Deflagellation of *Chlamydomonas reinhardtii* Follows a Rapid Transitory Accumulation of Inositol 1,4,5,-trisphosphate and Requires Ca^{2+} Entry

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Abstract. *C. reinhardtii* sheds its flagella in response to acidification. Previously, we showed correlations between pH shock, deflagellation, and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] production, but 100% of cells deflagellated by 5 s, which was the earliest that Ins(1,4,5)P_3 accumulation could be accurately measured by techniques available to us at that time (Quarmby, L.M., Y. G. Yueh, J. L. Cheshire, L. R. Keller, W. J. Snell, and R. C. Crain. *J. Cell Biol.* 1992. 116:737-744). To learn about the causal relationship between Ins(1,4,5)P_3 accumulation and deflagellation, we extended these studies to early times using a continuous-flow rapid-quench device. Within 1 s of acidification to pH 4.3–4.5, 100% of cells deflagellated. A transient peak of Ins(1,4,5)P_3 was observed 250–350 ms after pH shock, preceding deflagellation. Preincubation with 10 μM neomycin, which prevents hydrolysis of phosphatidylinositol 4,5-bisphosphate, inhibited both the transient production of Ins(1,4,5)P_3 and the subsequent deflagellation. The nonspecific Ca^{2+} channel blockers La^{3+} and Cd^{2+} prevented flagellar excision induced by mastoparan without inhibiting rapid Ins(1,4,5)P_3 production. Likewise, the Ins(1,4,5)P_3-gated channel inhibitors ruthenium red and heparin blocked deflagellation in response to mastoparan. These studies were extended to mutants defective in flagellar excision. Fa-1, a mutant defective in flagellar structure, produced Ins(1,4,5)P_3 but failed to deflagellate. These results support a model in which acid pH activates a putative cellular receptor leading to G-protein dependent activation of phospholipase C and accumulation of Ins(1,4,5)P_3. These events are upstream of Ins(1,4,5)P_3-dependent Ca^{2+} entry from the medium, and of deflagellation.

THE biflagellated unicellular green alga, *Chlamydomonas reinhardtii*, sheds its flagella when confronted with a variety of environmental or artificial stresses including acid, heat, ethanol, and mechanical shear (4, 10). Ca^{2+} elicits flagellar excision in detergent-permeabilized cell models (45, 46) and intracellular [Ca^{2+}] increases after receipt of a deflagellation signal (45) as measured by the length of contractile fibers in the nucleus-basal body connector. These observations led Salisbury to propose that increased intracellular Ca^{2+}, via its effects on the Ca^{2+}-binding protein centrin, mediates deflagellation (46).

We previously showed that acid-induced deflagellation is associated with phosphatidylinositol 4,5-bisphosphate (PtdIns-[4,5]P_2)[1] turnover and production of inositol 1,4,5-trisphosphate (Ins[1,4,5]P_3), probably via activation of a G-protein linked phospholipase C (41). We also found that depletion of extracellular Ca^{2+} inhibited not only deflagellation but also Ins(1,4,5)P_3 production. The present study was undertaken to elucidate the causal relationship between Ins(1,4,5)P_3 accumulation, Ca^{2+} entry, and deflagellation. In an attempt to determine the primary event in the deflagellation process, we have resorted to continuous-flow rapid-quench techniques to measure early events in the process (24). In our earlier studies, deflagellation was complete by 5 s, which was the earliest time point for which we could obtain accurate measurements of Ins(1,4,5)P_3 production by the techniques available to us at that time (41). With the continuous-flow technique, we find that deflagellation is very rapid, essentially complete within a second, but that it is preceded by an even more rapid initial accumulation of Ins(1,4,5)P_3. Entry of extracellular Ca^{2+}, which is required for deflagellation, is not required for the rapid, transitory production of Ins(1,4,5)P_3.

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1. *Abbreviations used in this paper*: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; ms, millis; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; TMB-8, 3,4,5-trimethoxylbenzoic acid 8-(diethlyamino)octyl ester.

Materials and Methods

Cells and Culture Conditions

Wild-type *C. reinhardtii* 137c (strain cc124 mt^-) and mutant strains fa-1 (cc1370 mt^+), bald-2 (cc-478 mt^+) and cw-92 (cc503 mt^+) were obtained from E. Harris (Chlamydomonas Genetics Center, Duke University, Durham, NC). All cells were grown at 25°C in a cycle of 12 h dark and 12 h of illumination with cool white fluorescent light (photon fluence, 40 μmol $m^{-2}s^{-1}$) while being shaken at 120 rpm on a rotary shaker (B. Braun Instruments, Burlingame, CA). Cw-92 mutant cells were grown in Sueoka medium (50) modified by addition of sodium acetate to give a final concen-

tration of 14.7 mM (22) and sorbitol to a final concentration of 1% wt/vol (25). Fa-1 and bald-2 mutant cells were grown in media defined previously (23). Wild-type cells were grown in the same media, titrating to pH 7.0 with concentrated HCl instead of glacial acetic acid; cultures were continuously bubbled with 5% CO_2 in air. Cells were used in early- to mid-log phase growth and were concentrated by low speed centrifugation (10 min, 500 *g*, room temperature). All experiments were performed between hours 3 and 6 of the light period.

