


Figure 8. Comparison of mastoparan-induced ^{45}Ca accumulation with benzoate-induced ^{45}Ca accumulation. (*a*) Benzoate-induced accumulation (○) is the same data as presented in Fig. 6. The mastoparan-induced accumulation (●) was measured on the same day, using the same culture, as the benzoate experiment. Cells were treated as described in the legend to Fig. 6, except that 10 μM mastoparan was used instead of benzoate to induce influx. Control accumulation was obtained with ^{45}Ca in 10 mM Hepes (▲). (*b*) The data in *a* were normalized to the ^{45}Ca accumulation at 23 s (*c*) mastoparan-induced influx (●) was done as described above, except that the cells were incubated in 5 μM [Ca^{2+}] (instead of 50 μM [Ca^{2+}]) and the [^{45}Ca] was also 5 μM. Benzoate-induced accumulation (○) was measured at 50 μM [Ca^{2+}]. Data are the mean of duplicates in a single experiment. The fivefold difference mastoparan- and benzoate-induced total flux at ~30 s was observed in five independent experiments. Hyperbolic kinetics for benzoate-induced accumulation were observed in seven independent experiments. The characteristic lag for the mastoparan-induced accumulation was observed in 15 independent experiments under a variety of conditions.

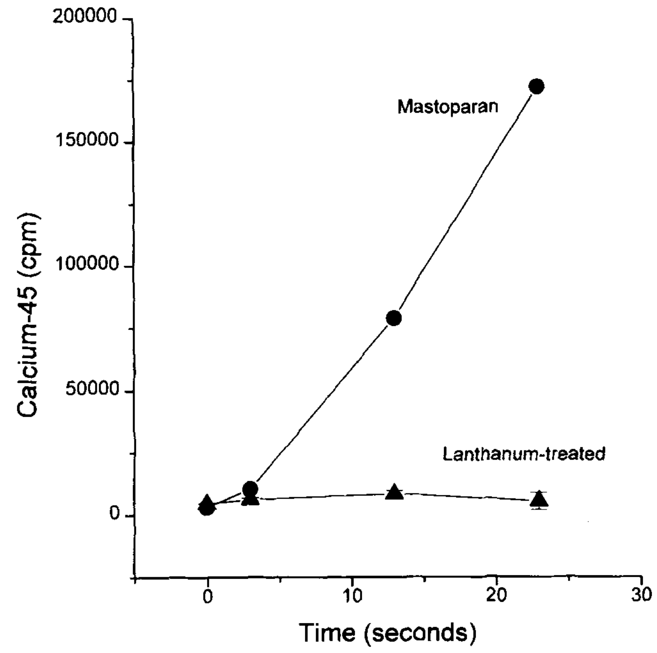


Figure 9. Effect of La^{3+} on mastoparan-induced ^{45}Ca accumulation. The experiment was done as described in Materials and Methods and the legend to Fig. 8, except that 20 μM La^{3+} was used instead of Cd^{2+}. The data are the mean of duplicates in a single experiment. Similar results were obtained in 1 other experiment. The data shown in this figure were obtained on the same day, with the same culture, as the experiment reported in Fig. 7.

et al., 1990). We previously reported that, although mastoparan induces deflagellation, mas-17 does not (Quarmby et al., 1992). Fig. 10 (inverted triangles) shows that mas-17 does not induce ^{45}Ca accumulation. Although mastoparan-induced Ca^{2+} influx is not the cause of deflagellation (Fig. 9), these data provide further correlative evidence for a relationship between mastoparan-induced deflagellation and Ca^{2+} influx.

