Ca\textsuperscript{2+} signaling in the Chlamydomonas flagellar regeneration system: cellular and molecular responses

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SUMMARY

In response to certain extracellular stimuli, Chlamydomonas reinhardtii cells excise their flagella, induce expression of more than 200 different flagellar mRNAs, and assemble a new flagellar pair. Normally, flagellar excision, gene induction and outgrowth are tightly coupled temporally. Our previous studies showed that uncoupling the cellular response of flagellar excision from flagellar outgrowth resulted in submaximal flagellar gene induction, and led us to propose that normal flagellar gene induction is a composite response. The present study extends these observations by measuring flagellar gene induction in Chlamydomonas cells stimulated under conditions where both flagellar excision and flagellar outgrowth are blocked. We find that the flagellar genes are induced in a Ca\textsuperscript{2+}-dependent manner in response to stimulation in the absence of flagellar excision and outgrowth. Flagellar gene induction is therefore independent of flagellar excision and outgrowth but sensitive to extracellular Ca\textsuperscript{2+} levels. Thus, flagellar excision, flagellar outgrowth and flagellar gene induction are three responses to a common stimulus that are related by their requirement for extracellular Ca\textsuperscript{2+}.

Key words: Ca\textsuperscript{2+}, gene expression, flagellar regeneration, Chlamydomonas

INTRODUCTION

In the Chlamydomonas reinhardtii flagellar regeneration system, cells respond to certain extracellular signals by excising their flagella, inducing expression of a large set of specific genes (some known to encode structural components of the flagellum), and assembling a new flagellar pair (Johnson and Rosenbaum, 1993). The synchrony, rapidity and complexity of these responses raise questions concerning the nature of the signals that regulate each response and whether the responses are independently regulated. One approach to addressing these questions is to examine the effects of blocking the individual responses or uncoupling their normal sequence of occurrence.

Previous studies of Chlamydomonas flagellar regeneration demonstrated that the cellular responses of flagellar excision and flagellar outgrowth can be uncoupled experimentally (Lefebvre et al., 1978; Quader et al., 1978; Rosenbaum et al., 1969). Our previous work demonstrated that temporally uncoupling flagellar excision from flagellar outgrowth resulted in a flagellar gene induction response that was submaximal (Cheshire and Keller, 1991; Schloss and Keller, unpublished data). We also found that delaying flagellar outgrowth resulted in a flagellar gene induction response that was submaximal (Cheshire and Keller, 1991). From these data, we proposed that the mRNA abundance change associated with normal flagellar regeneration is not a fixed, quantal response, but instead results from multiple gene regulatory events.

Both flagellar excision and gene induction require extracellular Ca\textsuperscript{2+}. Several studies have demonstrated the Ca\textsuperscript{2+} requirement for flagellar excision in vivo (Huber et al., 1986; Quarmby and Hartzell, 1994; Salisbury et al., 1987; Yueh and Crain, 1993), and in vitro in permeabilized cells (Salisbury et al., 1987; Sanders and Salisbury, 1989). Our previous studies demonstrated that flagellar gene induction also requires extracellular Ca\textsuperscript{2+}, because low extracellular Ca\textsuperscript{2+} (buffered to 10^{-7} M) reversibly inhibited flagellar gene induction in cells stimulated by mechanical shear (Cheshire and Keller, 1991). Previous studies also demonstrated a requirement for Ca\textsuperscript{2+} in flagellar outgrowth (Cheshire and Keller, 1991; Quader et al., 1978, Salisbury et al., 1987). Uncovering the Ca\textsuperscript{2+} dependencies of flagellar excision, outgrowth and gene induction raises questions concerning the role of Ca\textsuperscript{2+} in regulating and integrating these cellular and molecular responses to stimulation.

The present study further examines the interdependence of flagellar excision, flagellar outgrowth and flagellar gene induction. Here, we measure flagellar gene induction in extracellular Ca\textsuperscript{2+} concentrations between 10^{-3} and 10^{-6} M, and under conditions in which both flagellar excision and outgrowth are blocked. We find that the flagellar genes are induced in response to cell stimulation in the absence of flagellar excision and flagellar outgrowth in flagella-less cells, in mutants that are defective in flagellar excision, and in normal cells that have been blocked from excising (and therefore regrowing) their flagella. In addition, we find that flagellar gene
induction is sensitive to extracellular Ca$^{2+}$ levels. Thus, flagellar excision, gene induction and outgrowth during flagellar regeneration are three discrete responses to stimulation that are related by a requirement for extracellular Ca$^{2+}$.