Rapid Kinetics of Deflagellation and Ins(1,4,5)P_3 Accumulation Using a Continuous-flow Rapid-quench Device

A continuous-flow rapid-quench device (24) was used to measure ms changes in deflagellation and Ins(1,4,5)P_3 concentration. Two syringes, the first containing the cells (about 9 × 10^6 cells/ml) in culture medium and the second containing culture medium adjusted to pH 4.0 with concentrated acetic acid, were connected to a microliter mixing chamber. Acid-induced deflagellation was initiated by mixing equal volumes of the contents of the two syringes (final pH 4.3–4.5); the reaction mixture was allowed to flow through 0.32 cm diam tygon tubing, the end of which was immersed in quenching solution (24). Based on the rate of flow (either 1.575 or 2.845 ml/s), the time between mixing and quenching was calculated for lengths of tubing between 1.59 and 15.85 cm. Ice cold TCA (final concentration 5% wt/vol) was used to quench reactions for Ins(1,4,5)P_3 measurements, tincture of iodine was used to quench reactions for measurement of deflagellation (41). The quenched samples were collected and kept on ice until analysis. The continuous-flow rapid-quench device used was capable of quenching samples at time intervals ranging from 44 to 800 ms with a 6% standard error for time measurement.

Calcium Depletion

Cells were depleted of extracellular Ca^{2+} by washing repeatedly in Ca^{2+} free Hepes/EGTA buffer (10 mM Hepes, 1 mM EGTA, pH 7.0). Hepes buffer was stirred with carboxymethyl cellulose (Whatman CM52), for 1 h and filtered through Whatman filter paper (#1 qualitative) to remove Ca^{2+}. EGTA was added to Hepes buffer to a final concentration of 1 mM, the Hepes/EGTA buffer was readjusted to pH 7.0.

Determination of Ins(1,4,5)P_3 Concentration

The quenched, chilled samples were centrifuged for 10 min at 650 *g*, 4°C. The supernatants were collected and extracted four times with five volumes of water-saturated ether to remove the TCA. The final aqueous extract was neutralized with 16% sodium carbonate (wt/vol) to pH 7.5 (9) and aliquots were lyophilized in a speed-vac. Concentrations of Ins(1,4,5)P_3 were determined on samples reconstituted with distilled water by a radio-receptor assay as previously described (41).

Other Materials and Methods

Deflagellation was determined on iodine-quenched cells by phase contrast microscopy using an Axioscope (Carl Zeiss, Inc., Thornwood, NY). All chemicals were from Sigma Chem. Co. (St. Louis, MO), unless indicated otherwise. Stock solutions of 5 mM $CdCl_2$, 10 μM ω-conotoxin, 100 mM EGTA, 50 mg/ml heparin sulfate (Fisher Scientific Co., Springfield, NJ), 100 mM $LaCl_3$, 1 mM mastoparan, 1 mM neomycin, 200 mM verapamil, and 1 mM ruthenium red were prepared in distilled water. A stock solution of 100 mM EGTA was prepared in 300 mM KOH. Stock solutions of 100 mM nifedipine and 1 mM thapsigargin (LC Laboratories, Woburn, MA) were prepared in DMSO. Intact pertussis toxin (100 μg/ml) was dissolved in 0.5 M NaCl and stored at 4°C. TMB-8 was prepared as a 50-mM stock solution in ethanol. A stock solution of 40 μM D-Ins(1,4,5)P_3 (LC Laboratories) was prepared in distilled water and stored in 100 μl aliquots at −20°C. Before assay, it was diluted 1:10.

Results

Ins(1,4,5)P_3 Accumulates in Advance of Deflagellation Induced by Low pH

We previously showed that a 5–10-fold increase in Ins(1,4,5)P_3 levels in *C. reinhardtii* occurred 50 s after the pH was adjusted to 4.5 with acetic acid (41). We now have examined Ins(1,4,5)P_3 levels at much earlier times and have found that a rapid initial accumulation of Ins(1,4,5)P_3 is triggered by acidification. Two typical profiles of accumulation of Ins(1,4,5)P_3 after adjusting the pH to 4.3–4.5 are shown in Fig. 1, *a* and *b*. Despite some variability in time intervals before Ins(1,4,5)P_3 accumulation as well as before extensive deflagellation (compare Fig. 1, *a* and *b*), a 1.6–2-fold increase of Ins(1,4,5)P_3 was always observed to take place before extensive deflagellation. Although some deflagellation was occasionally observed immediately after acidification (see Fig. 1 *a*), it showed no further increase until after Ins(1,4,5)P_3 had started to accumulate. Finally, it should be noted that the rapid initial increase of

