Inositol Phospholipid Metabolism May Trigger Flagellar Excision in *Chlamydomonas reinhardtii*

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Abstract. *Chlamydomonas reinhardtii* cells shed their flagella in response to environmental stress. Under favorable conditions, flagella are quickly regrown. To learn more about the signals that trigger flagellar excision and regrowth we have investigated inositol phospholipid metabolites, molecules implicated in signal transduction in several other systems. After deflagellation by low pH or mastoparan, a potent activator of G proteins, there was a rapid increase in levels of inositol 1,4,5-trisphosphate measured by use of receptor-binding assays and HPLC. This increase was concomitant with a decrease in levels of phosphatidylinositol 4,5-bisphosphate and was followed by an increase in phosphatidic acid, results consistent with activation of phospholipase C and diacylglycerol kinase. Additional experiments suggest that this activated phospholipase C is not important for flagellar regrowth but plays a role in informing the excision apparatus of the environmental stress. Addition of neomycin (an inhibitor of phospholipase C) before exposure of cells to low pH or mastoparan prevented the increase in inositol 1,4,5-trisphosphate and also prevented deflagellation. Addition of neomycin after deflagellation blocked increases in inositol 1,4,5-trisphosphate that normally followed deflagellation, but did not block flagellar assembly. Furthermore, a flagellar excision-defective mutant, fa-1, did not shed its flagella in response to low pH or mastoparan, yet both of these agents activated phospholipase C in these cells. The results suggest that activation of phospholipase C, possibly via a G protein, is a proximal step in the signal transduction pathway inducing deflagellation in *Chlamydomonas.*

The unicellular green alga *Chlamydomonas reinhardtii* sheds its two flagella when confronted with environmental stresses such as pH shock or mechanical shear (38, 39). Deflagellation is quickly followed by increased synthesis of flagellar mRNA (2, 27, 31) and protein (29) and regrowth of flagella, which reach full length ~90 min after deflagellation. Little is known about the biochemical pathways that couple environmental stress to deflagellation and the subsequent synthesis and assembly of flagellar components. Initially, the environmental stress (e.g., pH shock) is communicated to the molecular apparatus responsible for deflagellation, and subsequently the deflagellated condition of the cell is communicated to the elements controlling relevant gene expression and flagellar assembly. The involvement of Ca²⁺ in control of deflagellation (14, 40, 42), flagellar mRNA accumulation (12a), and flagellar assembly (29) led us to postulate that products of inositol phospholipid hydrolysis might be components of signal transduction in this system. During signal transduction via the inositol phospholipid pathway in other systems, signal-activated phospholipase C hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) yielding the “second messengers” inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and 1,2-sn-diacylglycerol (DAG). The Ins(1,4,5)P₃ and DAG initiate synergistic cascades of metabolic activity; Ins(1,4,5)P₃ binds cooperatively to a receptor/channel causing release of Ca²⁺ into the cytosol (6), while diacylglycerol, in concert with Ca²⁺, activates protein kinase C (21, 28). Not surprisingly, many variations on this theme have been reported (for example, references 5, 6, 18, 20, 23, 30, 32, 35, 46). The inositol phospholipids are present in *Chlamydomonas* (9, 25, 37) but a signal transducing activation of phospholipase C has not been demonstrated.

We initiated studies to determine if *Chlamydomonas* uses this signaling system during flagellar excision or regrowth. We observed increased inositol phospholipid metabolism within seconds of pH shock or treatment with mastoparan.

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1. Abbreviations used in this paper: DAG, 1,2-sn-diacylglycerol; Ins(1,4,5)-P₃, inositol 1,4,5-trisphosphate; Ins(3,4,5)-P₃, inositol 1,3,4,5-tetrakisphosphate; PtdInsP, phosphatidylinositol phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol 4,5-biphosphate; PtdOH, phosphatidic acid; t, time.

