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 \(\mu\)M 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 \(\mu\)M 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 (cell70; 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.
Quantification of Deflagellation

For deflagellation experiments, 5 x 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 MgCl2 (Hartzell et al., 1993) or 10 μM mastoparan in 10 mM Heps, 1 mM MgCl2 (Quarmby et al., 1992). Deflagellation-inducing solutions also contained CaCl2 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 Ca2+ 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 Ca2+ 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 Ca2+ 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 Ca2+ 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 CaCl2 to produce the desired [Ca2+]. A computer program (Fabiato, 1988) that takes into account the binding of Ca2+, Mg2+, 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 Heps 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 Ca2+-free; additions of a calibrated CaCl2 stock solution were used to produce a range of final [Ca2+].

45Ca Flux

For 45Ca influx experiments, cells were harvested by centrifugation (10 min, 2000 x g; 4°C) and resuspended in 10 mM Na-Heps (pH 7.0), 1 mM MgCl2 and CaCl2 (5 or 50 μM). Cell concentration was adjusted to 2 x 10^6 cells/ml and the cells were stored in this buffer solution for 1 h. 250 μl of a solution containing 45Ca (5 or 50 μM; ~0.4 mCi/μmol), 1 mM MgCl2, and either 100 mM Na-Benzoate (pH 6.0) or 10 μM mastoparan in 10 mM Heps (pH 7.0) was aliquoted into test tubes. 250 μl of the cell suspension in the same [Ca2+] was pipetted at intervals into the 45Ca 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 CaCl2; 1 mM MgCl2, and 10 mM Na-Heps, pH 7.0) to all of the tubes. To obtain an estimate of "time-zero" binding of 45Ca to the cells, an aliquot of cells was added to 45Ca immediately after the addition of wash buffer. The cells were then immediately (within 1 s) separated from the solution by filtration (using aCell 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 Beckmann liquid scintillation counter.

The cell wall of Chlamydomonas has a high capacity for binding Ca2+. 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 45Ca. We determined that the wash protocol described above reduced the amount of wall-bound 45Ca 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). 45Ca (21.0 mCi/μg of Ca) was from DuPont NEN (Boston, MA).

Results

Requirement for Extracellular Ca2+

We first determined the extracellular Ca2+ requirement for deflagellation induced in vivo by either acid or mastoparan. We made solutions of defined [Ca2+] using BAPTA, which is an effective Ca2+ 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 Heps (pH 7) to induce deflagellation as a function of extracellular [Ca2+] (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 E<sub>50</sub> for [Ca2+] of ~100 μM. In contrast, mastoparan (10 μM) caused a significant proportion of the cells (60%) to deflagellate at [Ca2+] as low as ~0.1 nM. Deflagellation was efficient in response to either benzoate or mastoparan at high [Ca2+] (~1 mM); however, when [Ca2+] was buffered at 1 μM mastoparan induced deflagellation, but benzoate did not (Fig. 1). Thus, the [Ca2+] 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 CaCl2 in Heps 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 [Ca2+], 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 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 [Ca²⁺] or 1 mM [Ca²⁺] 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.

Figure 3. The effect of Ca²⁺ applied at different times relative to the deflagellation stimulus. (a) Benzoate-induced deflagellation was done as described in Fig. 1 except that 1 mM Ca²⁺ 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 Ca²⁺ 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.

Effects of Ca²⁺ Channel Blockers

We found that several Ca²⁺ channel blockers known to inhibit other Ca²⁺-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), Ni²⁺ (up to 1 mM), and Co²⁺ (up to 1 mM) (data not shown). However, two inorganic Ca²⁺ channel blockers, Cd²⁺ and La³⁺, which interfere with other flagellar signaling pathways in Chlamydomonas (Goodenough, 1993; Saito et al., 1993) did inhibit deflagellation, as described below.

Consistent with our hypothesis that benzoate induces deflagellation via influx of extracellular Ca²⁺ whereas mastoparan triggers deflagellation by releasing internal Ca²⁺ stores, we observed that ~80% of cells deflagellate if 1 mM Ca²⁺ is added 30 s after the cells are resuspended in low [Ca²⁺] regardless of whether mastoparan is added at the time of resuspension or 60 s later (Fig. 3 b).