Mutant Strains

T. Saito and U. Goodenough (Washington University, St. Louis, MO) recently found that the *imp-4* strain of *C. reinhardtii* (originally isolated for a defect in mating; Goodenough et al., 1976) carried a second mutation causing a defect in acid-induced deflagellation but not in the machinery of deflagellation (U. Goodenough, personal communication). The Goodenough laboratory crossed the *imp-4* strain to a wild-type strain (cc620/621), isolated an *adf-1* segregant (*a*cid *def*lagellation) that mates normally (*adf*$^-$, *imp*$^+$), and

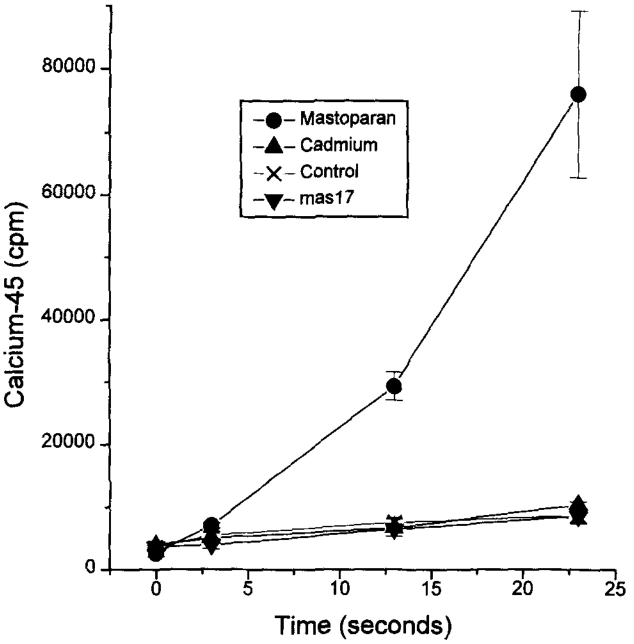


Figure 10. Effect of Cd^{2+} and mas-17 on ^{45}Ca accumulation induced by mastoparan. Cells were pre-treated with 100 μM Cd^{2+} for 1 min before the addition of mastoparan/^{45}Ca (□). In two runs, 10 μM mas-17 was used instead of mastoparan. The data shown are the mean of duplicates in a single experiment. The mas-17 result was observed in two independent experiments, the Cd^{2+} effect repeated in three independent experiments, and the mastoparan-induced accumulation was observed in seven independent experiments. The data shown in this figure were obtained on the same day, with the same culture, as the experiment reported in Fig. 6.