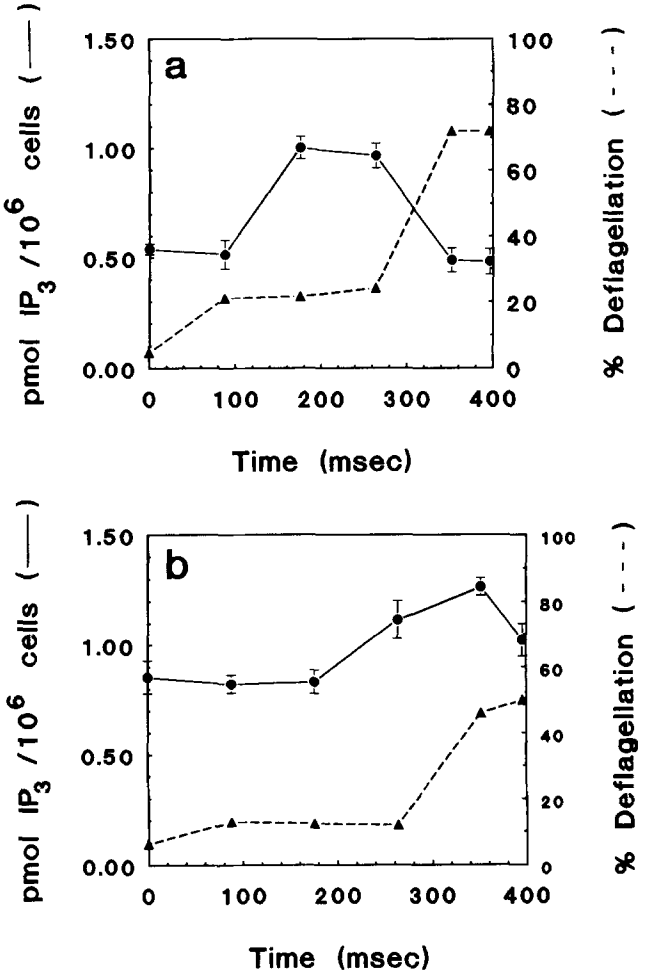

Figure 1. Rapid accumulation of Ins(1,4,5)P_3 precedes deflagellation induced by acidification. Wild-type *C. reinhardtii* was adjusted to pH 4.3–4.5 by addition of acetic acid and levels of Ins(1,4,5)P_3 (*closed circle*) and deflagellation (*closed triangle*) recorded as a function of time using a continuous-flow rapid-quench device. Determination of Ins(1,4,5)P_3 levels and the percent of cells that had lost both flagella were determined as described in Materials and Methods. Bars represent standard errors of five replicate determinations in samples from a single experiment. Time courses of Ins(1,4,5)P_3 accumulation and deflagellation are shown for two experiments (*a* and *b*), which represent typical responses of the four independent experiments that were performed.

$Ins(1,4,5)P_3$ does not persist but tends to return toward basal levels within 150 ms after peaking although, as observed earlier (41), a much larger increase in $Ins(1,4,5)P_3$ levels (5–10-fold) is ultimately observed 50 s after acidification.

Neomycin Blocks $Ins(1,4,5)P_3$ Production and Subsequent Flagellar Excision Whereas Pertussis Toxin Has No Effect

The amino glycoside antibiotic neomycin is an effective inhibitor of phospholipase C hydrolysis of $PtdIns(4,5)P_2$ (20). We previously showed that neomycin, after a 10-s preincubation, inhibited accumulation of $Ins(1,4,5)P_3$ observed 30 s after pH shock (41). In the present experiment, preincubation of wild-type cells with 10 μM neomycin for 15 s, was observed to inhibit the rapid, initial accumulation of $Ins(1,4,5)P_3$ as well as deflagellation (Fig. 2). These data further suggest that $Ins(1,4,5)P_3$ is acting as a second messenger and that its accumulation is upstream of deflagellation.

We previously demonstrated a stimulation of deflagellation and $Ins(1,4,5)P_3$ production by the G-protein activator, mastoparan (41). To further address the role of G-proteins in acid activation of phospholipase C, wild-type cells were incubated for 16 h with 1.33 μg/ml pertussis toxin, a potent inhibitor of some classes and subtypes of G-protein (27). However, the pertussis toxin treatment did not inhibit deflagellation induced by either acid or mastoparan nor did it inhibit the elevation of $Ins(1,4,5)P_3$ levels after these treatments (data not shown). Since this lack of an inhibitory effect could be caused by the cell wall of *C. reinhardtii* which may restrict access of the toxin to the membrane, a cell wall-less mutant, cw-92, was pretreated with 1.33 μg/ml pertussis toxin for 24 h. As in the case with wild-type cells, the toxin treatment of the cw-92 mutant cells had no effect on deflagellation induced by mastoparan (data not shown).