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Figure 4. Inhibition of deflagellation by La³⁺. (a) Cells were resuspended in 10 mM Hepes with 1 mM [Ca²⁺] and the specified [La³⁺] and then incubated for 1 min before the addition of an equal volume of 100 mM Na benzoate, pH 6.0 (ń) or 20 µM mastoparan, pH 7.0 (ą). 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 [Ca²⁺] and incubated for 1 h before the addition of the specified [La³⁺]. Cells were incubated for 1 min in La³⁺, then an equal volume of 100 mM Na benzoate, pH 6.0 (ń) or 20 µM mastoparan, pH 7.0 (ą) was added. Data are the mean of two independent experiments each done in triplicate.

Because Cd²⁺ also blocks many plasma membrane Ca²⁺ channels, we predicted that Cd²⁺ would behave like La³⁺ and inhibit benzoate-induced deflagellation but not mastoparan-induced deflagellation. Surprisingly, Cd²⁺ did not inhibit benzoate-induced deflagellation, but did inhibit mastoparan-induced deflagellation (Fig. 5). This was true both in the presence of 1 mM Ca²⁺ (Fig. 5 a) or 50 µM Ca²⁺ (Fig. 5 b). The IC₅₀ for Cd²⁺ inhibition of mastoparan-induced deflagellation was 180 µM in the presence of 1 mM Ca²⁺ and 25 µM in the presence of 50 µM Ca²⁺. Because mastoparan-induced deflagellation is relatively insensitive to La³⁺ (Fig. 4) and Co²⁺ (not shown), and is relatively insensitive to extracellular [Ca²⁺] (Figs. 1 and 2) we hypothesize that Cd²⁺ is not inhibiting the mastoparan pathway by blockage of a plasma membrane Ca²⁺ channel, but rather is inhibiting some other step in the mastoparan pathway.

4⁵Ca Influx

To test the hypothesis that acid was activating a La³⁺-sensitive, Cd²⁺-insensitive Ca²⁺ channel, we measured Ca²⁺ influx using ⁴⁵Ca. ⁴⁵Ca (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 [Ca²⁺]) was added at t = 0. Influx was terminated by the addition of ice-cold, 25 mM [Ca²⁺] buffer (see Materials and Methods). In these experiments we are measuring the accumulation of ⁴⁵Ca. Because the ⁴⁵Ca is added at the same time as the stimulus, accumulation is a minimal estimate of influx. Benzoate produced a dramatic stimulation ⁴⁵Ca accumulation (compare circles and triangles, Fig. 6). In cells pre-treated with 100 µM [Cd²⁺] 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 Cd²⁺ did not inhibit either benzoate-induced deflagel-
Figure 6. Induction of 45Ca 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 [45Ca] in 100 mM Na benzoate, pH 6.0 (○) and influx quenched as described in Materials and Methods. The control accumulation was obtained with 45Ca 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/45Ca (△). The data are the mean of duplicates in single experiment. Similar results were obtained in three independent experiments.

lation or the rapid initial accumulation of 45Ca 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 45Ca influx. Indeed, La\(^{3+}\) completely inhibited benzoate-induced 45Ca accumulation (Fig. 7). The observation that La\(^{3+}\) inhibited both the fast and the slow components of benzoate-induced 45Ca 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 45Ca (Fig. 8 a). However, there are substantial differences between mastoparan- and benzoate-induced 45Ca accumulation. First, in each of seven independent experiments, accumulation of 45Ca 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 45Ca accumulation was maximal by 3 s, whereas the mastoparan-induced 45Ca 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 time-course was comparable. Fig. 8 c compares the accumulation of 45Ca in response to acid treatment at 50 μM [Ca\(^{2+}\)] with mastoparan-induced 45Ca accumulation at 5 μM [Ca\(^{2+}\)]. Differences in the timecourses of stimulation of 45Ca 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 45Ca accumulation. We conclude that mastoparan-induced Ca\(^{2+}\) influx is not the trigger for mastoparan-induced deflagellation.

Mastoparan-induced 45Ca 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 45Ca 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 45Ca 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...
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 (acid deflagellation) that mates normally (*adf^-, imp^+*), and

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**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 10. Effect of Cd²⁺ and mas-17 on ⁴⁵Ca accumulation induced by mastoparan. Cells were pre-treated with 100 μM Cd²⁺ for 1 min before the addition of mastoparan/⁴⁵Ca (□). 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 Cd²⁺ 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.