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Although Ins(1,4,5)P₃ accumulates to high levels after deflagellation, experiments with neomycin suggest that this accumulation may not be necessary for normal flagellar regeneration. Instead, our data suggest that a G protein-mediated activation of phospholipase C triggers the deflagellation event itself.

**Materials and Methods**

**Cells and Culture Conditions**

*C. reinhardtii* 137c mt-(cc 124) and mutant fa-1 (cc 1370) were obtained from E. Harris (Chlamydomonas Genetics Center, Duke University, Durham, NC). Cells were grown at 25°C in the media described by Gorman and Levine (19), modified by omitting the glacial acetic acid, and titrating to pH 7.0 with concentrated HCl (22). A light to dark cycle of 12:12 with cool white fluorescent light (photons fluence, 40 μmol m⁻² s⁻¹) illuminated cultures continuously bubbled with 5% CO₂ in air and shaken at 115 rpm on a rotary shaker (B. Braun Instruments, Burlingame, CA). Cells were used in early- to mid-log phase growth. When necessary, cells were concentrated by low speed centrifugation (5 min, 500 g, at room temperature).

**Experimental Protocol for Deflagellation by pH Shock**

Beginning at time (t) = 0, 1 N acetic acid was added dropwise to 30 ml of cells (2 x 10⁶ cells/ml) and mixed with a magnetic stir bar. At t = 50 s, 1 N potassium hydroxide was added dropwise to return the culture to its initial pH. At specified times, 1-ml aliquots of cells were added to five drops of tincture of iodine. Real time observation demonstrated that this preservative prevented deflagellation when added immediately after acidification.

**Preparation of Samples for Biochemical Analysis**

Aliquots of the cultures, treated as described in the legends to figures, were mixed with 1/20th vol of ice-cold 100% TCA (wt/vol). The precipitate was removed by centrifugation (10 min, 650 g, at 4°C). TCA was removed from the supernatant by extracting four times with five volumes of water-saturated ether. The final aqueous extract was analyzed for inositol phosphates as described below. Lipids were extracted from the TCA pellet by a variation of the method of Folch (16). The pellets were mixed with 2:1 chloroform/methanol (vol/vol). After 15 min at room temperature, an aqueous solution containing 0.15 N HCl and 0.6% NaCl was added to a final ratio of chloroform/methanol/water of 8:4:3 (vol/vol/vol). The phases were mixed by vortex and separated by centrifugation (10 min, 650 g, at room temperature). The organic phase was removed to a clean glass test tube and washed once with 1 ml of pure upper phase (chloroform:methanol:0.15 N HCl, 0.6% NaCl, 3:48:47, vol/vol/vol). The lipid extract was evaporated to dryness under a stream of nitrogen and stored in 2:1 chloroform/methanol at -20°C.

**Quantitation of Ins(1,4,5)P₃**

Concentrations of Ins(1,4,5)P₃ were determined by isotope dilution using the commercially available radioreceptor kit (Amersham Corp., Arlington Heights, IL) or receptor preparations of Van Haastert (49). Samples prepared as described above were neutralized to pH 7.5 before the assay by the addition of 16% sodium carbonate. In some experiments Ins(1,4,5)P₃ was separated from other metabolites by HPLC before assay. To do this, samples were loaded onto a Partisil-10-SAX anion exchange column (Whatman Laboratory Products, Inc., Clifton, NJ) and eluted with a linear gradient of ammonium formate brought to pH 7.7 with phosphoric acid (13). Identification of the fractions contained Ins(1,4,5)P₃ was based on coelution with [³²P]inositol-labeled Ins(1,4,5)P₃ standard (New England Nuclear, Boston, MA).

**Separation and Quantitation of Ins(1,3,4,5)P₃**

Aqueous extracts from cells labeled in vivo with [³²P]PP, were analyzed by HPLC using a gradient of ammonium formate as described by Batty and co-workers (4). Tritiated Ins(1,3,4,5)P₃ standard was purchased from New England Nuclear.