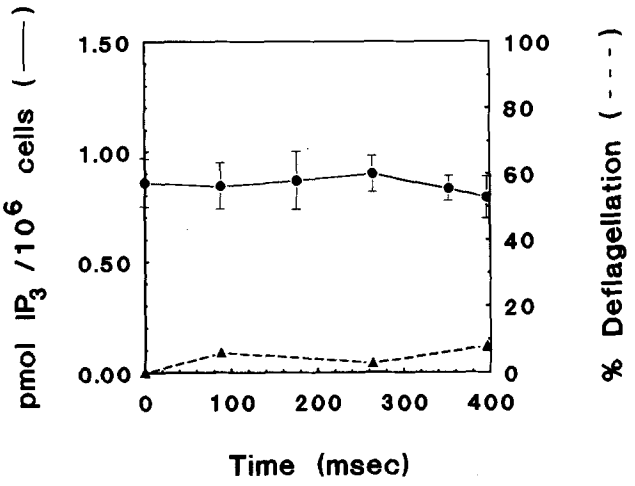

Figure 2. Neomycin inhibits both $Ins(1,4,5)P_3$ accumulation and deflagellation. Cells were incubated with 10 μM neomycin for 15 s before mixing with acetic acid and levels of $Ins(1,4,5)P_3$ (*closed circles*) and the percent of cells losing both flagella (*closed triangles*) was determined as in Fig. 1. Bars represent standard errors of five replicate determinations in samples from a single experiment which is representative of three independent experiments that were performed.

Extracellular Ca^{2+} Is Required For Deflagellation and Post-Deflagellation $Ins(1,4,5)P_3$ Accumulation

In preliminary attempts to address the role of external Ca^{2+} in deflagellation, cells, washed several times with Ca^{2+} free buffer, were found to exhibit slower rates of deflagellation. However, the final extent of deflagellation remained high (data not shown). Inclusion of 1 mM EGTA in the wash further slowed deflagellation; 50% of the cells were observed to be deflagellated 5 s after acidification (data not shown) which can be compared to a similar extent of deflagellation observed at 400 ms in control cells (Fig. 1). However, the extensive time required to deplete Ca^{2+} by washing in the presence of EGTA caused changes in cell morphology and motility (data not shown). Unfortunately, the removal of free Ca^{2+} by short exposures to EGTA without washing is not possible in these experiments because the chelated Ca^{2+} would be released by protonation of EGTA that would occur during acidification of the extracellular medium.

In an attempt to circumvent these problems, deflagellation was induced by mastoparan which has been found to cause deflagellation of *C. reinhardtii* in medium of neutral pH (41). Preincubation of cells for 2 min with millimolar EGTA, which inhibited deflagellation (see legend to Fig. 3) induced by 5 μM mastoparan, also inhibited the accumulation of $Ins(1,4,5)P_3$ observed 15–100 s after mastoparan treatment (Fig. 3). This EGTA effect supports our suggestion that extracellular Ca^{2+} is involved in flagellar excision and suggests that the production of $Ins(1,4,5)P_3$ observed between 10 and 100 s requires extracellular Ca^{2+}.

To investigate the role of Ca^{2+} channels in these re-

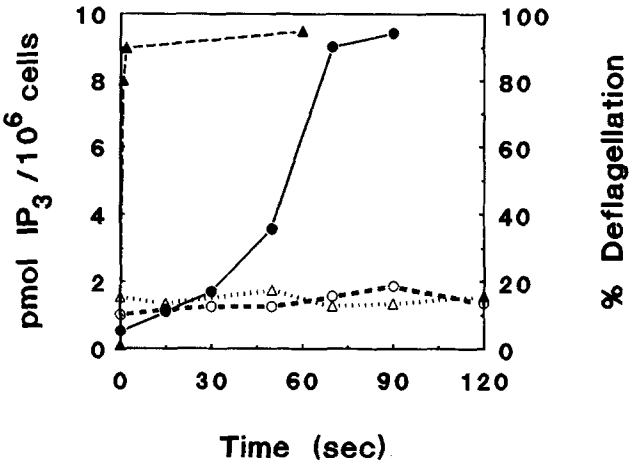

Figure 3. EGTA inhibits mastoparan induced $Ins(1,4,5)P_3$ accumulation. Cells were incubated for 2 min in the absence (*closed circles*) or presence of 1 mM (*open triangles*) or 2 mM (*open circles*) EGTA. Accumulation of $Ins(1,4,5)P_3$ was determined as a function of time after treatment of these cells with 5 μM mastoparan. Deflagellation (*closed triangles*) was recorded as a function of time for cells treated with mastoparan in the absence of EGTA. Approximately 60% of the cells still had flagella 1 min after mastoparan addition when the cells were preincubated with 1 mM EGTA; ~90% of the cells still had flagella 1 min after mastoparan addition when the cells were preincubated with 2 mM EGTA. Results are representative of two independent experiments assayed in duplicate.