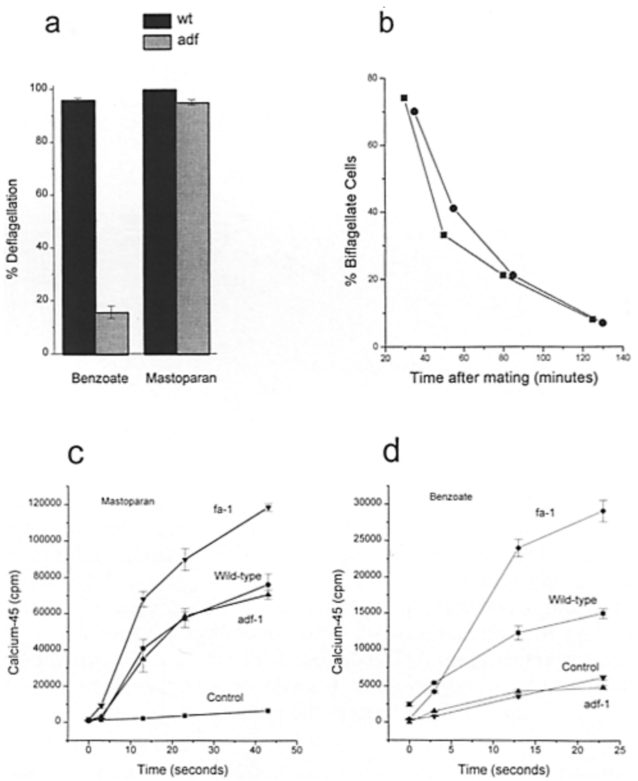


Figure 11. Deflagellation and ^{45}Ca accumulation in two *C. reinhardtii* mutant strains. (*a*) *Adf-1* cells were treated as described in the legend to Fig. 1, except that 1 mM $CaCl_2$ was included in all solutions and BAPTA was omitted. This experiment was repeated four times with similar results. In one experiment [Ca^{2+}] was varied from 500 μM to 10 mM, in no case did *adf-1* cells deflagellate in response to acid. (*b*) This experiment was done by U. Goodenough (Washington University). Both mating types of the *Adf-1* and wild-type (cc620 and cc621) strains were differentiated into gametes as previously described (Martin and Goodenough, 1975). Cells were suspended in 10 mM Pipes, pH 7.4, 100 μM $CaCl_2$ at 10^7 cells/ml. Cultures of gametes of opposite mating type were mixed at time zero. Mating cultures were acidified at the indicated times by the addition of 0.1 N acetic acid to a final pH value of 4.5. After ~10 s at pH 4.5, cultures were neutralized with 0.1 N KOH and immediately fixed by the addition of an equal volume of 4% glutaraldehyde. The percent of cells that retained two flagella after acid treatment are reported. Squares are *adf-1*(mt+) X cc621(mt-); circles are cc620(mt+) X *adf-1*(mt-). (*c* and *d*) ^{45}Ca accumulation was measured in response to mastoparan (*c*) and acid (*d*) as described in the legends to Figs. 8 and 6, respectively.

provided us with this strain. Although *adf-1* cells do not deflagellate in response to acid, we discovered that they do deflagellate in response to mastoparan (Fig. 11 *a*).

After mating, zygotes are temporarily quadraflagellate. Immediately after *adf-1* gametes were mated with wild-type gametes, two of the flagella (presumably derived from the wild-type gamete) were readily shed upon acid treatment whereas two (presumably derived from the *adf-1* gamete) are retained by the zygote (Fig. 11 *b*). Older zygotes shed all four flagella in response to acidification. These results are shown in Fig. 11 *b*, plotted as the percent of cells retaining two flagella after acid treatment. No uni- or triflagellate cells were observed. We conclude that the wild-type gamete can rescue the deflagellation defect of the *adf-1* flagella.

Adf-1 cells exhibit wild-type mastoparan-induced ^{45}Ca accumulation (Fig. 11 *c*), but ^{45}Ca accumulation is not stimulated by acid (Fig. 11 *d*). We conclude that the *adf-1* mutant strain is specifically defective in the acid-activated signalling pathway.

The *fa-1* strain is also defective in deflagellation but, unlike the *adf-1* strain, *fa-1* cells do not deflagellate in response to any known signal, nor are the flagella shed when the cells are permeabilized in the presence of Ca^{2+} (Lewin and Burrascano, 1983; Sanders and Salisbury, 1989). This suggests that *fa-1* cells are defective in the machinery of deflagellation. Therefore we hypothesized that these cells would exhibit normal ^{45}Ca accumulation in response to acid and mastoparan. In *fa-1* cells, both acid and mastoparan stimulate ^{45}Ca accumulation to even greater levels than wild-type cells (Fig. 11, *c* and *d*). We conclude that both transduction pathways leading to Ca^{2+} influx are intact in *fa-1* cells.

Discussion

We have demonstrated that acid and mastoparan induce deflagellation via distinct pathways. However, both agents stimulate the accumulation of ^{45}Ca. In the discussion below we interpret stimulation of ^{45}Ca accumulation as stimulation of Ca^{2+} influx rather than as an inhibition of Ca^{2+} efflux. Although we cannot formally distinguish these possibilities, effects on efflux are unlikely because: (*a*) in order for Ca^{2+} to be an effective intracellular signal, basal permeability to Ca^{2+} is generally very low; and (*b*) acid-induced