**Separation, Quantitation, and Identification of Phospholipids**

Phospholipids were labeled in vivo with [³²P]Pi, as described in the relevant figure legends. The lipids were separated by TLC (Silica gel H with 1% sodium oxide; AnalTech, Newark, DE) developed in 90:70:20 chloroform/methanol/fresh 4 N NH₄OH (13). Spots were visualized by autoradiography and scraped for liquid scintillation counting. [³²P]PP-labeled lipid samples were identified by comigration with authentic [³²H]inositol-labeled standards (New England Nuclear) on TLC, followed by monomethylamine deacylation (8) and HPLC analysis (51).

**Results**

**Deflagellation Is a Rapid Response**

To study signal transduction in this system, it was important initially to determine the time course of flagellar loss at low pH. The times of deflagellation and pH change in a typical experiment, shown in Fig. 1a, demonstrated that deflagellation was a very rapid event. From Fig. 1b, it can be estimated that a pH of ~5.0 was required for deflagellation by 95% of the cells.

**Ins(1,4,5)P₃ Accumulates Subsequent to pH Shock**

We observed a rapid and large increase in Ins(1,4,5)P₃ (detected by radioreceptor assay) upon acidification (Fig. 2). In six independent experiments, the peak in putative Ins(1,4,5)P₃ was ~fivefold higher than basal. Photosynthetic organisms synthesize many novel inositol metabolites with unknown affinity for the Ins(1,4,5)P₃ receptor. To verify that the Ins(1,4,5)P₃ receptor binding measured in the isothe dilution assay was actually Ins(1,4,5)P₃, HPLC methods were used. The data in Table I demonstrate that receptor cross-reactive activity in our extracts coeluted with [³²H]Ins(1,4,5)P₃ standard on HPLC (see Materials and Methods).

Although only the data from a single HPLC fraction is shown, the [³²H]Ins(1,4,5)P₃ standard eluted in two adjacent fractions, with almost equal counts in each (the ratio of standard in the two fractions was 1.0:1.2); assayed Ins(1,4,5)P₃ levels showed the same detailed elution profile (the ratio in the same two fractions was 1.0:1.3). The identification of Ins(1,4,5)P₃ also was corroborated by radiolabeling the cells in vivo. Because C. reinhardtii incorporates inositol poorly (<0.08% of added radioactivity is incorporated into lipid after a 43-h incubation; reference 37), cells were labeled with [³²P]Pi. When aqueous extracts from such cells were separated by HPLC, a large number of fractions contained peaks of radioactivity. [³²H]Ins(1,4,5)P₃ standard eluted with a shoulder just after a larger peak of radioactivity of [³²P]Pi-labeled compounds. The increase in the [³²P]Pi coeluting with [³²H]Ins(1,4,5)P₃ standard in deflagellated cells relative to control cells was in good agreement with the
measured increase in Ins(1,4,5)P₃ levels based on receptor binding (Table 1).

**Ins(1,3,4,5)P₄ Does Not Accumulate After pH Shock**

In many systems, accumulations of Ins(1,3,4,5)P₄ and DAG accompany or immediately follow the production of Ins(1,4,5)P₃ (6). In *Chlamydomonas* however, authentic [³H]-Ins(1,3,4,5)P₄ standard, mixed with extracts from either control or deflagellated cells labeled to isotopic equilibrium with [³²P]Pi, eluted as a single peak cleanly resolved from all fractions containing detectable levels of [³²P]Pi, (data not shown). This demonstrated that *C. reinhardtii* cells contained little or no basal Ins(1,3,4,5)P₄ and that increased labeling of this compound on deflagellation was not observed. In this regard, Ins(1,4,5)P₃ metabolism by *C. reinhardtii* may be similar to the pathways elucidated for Dictyostelium (49).

**Figure 1.** Characterization of deflagellation response. (a) Deflagellation and pH changes after onset of addition of acetic acid (see Materials and Methods). The open circles indicate measured pH; the extent of deflagellation in the culture (●) is expressed as the percentage of cells that had shed both flagella. (b) Effect of pH on extent of deflagellation. Cultures were rapidly brought to the indicated pH and after 60 s were fixed with tincture of iodine. In both a and b, at least 100 cells were counted for each determination.