sponses, the effects of calcium channel blockers, Cd^{2+} (18), La^{3+} (35), and verapamil, nifedipine, ω-conotoxin, TMB-8, and ruthenium red (40) on deflagellation were investigated (Table I). Cd^{2+} at 300 μM and La^{3+} at 50 μM inhibited deflagellation and the extensive accumulation of Ins(1,4,5)-P_3 observed at 60 and 30 s, respectively, in response to mastoparan treatment. La^{3+} at 300 μM also inhibited deflagellation and Ins(1,4,5)P_3 accumulation in response to pH shock. On the other hand, the channel blockers verapamil, nifedipine, and ω-conotoxin, and the cell permeable intracellular Ca^{2+} channel blocker TMB-8 had no effect on deflagellation. In contrast, ruthenium red, a blocker of an Ins(1,4,5)P_3-gated Ca^{2+} channel in olfactory cilia and sarcoplasmic reticulum (39, 42, 49), completely blocked deflagellation induced by either acidification or mastoparan. Finally, preincubation for 30 min with thapsigargin, a Ca^{2+} ATPase inhibitor (28) was observed to have no inhibitory effect; deflagellation was stimulated by low pH or mastoparan to the same extent as control cells (data not shown).

Cd^{2+} and La^{3+} Prevent Rapid Deflagellation Without Inhibiting Rapid Ins(1,4,5)P_3 Accumulation

Wild-type cells were preincubated for one min with 50 μM La^{3+} (in culture medium modified by omitting phosphate which precipitates La^{3+}). Ins(1,4,5)P_3 exhibited a normal elevation of about twofold 300 ms after acidification, while both the rapid (Fig. 4 *a*) and long term (Table I) stimulation of deflagellation by acidification were greatly inhibited. Ins(1,4,5)P_3 levels returned to basal values within 150 ms after the peak as was observed in the absence of La^{3+} (Fig. 1). Cd^{2+}, also inhibited rapid deflagellation without inhibiting the rapid, transient accumulation of Ins(1,4,5)-P_3 (Fig. 4 *b*), though deflagellation did occur after 30 s (data not shown).

*Table I. Effect of Calcium Channel Blockers on Deflagellation and Ins(1,4,5)P_3 Accumulation in Response to pH Shock or Mastoparan**

Pretreatment	Deflagellation stimulus	Deflagellation‡ (%)	Ins(1,4,5)P_3§ (% *control*)
None	acid	100	700
None	mastoparan	>90	300
$LaCl_3$‖	acid	10	59
$LaCl_3$‖	mastoparan	11	41
$CdCl_2$¶	mastoparan	16	13
Verapamil + nifedipine**	acid	100	868
Verapamil + nifedipine**	mastoparan	>90	191
TMB-8‡‡	acid	100	–
TMB-8‡‡	mastoparan	>90	–
ω-conotoxin§§	acid	100	–
ω-conotoxin§§	mastoparan	>90	–
Ruthenium red‖‖	acid	10	–
Ruthenium red‖‖	mastoparan	2.6	121

* At time 0, either cells were adjusted to pH 4.3–4.5 by dropwise addition of 2 N acetic acid or mastoparan was added to the cell suspension to a concentration of 5 μM. Cells were stirred with a magnetic stir bar at 22°C.
‡ An aliquot of cells was added to five drops of tincture of iodine 60 s after acidification or mastoparan treatment. Percent of cells deflagellated indicates cells that lost both flagella. Two hundred cells were counted for each treatment.
§ A 1-ml aliquot of cells was quenched with 1/20th vol of ice-cold 100% TCA (wt/vol) 30 s after acidification or mastoparan treatment. Ins(1,4,5)P_3 was assayed as described in Materials and Methods. Values are expressed as percent of Ins(1,4,5)P_3 in control cells before additions.
‖ $LaCl_3$ (50 μM) was added 1 min before acidification or mastoparan treatment.
¶ $CdCl_2$ (300 μM) was added 1 min before treatment with 5 μM mastoparan.
** Verapamil (100 μM) and nifedipine (50 μM) was added together 1 min before acidification or mastoparan treatment. Neither verapamil nor nifedipine individually had any inhibitory effect on deflagellation in response to pH shock or mastoparan treatment (data not shown).
‡‡ TMB-8 (800 μM) was added 1 min before acidification or mastoparan treatment.
§§ ω-conotoxin (1 μM) was added 15 min before acidification or mastoparan treatment.
‖‖ Ruthenium red (10 μM) was added 15 min before acidification or mastoparan treatment.