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 ⁴⁵Ca accumulation (Fig. 11 c), but ⁴⁵Ca 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 Ca²⁺ (Lewin and Barrassano, 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 ⁴⁵Ca accumulation in response to acid and mastoparan. In fa-1 cells, both acid and mastoparan stimulate ⁴⁵Ca accumulation to even greater levels than wild-type cells (Fig. 11, c and d). We conclude that both transduction pathways leading to Ca²⁺ 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 ⁴⁵Ca. In the discussion below we interpret stimulation of ⁴⁵Ca accumulation as stimulation of Ca²⁺ influx rather than as an inhibition of Ca²⁺ efflux. Although we cannot formally distinguish these possibilities, effects on efflux are unlikely because: (a) in order for Ca²⁺ to be an effective intracellular signal, basal permeability to Ca²⁺ is generally very low; and (b) acid-induced
deflagellation is efficient under conditions which inhibit Na+/Ca²⁺ 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 Ca²⁺ channel/transporter, causing an influx of Ca²⁺ which in turn triggers deflagellation. Because acidification also activates phospholipase C, acid may open a plasma membrane Ca²⁺ channel/transporter via production of IP₃ (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 Ca²⁺ is necessary for deflagellation (Fig. 1), deflagellation is inhibited by the potent Ca²⁺ channel blocker, La³⁺ (Fig. 4), acid treatment induces a rapid accumulation of ⁴⁵Ca which is inhibited by La³⁺ (Fig. 7). Cd²⁺ does not inhibit either deflagellation or rapid ⁴⁵Ca accumulation induced by acid (Figs. 5 and 6).

Comparison of the amount of Ca²⁺ required for in vitro deflagellation with the accumulation of ⁴⁵Ca induced by acid in vivo lends further support to our model of acid-induced deflagellation. Sanders and Salisbury (1989) reported that 1 μM [Ca²⁺] is necessary and sufficient to trigger excision of flagella in detergent permeabilized cells. We estimate the rapid initial accumulation of Ca²⁺ in response to acid to be on the order of 0.1 pmol/10⁶ cells/s. If we assume an average cell volume of 0.05 μl, then the accumulation is 2 nmoles/pl/s. This calculation demonstrates that the measured accumulation of Ca²⁺ could yield a 1 μM increase in total [Ca²⁺], within 500 ms. This is sufficiently rapid that acid-induced influx of Ca²⁺ 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 [Ca²⁺] do not deflagellate in response to acid. Superficially this is consistent with our findings, however, at pH 4.3 EGTA does not chelate Ca²⁺ very well and the concentration of free Ca²⁺ 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 IP₃ which mobilizes intracellular Ca²⁺ and thereby triggers deflagellation. Cd²⁺ 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.

In our model, mastoparan-induced deflagellation is followed by an influx of extracellular Ca²⁺, perhaps serving to refill depleted internal stores. The lag that occurs before mastoparan-induced Ca²⁺ influx (Fig. 8b and c and Fig. 11c) is strong support for the idea that Ca²⁺ 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 mastoparan pathway (Fig. 11a). 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. 11d. A second Chlamydomonas mutant strain, fa-I, 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-I cells. Fig. 11 (c and d) illustrates the robust activation of ⁴⁵Ca influx in fa-I 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 Ca²⁺ channel or transporter that can be activated by acid. We are interested to
learn how acid activates this flux of Ca\textsuperscript{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. reinhardtii (to be described elsewhere) from cells mutagenized by the insertion of plasmid DNA in order to facilitate subsequent cloning (Tam and Lefebvre, 1993).

We are deeply indebted to T. Saito and U. Goodenough for providing us with \textit{adl-1} cells. We also thank U. Goodenough for providing the heterokaryon analysis presented in Fig. 11 \textit{b} and for constructive criticism of the manuscript. We are grateful to Dr. R. B. Gunn for his insightful comments and for the use of his research facilities. M. Sanders and J. Salisbury generously shared unpublished data with us.

The Emory University Research Council provided financial support for this project.

Received for publication 8 September 1993 and in revised form 29 November 1993.

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