**MATERIALS AND METHODS**

**Cell cultures**

*Chlamydomonas reinhardtii* wild-type strain 137c (mt$^{-}$) (CC-124), mutant bald-2 (mt$^{+}$) (CC-479), and mutant fa-1 (mt$^{-}$) (CC-1370) were obtained from E. Harris at the Chlamydomonas Genetics Center, Duke University, Durham, NC. Vegetative cells were cultured at 25°C in Medium I of Sager and Granick (1953) with continuous aeration on a cycle of 14 hours light/10 hours dark. Cultures were grown to a density of 5×10$^{5}$ to 4×10$^{6}$ cells/ml. Cells density was determined using a Neubauer counting chamber (Clay-Adams, Parsippany, NJ).

**General experimental procedure**

Wild-type and fa-1 cells were harvested by low-speed centrifugation (900 × g for 5 minutes) and resuspended in fresh Medium I at a final cell concentration of 1×10$^{7}$ cells/ml. Cells were allowed to recover from centrifugation for 1.5 to 2 hours while stirring gently under fluorescent light at room temperature. At times before and after cells were exposed to an experimental condition, cell samples were collected for RNA isolation and microscopic observation.

bald-2 cells form large aggregates or palmelloid colonies during growth due to their inability to hatch from their mother cell walls (Schloss et al., 1984). These cell walls contaminate RNA samples, and interfere with quantitative S$_1$ nuclease analysis. To reduce this contamination, bald-2 cells were harvested by low-speed centrifugation (900 × g for 5 minutes), resuspended in 200 ml of Medium I, homogenized for 1 minute at setting 30 on a VirTis homogenizer (The VirTis Co., Gardiner, NY) and then centrifuged at 600 × g for 2 minutes. The supernatant containing many disrupted mother cell walls was decanted and the procedure was repeated. Cells suspended in fresh Medium I at a final concentration of 1×10$^{7}$ cells/ml recovered from this treatment while being stirred gently under fluorescent light at room temperature for 1.5 to 2 hours.

For experiments performed in nominally Ca$^{2+}$-free medium, cells were collected and gently washed twice in 10 mM PIPES-KOH, pH 7.3, and then resuspended in fresh 10 mM PIPES-KOH, pH 7.3, at a concentration of 1×10$^{7}$ cells/ml. For other experiments in this series, cells were washed twice and resuspended in 10 mM PIPES-KOH, pH 7.3, and 10-200 µM CaCl$_2$ was added before stimulation by pH shock.

Flagellar lengths were measured as described previously (Cheshire and Keller, 1991).

**Procedures for cell stimulation**

To stimulate cells using mechanical shear, cells were deflagellated by homogenization in a VirTis homogenizer for 45-60 seconds at a setting of 30-35, in order to deflagellate >99% of the cell population. To stimulate cells using pH shock, 1 M acetic acid was added dropwise to cell cultures, which were stirring gently with a magnetic stir bar until a pH of 4.3 was reached (~10 seconds). Twenty seconds after the start of acetic acid addition, 1 M potassium hydroxide was added dropwise until the culture returned to its initial pH of ~6.8 to 7.0 (Witman et al., 1978).

**RNA isolation**

At specific times before and after cells were exposed to an experimental condition, total RNA was extracted from 1×10$^{8}$ cells using a modification of the procedure by Wagner et al., as previously described (Cheshire and Keller, 1991). Briefly, cells were collected and lysed, and RNA was extracted once with hot phenol (55°C), twice with phenol/chloroform, once with chloroform/isooamyl alcohol, and then precipitated with 2 volumes of ethanol.

**Preparation of probes**

3’ End-labeled probes were prepared from cDNA or genomic clones encoding β-2-tubulin, radial spoke protein 3 (Rsp3), and the constitutively expressed G-protein β-like subunit pc8-13. The β-2-tubulin probe was derived from the cDNA clone pc8-31 (gift from J. Schloss), and consisted of the BamHI/SacI fragment subcloned into the BamHI and SacI sites in the polynucleotide of pGEM3Zf(−). The Rsp3 clone was derived from the Rsp3EB genomic clone constructed by Williams et al. (1986), and consisted of the SacI/SalI fragment subcloned into the SacI and HindIII sites in the polynucleotide of pGEM3Zf(−). The G protein β-like subunit probe (p31gt18-13-2-7) was the pc8-13 cDNA subcloned into pBluescript (gift from J. Schloss; see Schloss, 1990).