**Figure 2.** Ins(1,4,5)P₃ accumulation subsequent to deflagellation by pH shock. Ins(1,4,5)P₃ levels were quantified by isotope dilution radio-receptor assay as described in Materials and Methods. Bars are the range of duplicate samples in the same experiment. The time course of accumulation shown is typical of results obtained in six independent experiments.

**Metabolism of Inositol Phospholipids Accompanies the Increased Levels of Ins(1,4,5)P₃**

To further elucidate the biochemical events associated with deflagellation, we identified and quantified changes in the levels of lipids implicated in this signaling pathway: the inositol phospholipids, phosphatidic acid (PhDiOH), and DAG. The quantification of phospholipids described below was based on identification by comigration of *C. reinhardtii* lipids with [³H]-labeled authentic standards on TLC. Before routine use, this procedure was validated by monomethylamine deacylation (8) of lipids initially separated by TLC. In each case, TLC-purified, in vivo [³²P]-labeled, *C. reinhardtii* lipids yielded deacylation products which coeluted with deacylated authentic [³H]-labeled standards on HPLC.

**Table I. Verification That Ins(1,4,5)P₃ Is the Cross-Reactive Agent in the Isotope Dilution Assay**

|                  | Ins(1,4,5)P₃ receptor binding | [³²P]Ins(1,4,5)P₃ by HPLC |
|------------------|------------------------------|--------------------------|
| Total extract HPLC fraction 29 |                 | 1120                     |
| HPLC fraction 29 | 60 s                         | 6500                     |
| Fold increase    | 6.0                          | 5.8                      |

10 ml of aqueous extract from control cells and from cells 60 s after the onset of pH shock were prepared as described in Materials and Methods. The extract was liophilized, and each was dissolved in 1 ml of water with 0.01 µCi of [³H]Ins(1,4,5)P₃ standard. The samples were separated by HPLC (see Materials and Methods) with 15 s fractions collected during elution of InsP₃. Fractions containing Ins(1,4,5)P₃ (fractions 28 and 29) were identified by liquid scintillation counting of aliquots. Ammonium formate was removed from the eluate by two cycles of lyophilization. The samples were dissolved in water, neutralized with concentrated sodium hydroxide, and assayed for Ins(1,4,5)P₃ by isotope dilution. To control for the effects of the HPLC buffer on the assay, a standard curve was prepared with corresponding HPLC elute (treated as described) from a run with no sample injected. The data in the third column show the corresponding HPLC separation of Ins(1,4,5)P₃ from cells labeled in vivo with [³²P]Pi (see legend to Fig. 3 for labeling protocol).
For phosphatidylinositol (PtdIns), PtdOH, and PtdIns-(4,5)-P₂, single peaks of ³²P were observed, in each case coeluting with the corresponding ³H standard. This indicated that, for these lipids, the TLC separation was clean. In the case of PtdInsP, collection of 10-s fractions during the anticipated elution of glycerol-phosphoinositol 4-phosphate (PtdIns[4]P), the other eluted just before the standard, in the position expected for glycerophosphoinositol 3-phosphate (1, 51). We observed preferential labeling of PtdIns[4]P in short-term (10-min) labeling experiments (~80% of PtdInsP is PtdIns[4]P whereas high levels of putative PtdIns(3)P (~50%) were detected after long-term (46 h) labeling. As the respective lipid isomers corresponding to these deacylation products comigrated on TLC, we have used the generic label “PtdInsP” in this article to describe the compound quantitated by TLC.