Heparin Inhibits Deflagellation

Cells were incubated with 100 μg/ml heparin for 5 min be-

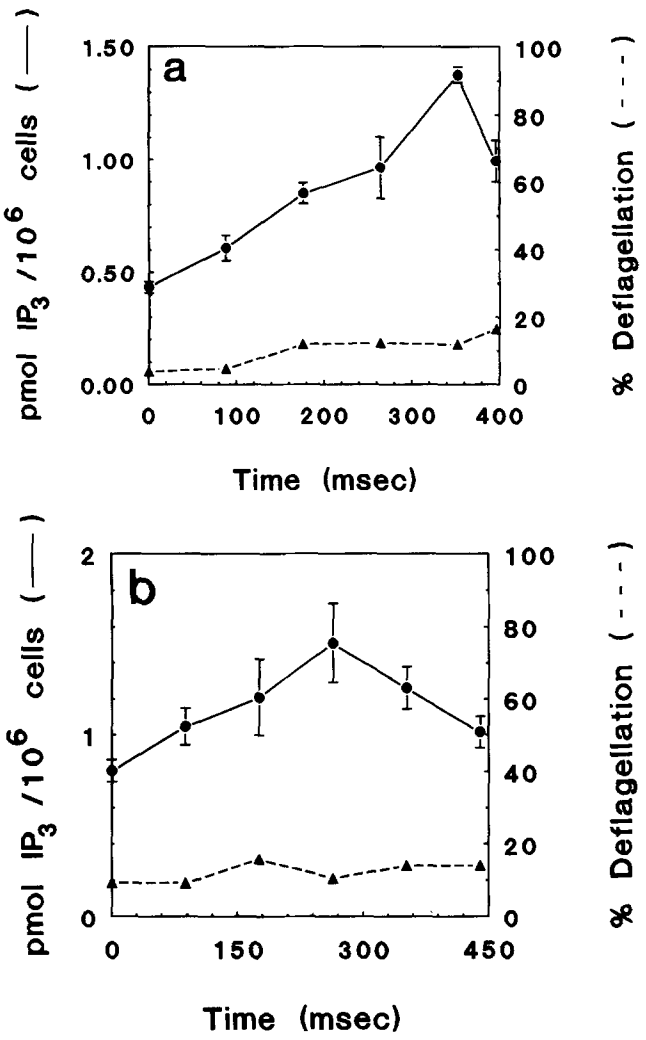

Figure 4. Non-specific Ca^{2+} channel blockers inhibit flagellar excision without affecting rapid Ins(1,4,5)P_3 accumulation. (*a*) Cells were preincubated with 50 μM La^{3+} ions for 1 min before adjusting to pH 4.3. Determination of Ins(1,4,5)P_3 levels (*closed circles*) and the percent of cells losing both flagella (*closed triangles*) was determined as in Fig. 1. (*b*) As in *a*, except cells were preincubated with 300 μM Cd^{2+} ions for 1 min before acid. Ins(1,4,5)P_3 levels (*closed circles*) and percent cells losing both flagella (*closed triangles*) were determined as in Fig. 1. Bars represent standard errors of five replicate determinations in samples from a single experiment which is representative of two independent experiments that were performed.

fore mastoparan treatment. Since heparin is unlikely to gain access to the cell interior, poly-L-lysine was included at a concentration of 30 μg/ml as a permeabilizing agent (36). Poly-L-lysine, at this concentration, had no effect on deflagellation or cell morphology. Approximately 70% inhibition of mastoparan-induced deflagellation was observed in cells treated with heparin alone, but deflagellation induced by mastoparan was completely inhibited when cells were treated with heparin in the presence of poly-L-lysine (data not shown).

Rapid Accumulation of Ins(1,4,5)P_3 Is Induced by Low pH in fa-1 Cells and bald-2 Cells Without Deflagellation

Fa-1, a flagellar excision-defective mutant, did not deflagellate in response to low pH but did accumulate Ins(1,4,5)P_3 (Fig. 5), although the accumulation was somewhat delayed compared to the response of wild-type cells (compare Figs. 1 and 5). Furthermore, bald-2, a mutant that lacks flagella, also accumulated Ins(1,4,5)P_3 in response to acidification though it clearly could not deflagellate (data not shown). These results further support the conclusion that Ins(1,4,5)-P_3 accumulation is not a response to deflagellation.

Discussion

Rapid, Transient Accumulation of Ins(1,4,5)P_3 Precedes Deflagellation

Our earlier finding that acid-induced deflagellation of *C. reinhardtii* is associated with PtdIns(4,5)P_2 breakdown and an accumulation of Ins(1,4,5)P_3 and phosphatidic acid (41) suggests that Ins(1,4,5)P_3 may be a second messenger mediating signals that cause deflagellation, perhaps by mobilizing Ca^{2+}, which has been shown by Salisbury to induce deflagellation (45, 46). To test this hypothesis, our first objective was to determine if the accumulation of Ins(1,4,5)P_3 is an early event in the process. Unfortunately, in our early study, deflagellation was complete within 5 s after acidification which was the earliest time that Ins(1,4,5)P_3 accumulation could be accurately measured by the techniques available to us at that time.