A 5 µg sample of each plasmid was linearized with the appropriate restriction enzyme (pc8-31BS with BamHI; Rsp3BS with NotI; p31gt18-13-2-7 with BglII) and then treated with 5 units of Klenow fragment (Promega Biotec, Madison, WI) and 50 µCi of [α-32P]dATP or [α-32P]dCTP (3000 Ci/mmol, NEN Research Products, Boston, MA) for 30 minutes at room temperature. The reaction was stopped by the addition of 0.5 M EDTA (25 mM final concentration), and the sample was extracted sequentially with phenol, pH 7.0, and chloroform/isooamyl alcohol (96:4, v/v). To separate unincorporated nucleotides from the labeled DNA, the samples were passed through 5 ml Sephadex G-50 columns (obtained from Sigma Chem. Co., St Louis). The specific activities of the probes in all experiments were 2×10$^{6}$ to 6×10$^{6}$ dpm/µg.

**S$_1$ nucleic acid protection analysis**

As described previously, a modification of the S$_1$ nucleic acid protection analysis of Weaver and Weissman (1979) was used to detect changes in the levels of β-2-tubulin, Rsp3 and G-protein β-like subunit mRNAs. Hybridization reactions contained 2.5 to 10 µg of total RNA and 20 ng of the appropriate probe; any experimental set of samples analysed in the same S$_1$ reaction contained the same amount of RNA. Hybridization temperatures were 58°C for β-2-tubulin and G-protein β-like subunit mRNAs, and 64°C for Rsp3 mRNA. 100 units of the single-strand-specific nuclease S$_1$ (Gibco BRL Life Sciences, Gaithersburg, MD) was used in each digestion. Samples were electrophoresed on 7 M urea/7.5% polyacrylamide gels, which were dried and exposed to Kodak diagnostic X-ray film at room temperature.

The expected sizes of the β-2-tubulin, Rsp3 and G protein β-like subunit protected bands were 200, 383 and 376 nucleotides, respectively. By comparing densitometric signals of experimental samples hybridized with dilutions of labeled probe, we estimate that our hybridization reactions contained ≥5-fold excess of probe over complementary mRNA in samples from the peak of induction.

Changes in abundance of β-2-tubulin, Rsp3 and pc8-13 mRNAs were measured because each mRNA falls into a different class, based on the kinetics of their mRNA abundance change after flagellar excision (Schloss et al., 1984). β-2-Tubulin, a class IV mRNA, accumulates to high abundance by 15 minutes after flagellar excision, peaks in abundance at 45 minutes, and returns to basal levels of expression by 120 minutes. Rsp3 is a class II mRNA that peaks in abundance at 15-20 minutes and returns to basal expression levels by 60 minutes after flagellar excision. pc8-13, a class I mRNA, is constitutively expressed before and after flagellar excision. Thus, changes in expression of β-2-tubulin and Rsp3 represent changes in expression of the large set of flagellar genes, while pc8-13 expression serves as a control for non-specific gene induction.

**Densitometry**

Band intensities from the S$_1$ nuclease protection analyses were quantified by scanning densitometry of autoradiographs using Quantity One Version 2.2 software from protein+dna imageWare systems (pdi inc., Huntington Station, NY). Relative densities from two or more autoradiographic exposures were averaged for each experiment, and standard errors are indicated for each measurement.
RESULTS

Flagellar gene induction after stimulation of wild-type cells

Previous studies raised the question of whether flagellar excision and outgrowth are required for the stimulation of flagellar gene induction, or whether flagellar gene induction could be stimulated in the absence of flagellar excision and flagellar outgrowth. To determine whether the flagellar genes were induced in the absence of flagellar excision, we stimulated cells, examined them microscopically for flagellar morphology, and measured flagellar mRNA abundance changes by S1 nuclease protection analysis. In the experiments presented here, cells were stimulated using either mechanical shear or pH shock, two standard means of deflagellating cells (Harris, 1989; Witman et al., 1978). Stimulation of wild-type cells by either of these methods caused flagellar excision in virtually 100% of the cell population. By 120 minutes after stimulation with mechanical shear or pH shock, cells regrew flagella of nearly pre-stimulation length.