When phosphate-deprived cells were labeled in vivo with ³²P, for 46 h before pH shock, PtdIns(4,5)P₂ levels dropped rapidly and returned to basal levels after neutralization (Fig. 3 a). PtdOH levels also increased (Fig. 3 b) with a slight lag relative to the accumulation of Ins(1,4,5)P₃ (Fig. 2) and disappearance of PtdIns(4,5)P₂ (Fig. 3 a). A transient accumulation of PtdIns was apparent after deflagellation (Fig. 3 c). Data on turnover of PtdInsP is not shown because the presence of two isomers in the material separated by TLC (see above) made interpretation of the results difficult (37).

In a deflagellation experiment yielding an accumulation of 5 pmol of Ins(1,4,5)P₃/10⁶ cells above control levels of 1 pmol/10⁶ cells, <1 pmol of DAG/10⁶ cells accumulated (above a background of 3.1 pmol/10⁶ cells). Similar results were obtained in two other experiments. This is consistent with the accumulation of PtdOH reported above.

The metabolite changes subsequent to pH shock were suggestive of signal-responsive activation of phospholipase C and diacylglycerol kinase, but it was not clear at which step these metabolites were acting. For example, low pH could activate phospholipase C and initiate a cascade resulting in deflagellation. Alternatively, other mechanisms could induce deflagellation, and the loss of flagella could activate phospholipase C. These and other possible causes and consequences of phospholipase C activation were investigated as described below.

**Ins(1,4,5)P₃ Accumulation Occurs in Response to Low pH in the Absence of Deflagellation**

Two lines of evidence indicated that low pH plays a direct role in elevating levels of Ins(1,4,5)P₃. First, the beginning of decay of Ins(1,4,5)P₃ levels was coincident with the termination of the low pH treatment. Whether neutralization began at 20, 50, or 80 s after t = 0, the Ins(1,4,5)P₃ levels began to decrease within seconds of addition of the first few drops of base (Fig. 4). This suggested that Ins(1,4,5)P₃ accumulation might be directly influenced by pH. Further evidence that Ins(1,4,5)P₃ accumulation was a direct response to the low pH treatment was obtained using the fa-1 mutant. This C. reinhardtii mutant does not shed its flagella in response to pH shock, apparently because of a defect in the ex-
Ins(1,4,5)P3 Accumulation May Trigger Flagellar Excision

The aminoglycoside antibiotic neomycin, which binds PtdIns(4,5)P2, preventing its hydrolysis by phospholipase C (17), has been used as a pharmacologic tool for investigating signal transduction by the inositol phospholipid system (for example see references 11, 43, 47). Treatment of C. reinhardtii cells with micromolar levels of neomycin sulphate, for 10 s before \( t = 0 \), prevented both Ins(1,4,5)P3 accumulation and deflagellation in response to pH shock (Fig. 5). In a control experiment, sodium sulphate did not inhibit the deflagellation response (>98% of cells shed their flagella in response to 40 s at pH 4.3 in the presence of 300 \( \mu \)M sodium sulphate). These data suggest that phospholipase C activation plays a role in triggering the deflagellation response to pH shock.

The G-Protein Activator, Mastoparan, Induces Ins(1,4,5)P3 Accumulation and Deflagellation Independent of Low pH

To address the possibility that Ins(1,4,5)P3 accumulation and deflagellation might be independent responses to low pH, we used the wasp venom tetradecapeptide, mastoparan. Mastoparan is a potent activator of G proteins (24) and some isotypes of phospholipase C are responsive to activated G proteins (45, 50, 52). Fig. 6 illustrates that mastoparan stimulated accumulation of Ins(1,4,5)P3. In samples taken 30 s after the addition of mastoparan, 2 \( \mu \)M mastoparan led to a threefold increase in Ins(1,4,5)P3 levels; 8 \( \mu \)M led to a fivefold increase. These increases in Ins(1,4,5)P3 were comparable to increases observed with pH shock (Table I and Fig. 2). More importantly, these increases in Ins(1,4,5)P3 levels also were associated with a similar change in the extent of deflagellation. Samples treated with 2 \( \mu \)M mastoparan were approx. 50% deflagellated; 8 \( \mu \)M mastoparan resulted in complete deflagellation. The inactive mastoparan analogue, Mas-17 (24) did not cause either Ins(1,4,5)P3 accumulation or deflagellation: after 30 s of exposure to 15 \( \mu \)M Mas-17, Ins(1,4,5)P3 was 0.3 pmol/10^6 cells and 99% of the cells still were flagellated (compare with Fig. 6). Ins(1,4,5)P3 production, therefore, was not contingent upon either loss of flagella or acidification.