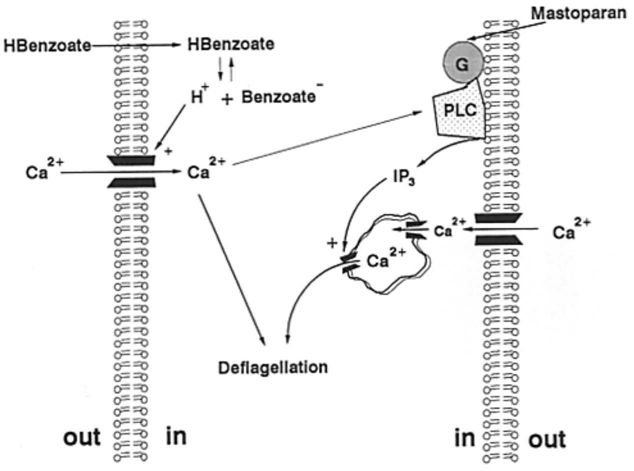


Figure 12. Working model for acid- and mastoparan-induced deflagellation (see Discussion). Intracellular acidification activates a La^{3+}-sensitive, plasma membrane. Ca^{2+} channel/transporter causing an influx of Ca^{2+} which in turn triggers deflagellation. Mastoparan activates a phospholipase C (*PLC*)-coupled G protein, leading to production of IP_3 which mobilizes intracellular Ca^{2+} and thereby triggers deflagellation. Cd^{2+} inhibition is specific to the mastoparan pathway. N.B. A single arrow in the diagram is not meant to imply a single step in the pathway.

deflagellation is efficient under conditions which inhibit Na^+/Ca^{2+} exchange (our unpublished data) thereby ruling out inhibition of efflux via this exchanger as the mechanism of acid-induced deflagellation.

Our working model for acid- and mastoparan-induced deflagellation is shown in Fig. 12. The acid pathway is shown on the left and the mastoparan pathway on the right. A protonated organic acid, highly soluble in the lipid bilayer, diffuses into the cell where protons are released (Hartzell et al., 1993). Intracellular acidification activates, either directly or indirectly, a plasma membrane Ca^{2+} channel or transporter, causing an influx of Ca^{2+} which in turn triggers deflagellation. Because acidification also activates phospholipase C, acid may open a plasma membrane Ca^{2+} channel/transporter via production of IP_3 (Quarmby et al., 1992; Yueh and Crain, 1993), but this remains to be proven. Our model for acid-induced deflagellation is supported by the following observations: acid influx is required for deflagellation (Hartzell et al., 1993), extracellular Ca^{2+} is necessary for deflagellation (Fig. 1), deflagellation is inhibited by the potent Ca^{2+} channel blocker, La^{3+} (Fig. 4), acid treatment induces a rapid accumulation of ^{45}Ca which is inhibited by La^{3+} (Fig. 7). Cd^{2+} does not inhibit either deflagellation or rapid ^{45}Ca accumulation induced by acid (Figs. 5 and 6).

Comparison of the amount of Ca^{2+} required for in vitro deflagellation with the accumulation of ^{45}Ca induced by acid in vivo lends further support to our model of acid-induced deflagellation. Sanders and Salisbury (1989) reported that 1 μM [Ca^{2+}] is necessary and sufficient to trigger excision of flagella in detergent permeabilized cells. We estimate the rapid initial accumulation of Ca^{2+} in response to acid to be on the order of 0.1 pmoles/10^6 cells/s. If we assume an average cell volume of 0.05 pl, then the accumulation is 2 nmoles/pl/s. This calculation demonstrates that the measured accumulation of Ca^{2+} could yield a 1 μM increase in total $[Ca^{2+}]_i$ within 500 ms. This is sufficiently rapid that acid-induced influx of Ca^{2+} is most likely the direct trigger of deflagellation (Quarmby et al., 1992; Yueh and Crain, 1993). Sanders and Salisbury (1989) report that cells in <0.1 μM [Ca^{2+}] do not deflagellate in response to acid. Superficially this is consistent with our findings, however, at pH 4.3 EGTA does not chelate Ca^{2+} very well and the concentration of free Ca^{2+} would increase upon acidification. Consequently, we wonder whether, under their conditions, deflagellation was inhibited by another mechanism.