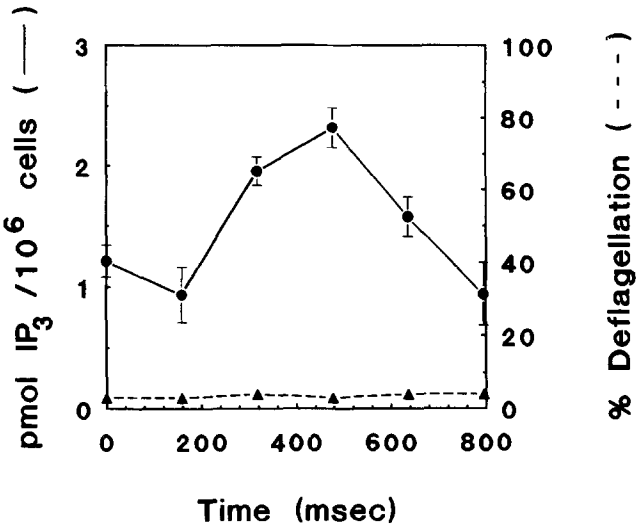

Figure 5. Excision-defective cells, fa-1, accumulated Ins(1,4,5)P_3 rapidly in response to pH shock but did not deflagellate. Cells were mixed with acetic acid to pH 4.5 and levels of Ins(1,4,5)P_3 (*closed circles*) and the percent of cells losing both flagella (*closed triangles*) was determined as in Fig. 1. Bars represent standard errors of five replicate determinations in samples from a single experiment which is representative of two independent experiments that were performed.

By means of a continuous-flow rapid-quench technique, we now find that the response of deflagellation to acidification is rapid, essentially complete within a second. More importantly, we also find that this response is preceded by an even more rapid initial accumulation of Ins(1,4,5)P_3. This accumulation, although small (1.6–2.0-fold) compared to the long term accumulation of Ins(1,4,5)P_3 after acidification (5–10-fold), is significant but only transitory with a peak being exhibited between 280 and 380 ms after acidification. This response is reminiscent of the rapid production of second messengers that have been reported in several animal systems. Stimulation of olfactory cilia by selected odorants elevates either cAMP or Ins(1,4,5)P_3 to peak levels within 100 ms (5, 6). Similarly, cGMP and Ins(1,4,5)P_3 act to transduce signals that elicit rapid cellular responses in vertebrate (33, 48) and invertebrate (19) visual transduction, respectively.

The conclusion that Ins(1,4,5)P_3 is responsible for mediating deflagellation rather than some unrelated signal transduction pathway is supported by the following observations: (*a*) Both deflagellation and the rapid, transient accumulation of Ins(1,4,5)P_3 are inhibited by neomycin, an inhibitor of phospholipase C catalyzed hydrolysis of PtdIns(4,5)P_2. (*b*) Both deflagellation and the rapid, transient accumulation of Ins(1,4,5)P_3 show a considerably slower response to mastoparan compared to acid treatment. (*c*) The accumulation of Ins(1,4,5)P_3 does not occur in response to acid treatment of imp-4 mutant cells (Yueh, Y. G., and R. C. Crain, unpublished results) which are defective in acid-induced deflagellation (Saito, T., and U. W. Goodenough, unpublished results). (*d*) Rapid accumulation of Ins(1,4,5)P_3 without deflagellation was observed after acidification of fa-1 cells, a flagellar excision mutant which, unlike wild-type cells, does not deflagellate when the detergent permeabilized cells are treated with Ca^{2+} (46). These cells can generate Ins(1,4,5)P_3 and presumably mobilize Ca^{2+}, but fail to deflagellate because sufficient contraction of fibers in the transition zone does not occur in response to elevated levels of Ca^{2+} (46).

Acid pH Activates a G-Protein Sensitive Phospholipase C

Since acidification of the cytosol is required to stimulate flagellar excision (26), we believe that variations in rates of flagellar excision observed in our experiments as seen in Fig. 1, may result from differences in $[H^+]$. In support of this postulate, we have found variations in the rate of Ins(1,4,5)P_3 accumulation and of deflagellation that correlate with the final pH of the medium. Below pH 4, deflagellation was very rapid, i.e., complete within 500 ms (unpublished results) whereas at pH 4.3–4.5, which is the condition used in the present experiments, deflagellation did not occur to a sig-

nificant extent until after 300 ms and was complete only after ~1 s. The signal transduction cascade leading to flagellar excision might therefore involve a direct activation of phospholipase C by acid or an indirect activation via G-protein regulated phospholipase C (3, 41). Our observation that the G-protein activator mastoparan, at micromolar concentrations, rapidly activates phospholipase C and stimulates deflagellation support the latter hypothesis. Though the G-protein inhibitor pertussis toxin (32) fails to inhibit the response it is quite possible that the phospholipase C in this system is insensitive to pertussis toxin, as has been reported for a number of other systems (3, 11, 37).