Moreover, this suggested that phospholipase C activation induced flagellar excision. Consistent with this idea, we found that a 10-s treatment with 10 \( \mu \)M neomycin completely inhibited both deflagellation and Ins(1,4,5)P3 accumulation.
Figure 7. Neomycin does not block flagellar regeneration. Neomycin at 20 μM (●), 10 μM (○), or buffer alone (■) was added to cells 10 s after the initial addition of acid. (a) Mean flagellar length measured from photomicrographs (at least 100 flagella were measured for each estimate). (b) Accumulation of Ins(1,4,5)P₃ in the same experiment as a.

Discussion

The changes in metabolite levels we report are consistent with activation of phospholipase C and DAG kinase. A rapid increase in mass levels of Ins(1,4,5)P₃ (Fig. 2) and a concomitant decrease in the amount of PtdIns(4,5)P₂ (Fig. 3) suggest the activation of a phospholipase C. Although these changes were not accompanied by a correspondingly large increase in DAG, they were followed by an accumulation of PtdOH (Fig. 5), similar to that seen during the response of the alga, Dunaliella salina, to hypotonic shock (15). This suggests that DAG kinase, which may be an important modulator of signal transduction (26), is activated in this system.

Although these were novel results, we were equally interested in learning what signals were activating phospholipase C and if this activation had any physiological consequences relevant to deflagellation or flagellar regeneration. The possible causes and consequences of phospholipase C activation are outlined in Fig. 8. The accumulation of Ins(1,4,5)P₃ in response to both pH shock and mastoparan in both wild-type cells and in a mutant that is unable to deflagellate (fa-1) demonstrated that activation of phospholipase C was not a consequence of deflagellation (eliminating possibilities 3 and 4 in Fig. 8). Coincident with neutralization of the cultures we observed dramatic increases in PtdInsP and PtdIns(4,5)P₂ (Fig. 3), and a drop in Ins(1,4,5)-
P3 (Fig. 4). These data suggest that low pH increases the activity of phospholipase C (in further support of possibilities 1, 2, and 5). PtdIns(4,5)P$_2$ hydrolysis in response to reduced extracellular pH has been observed in a number of mammalian cell types (44). When phospholipase C was inhibited by neomycin (added after deflagellation), C. reinhardtii cells regenerated their flagella normally, indicating that the large accumulation of Ins(1,4,5)P$_3$ was irrelevant to the process of flagellar regeneration (possibility 5, Fig. 8).

The simplest interpretation of our results is that Ins(1,4,5)P$_3$ production is required for deflagellation in invertebrates (7, 41, 52), indicate that rapid transduction by inositol phospholipid metabolites may be quite widespread.

We have demonstrated that both pH shock and mastoparan cause accumulation of Ins(1,4,5)P$_3$ in C. reinhardtii. It is likely that both agents are achieving this through the activation of phospholipase C, but it is unclear whether or not they are acting at the same point. For example, mastoparan might be activating a G protein or phospholipase C directly, whereas low pH could increase substrate availability. In any event, activation of phospholipase C appears to be the mechanism by which both mastoparan and low pH stimulate flagellar excision. This conclusion predicts a role for calcium in the deflagellation response. Sanders and Salisbury (40) have reported that the calcium-induced contraction of centrin mediates microtubule severing, a key event in flagellar excision. It remains to be demonstrated that Ins(1,4,5)P$_3$ accumulations are causing deflagellation by raising intracellular calcium. This system provides a rare opportunity to study a defined signal transduction pathway in an experimentally amenable photosynthetic organism.

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