In our model, mastoparan activates a phospholipase C-coupled G protein, leading to production of IP_3 which opens an intracellular Ca^{2+} channel thus releasing Ca^{2+} into the cytosol, thereby triggering deflagellation. Although the machinery of deflagellation is Ca^{2+} dependent (Sanders and Salisbury, 1989) very little, if any, extracellular Ca^{2+} is required for mastoparan-induced deflagellation is within the cells. Furthermore, mastoparan-induced deflagellation is relatively insensitive to La^{3+}, a very potent inorganic Ca^{2+}-channel blocker. In an earlier paper (Quarmby et al., 1992) we report that mastoparan activates PLC in *Chlamydomonas.* Therefore, the intracellular store implicated in mastoparan-induced deflagellation may be released by IP_3.

Cd^{2+} inhibited mastoparan-induced deflagellation, but because deflagellation requires little, if any, extracellular Ca^{2+} and is relatively insensitive to La^{3+}, it is unlikely that Cd^{2+} is inhibiting deflagellation by blocking a plasma membrane Ca^{2+} channel. Although Cd^{2+} is best known as a Ca^{2+} channel blocker, it can also activate calmodulin and have other effects as well (e.g., Kostrzewska and Sobieszek, 1990; Behra, 1993).

In our model, mastoparan-induced deflagellation is followed by an influx of extracellular Ca^{2+}, perhaps serving to refill depleted internal stores. The lag that occurs before mastoparan-induced Ca^{2+} influx (Fig. 8 *b* and *c* and Fig. 11 *c*) is consistent with this model. The observation that La^{3+} (20 μM) completely blocks mastoparan-induced ^{45}Ca influx (Fig. 9) yet inhibits mastoparan-induced deflagellation <25% (Fig. 4) is strong support for the idea that Ca^{2+} influx is a response to, rather than the cause of, deflagellation.

We have used two existing *Chlamydomonas* mutant strains to test the model presented in Fig. 12. First, if the two pathways are independent then it should be possible to identify a mutant strain with a defect in only one of the pathways. We have identified *adf-1* as defective in the acid, but not the matoparan pathway (Fig. 11 *a*). Because we calculate that the acid-induced influx of calcium is sufficient to directly activate the machinery of deflagellation, we predicted that a mutation unique to the acid pathway must prevent the rapid influx of calcium induced by acid. This hypothesis is validated by the data presented in Fig. 11 *d*. A second *Chlamydomonas* mutant strain, *fa-1*, does not deflagellate in response to either acid or mastoparan. In our model, only the calcium-responsive machinery of deflagellation is shared by the two pathways. We therefore predicted that both signal transduction pathways would be intact in *fa-1* cells. Fig. 11 (*c* and *d*) illustrates the robust activation of ^{45}Ca influx in *fa-1* cells responding to either acid or mastoparan. It is intriguing to consider that deactivation of the pathways may be lacking in a cell that does not shed its flagella.

We have demonstrated that *Chlamydomonas* cells express an abundant and/or high capacity Ca^{2+} channel or transporter that can be activated by acid. We are interested to learn how acid activates this flux of Ca^{2+} and whether the channel or transporter is specifically localized to either flagellar membranes or the flagellar transition zone. We also want to determine whether this pathway is used in other cells, perhaps for other purposes. To these ends, we have isolated new deflagellation-deficient mutant strains of *C. reinhardtti* (to be described elsewhere) from cells mutagenized by the insertion of plasmid DNA in order to facilitate subsequent cloning (Tam and Lefebvre, 1993).

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