To measure flagellar gene induction in response to stimulation, total RNA was collected from cells before and at specific times after stimulation, and subjected to S1 nuclease protection analyses. mRNA abundance changes induced in wild-type cells by pH shock are shown in Fig. 1. The mRNA abundance changes induced by pH shock stimulation of wild-type cells are similar in magnitude and kinetics to the abundance changes induced by mechanical shear shown previously (Cheshire and Keller, 1991). In control wild-type cells, β2-tubulin mRNA was induced ~9-fold by 15 minutes and returned to pre-stimulation levels by 90-120 minutes after pH shock stimulation (Fig. 1, lanes 1-3). Rsp3 mRNA abundance in wild-type cells increased ~6- to 8-fold after stimulation, and returned to pre-stimulation levels by 90-120 minutes. The abundance of these mRNAs decreased to pre-stimulation levels by 90 minutes. This abundance change was ~30 to 40% that of the abundance change in control wild-type cells stimulated by either pH shock or mechanical shear. The abundance change was specific for these flagellar genes, because no change in mRNA abundance of the constitutively expressed pcf8-13 gene was observed in these conditions.

bald-2 mutant cells

We also examined flagellar gene induction in the flagella-less genetic mutant strain bald-2, which fails to assemble flagella as a consequence of lacking functional basal bodies (Goodeough and St. Clair, 1975). Stimulation by pH shock or mechanical shear caused no detectable change in bald-2 cell morphology.

Stimulation of bald-2 cells by pH shock caused a transient ~3- to 4-fold increase in the abundance of β2-tubulin and Rsp3 mRNAs by 15 minutes after stimulation that returned to below pre-stimulation levels by 90 minutes (Fig. 2, lanes 4-6). The peak of this abundance change was only ~40% of that observed in wild-type cells deflagellated by mechanical shear, and mRNA abundance declined to basal levels more rapidly than in wild-type cells, by 60 minutes (J. Cheshire, unpublished observations). However, this abundance change was specific for the β2-tubulin and Rsp3 flagellar genes, because no change in expression of the constitutively expressed pcf8-13 gene was observed in these conditions. In contrast to the results of stimulation by pH shock, stimulation of bald-2 cells by mechanical shear resulted in no change in abundance of β2-tubulin, 5 minutes of deflagellation, measured by loss of cell refractivity using a phase-contrast microscope.

Stimulation of fa-1 cells by pH shock caused a transient ~2- to 3-fold increase in the abundance of β2-tubulin and Rsp3 mRNAs by 15 minutes after stimulation (Fig. 1). The abundance of these mRNAs decreased to pre-stimulation levels by 90 minutes. This abundance change was ~30 to 40% that of the abundance change in control wild-type cells stimulated by either pH shock or mechanical shear. The abundance change was specific for these flagellar genes, because no change in mRNA abundance of the constitutively expressed pcf8-13 gene was observed in these conditions.

Flagellar gene induction after stimulation of cells in which flagellar excision is blocked genetically

We stimulated cells of two genetic mutant strains, fa-1 (Lewin and Burrascano, 1983) and bald-2 (Goodenough and St. Clair, 1975), and compared flagellar morphology and flagellar mRNA abundance changes in these cell types with those of wild-type cells.

Fa-1 mutant cells

The fa-1 Chlamydomonas mutant strain retains its flagella in conditions that cause excision of wild-type cells (Lewin and Burrascano, 1983), but flagellar morphology is drastically altered by stimulation. For example, pH shock causes each fa-1 flagellum to coil within a balloon of flagellar membrane. However, these coiled flagella do not excise (M. Sanders, personal communication). In contrast to pH shock, stimulation of fa-1 cells by mechanical shear resulted in cell death within

Fig. 1. S1 nuclease protection analyses and quantification of RNAs from wild-type and fa-1 cells. Cells were stimulated by pH shock and RNA samples were collected at the times after pH shock indicated above each lane (lanes 1-6). RNA isolation, S1 assays, electrophoresis, autoradiography and densitometry were performed as described in Materials and Methods. mRNA abundance changes in β2-tubulin (s.e.m. ±0.36), rsp3 (s.e.m. ±0.30), and pcf8-13 (s.e.m. ±0.17) were standardized to RNA levels in non-treated cells (NT).
Rsp3 and pcf8-13 mRNAs (J. Cheshire, unpublished observations).

These results showing that the flagellar genes can be stimulated by pH shock of both fa-1 and bald-2 mutant cells indicate that flagellar excision and the accompanying flagellar outgrowth are not required for flagellar gene induction.

Flagellar gene induction after stimulation of cells in which flagellar excision and outgrowth are blocked chemically

The genetic lesions of the fa-1 and bald-2 strains used in the studies described above may affect their response to stimulation in other uncharacterized ways. To control for this possibility, we measured the response to stimulation of wild-type cells that either lack flagella, or were chemically blocked from excising their flagella.

Flagella-less wild-type cells
To prepare ‘flagella-less wild-type cells’, cells were deflagellated by mechanical shear in the presence of the antimicrotubule drug colchicine, which blocks flagellar outgrowth (Rosenbaum et al., 1969; Schloss and Keller, unpublished data), and stirred gently under illumination during recovery from deflagellation. When flagella-less wild-type cells were stimulated by pH shock, a transient ~2- to 3-fold increase in β2-tubulin abundance was observed by 15 minutes after stimulation (data not shown).