Extracellular Ca^{2+} Is Required to Achieve Deflagellation

The rapid, transient accumulation of Ins(1,4,5)P_3 appears necessary to achieve deflagellation, but it alone is not sufficient. The prevention or delay of deflagellation by the nonspecific Ca^{2+} channel blockers, Cd^{2+} and La^{3+}, suggests that entry of Ca^{2+} is also required. Mobilization of Ca^{2+} from an Ins(1,4,5)P_3-sensitive intracellular pool alone has been found to be insufficient to elicit a physiological response in many systems (1, 2, 29). Mechanistically, Ca^{2+}- or Ins(1,3,4,5)P_4-regulated Ca^{2+} entry may follow the initial accumulation of Ins(1,4,5)P_3 and mobilization of Ca^{2+} from internal stores (the vacuole or ER) (29), though we have no evidence for production of Ins(1,3,4,5)P_4 in response to acid (41). Alternatively, Ca^{2+} influx may be stimulated either by direct binding of Ins(1,4,5)P_3 to the plasma membrane (31, 42, 43), or by capacitative release of Ca^{2+} stores proposed to be close to the plasma membrane, initiated by Ins(1,4,5)P_3 (2).

These results suggest that entry of extracellular Ca^{2+}, downstream of "primer" Ins(1,4,5)P_3 accumulation, is required for deflagellation. The increased entry of Ca^{2+}, which in turn can directly activate phospholipase C to produce additional Ins(1,4,5)P_3, could thus further amplify or prolong the signal. Support for this conclusion is provided by the observation that La^{3+} had no effect on the ms accumulation of Ins(1,4,5)P_3 but totally blocked the 5–10-fold increase in Ins(1,4,5)P_3 that occurred 30–60 s after acid treatment. Similarly ruthenium red inhibited deflagellation induced by both acid and mastoparan and Ins(1,4,5)P_3 accumulation after acid treatment, again consistent with the involvement of Ins(1,4,5)P_3 in deflagellation. Ruthenium red, a calcium channel blocker has previously been shown to block the integrated electrical response of the olfactory epithelium of catfish to L amino acid (39, 42), also mediated by rapid accumulation of Ins(1,4,5)P_3. Finally, the lack of effect of other channel blockers, e.g., verapamil and nifedipine, is also consistent with our hypothesis for an Ins(1,4,5)P_3-sensitive calcium channel in the plasma membrane (40, 42).

Deflagellation Requires Ca^{2+} Entry through a Heparin Sensitive Channel

At least two different channels that mediate Ca^{2+} release from a nonmitochondrial intracellular pool have been described in animal systems (2, 12) but only one has been well characterized, an Ins(1,4,5)P_3-sensitive Ca^{2+} channel that has been purified and sequenced (38, 51) and its properties well described (8, 14, 15, 44). A similar channel has been described in plant vacuoles (47) and plant microsomal preparations (13). In both plant (7) and animal (40) systems, this channel is inhibited by micromolar concentrations of heparin. In view of our finding that flagellar excision in response to mastoparan is inhibited by heparin, this channel would appear to play an essential role in the deflagellation process.

We have further attempted to identify the involvement of Ca^{2+} mobilized from intracellular pools. Thapsigargin inhibits Ca^{2+} ATPases on intracellular membranes and thus prevents Ca^{2+} sequestration. Most reports have shown that thapsigargin depletes Ca^{2+} in Ins(1,4,5)P_3-sensitive pools (16, 21, 34, 52) though in one case, thapsigargin was found to deplete Ca^{2+} in an Ins(1,4,5)P_3-insensitive pool (17). We found that cells preincubated with thapsigargin for 30 min still undergo deflagellation in response to acid shock. This observation suggests that Ca^{2+} pools involved in the deflagellation response are unresponsive to thapsigargin. Although this may result because of thapsigargin specificity, we propose that Ins(1,4,5)P_3 acts directly to enhance Ca^{2+} entry, as has been observed in the olfactory system (39, 42) rather than through mobilization of Ca^{2+} from intracellular stores.

Concluding Remarks

Chlamydomonas sheds its flagella in response to a variety of chemical and mechanical treatments. Each flagellum is reproducibly excised near its base on the cell body, just distal to the transition zone, the region of the cell where the flagellar axoneme joins the basal body (46). Within minutes after deflagellation, the coordinate synthesis of tubulin and many other mRNAs is upregulated (reviewed in 30). These events give rise to assembly of the '9+2' axoneme; flagellar regeneration is essentially complete within 90 min. Little is known about the biochemical pathways that couple stress signals to deflagellation and that couple deflagellation to the subsequent synthesis and assembly of flagellar components. We hypothesize that Ins(1,4,5)P_3 acts as a second messenger which mediates deflagellation based on the temporal correlation of these two events and on the sensitivity of deflagellation to ruthenium red and other Ca^{2+} channel blockers. That Ins(1,4,5)P_3 is produced in response to the stress signal rather than the deflagellation event has been demonstrated by our studies on two mutants, fa-1 which does not deflagellate because of a defect that appears to be in the axoneme attachment to the basal body (46), and bald-2 which lacks flagella. In the future, we hope to use additional mutants to examine the putative "pH receptor," the G-proteins, and the Ins(1,4,5)P_3 receptor protein(s) involved in this process.

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