Neomycin-treated cells
Quarmby et al. (1992) observed that treating wild-type cells with 10 µM neomycin sulfate 10 seconds before pH shock blocked both flagellar excision and the normal accumulation of IPs after pH shock. In the present study, we confirmed these observations and also found that cells deflagellated by mechanical shear before or after treatment with 10 µM neomycin regrow their flagella with normal kinetics.

To determine the effects of neomycin treatment on flagellar gene induction, we examined flagellar mRNA abundance changes in neomycin-treated cells. As shown in Fig. 3A, flagellar gene induction occurred when 10 µM neomycin was added either before (lanes 3, 7) or after (lanes 4, 8) stimulation of wild-type cells by either pH shock (lanes 2-4) or mechanical shear (lanes 6-8). When wild-type cells were treated with concentrations of neomycin that block flagellar
excision by pH shock, β2-tubulin and Rsp3 mRNAs were elevated at 15 minutes after stimulation to levels in control samples not treated with neomycin (Fig. 3A, lane 3). However, the levels of induction in neomycin-treated cells rapidly decline, and do not reach wild-type levels at 45 minutes after deflagellation (J. Evans, unpublished observations). Similarly, when neomycin-treated bald-2 cells were stimulated by pH shock (Fig. 3B, lanes 3–6), the flagellar genes were induced to equivalent levels to those of untreated bald-2 cells stimulated by pH shock (Fig. 3B, lanes 1, 2). Expression of pcf8-13 mRNA remained at constitutive levels in all of these experiments, indicating that the responses were specific for the flagellar genes.

Cells in nominally Ca2+-free media

_Chlamydomonas_ gametes do not excise their flagella in response to pH shock when washed out of growth medium and held in 10 mM PIPES-KOH, pH 7.3, in the absence of added Ca2+ (Goodenough et al., 1993). We questioned whether vegetative cells also retain their flagella and examined flagellar gene induction in these conditions. Wild-type vegetative cells were washed into 10 mM PIPES-KOH, pH 7.3, and changes in flagellar mRNA abundance were measured in cell samples collected before and after stimulation by pH shock. Stimulation of cells by pH shock in this nominally Ca2+-free medium is distinguished from conditions used in our previous experiments (Cheshire and Keller, 1991), in which cells were stimulated by mechanical shear in growth medium in which extracellular free Ca2+ was buffered to 10−7 M by chelation with EGTA. We did not pH shock cells in Ca2+-free medium buffered with EGTA, because chelated Ca2+ is released as EGTA becomes protonated by acidification of the medium during pH shock.

No flagellar excision occurred when vegetative cells were stimulated by pH shock in 10 mM PIPES-KOH, pH 7.3. In contrast, cells deflagellated by mechanical shear stimulation in 10 mM PIPES-KOH, pH 7.3, regrew flagella with kinetics similar to wild-type cells in growth media (Fig. 4B).

β2-Tubulin and Rsp3 mRNAs were induced ~3-fold after pH shock of vegetative cells in 10 mM PIPES-KOH, pH 7.3 (Fig. 4, lanes 4, 5). For β2-tubulin, this represents an induction of ~23% of that of cells deflagellated by pH shock in medium containing 10 mM PIPES-KOH, pH 7.3, and 100 μM added Ca2+ (Fig. 4, lanes 4 and 10). Likewise, the abundance of β2-tubulin and Rsp3 mRNAs increased ~3- to 5-fold in wild-type cells deflagellated by mechanical shear in medium containing 10 mM PIPES-KOH, pH 7.3 (Fig. 4, lanes 3, 4, and 10). pcf8-13 mRNA was expressed at constitutive levels in all samples from this experiment, indicating that the responses are specific for the flagellar genes.

These results demonstrate that flagellar gene induction does not depend on flagellar excision and outgrowth. In fact, in nominally Ca2+-free medium, the flagellar genes are induced submaximally both in the absence of flagellar excision (after pH shock) and when flagellar excision and outgrowth occur (after mechanical shear). Here, β2-tubulin and Rsp3 mRNAs encoded by two of the flagellar genes are induced in response to stimulation, and apparently not in response to the process of flagellar excision itself.
Flagellar excision and outgrowth, and increases in the level of flagellar gene induction correlate with increases in the extracellular \( \text{Ca}^{2+} \) concentration

The experiments described in previous sections using cells that were genetically or chemically blocked from excising their flagella demonstrate that flagellar excision and outgrowth are not required for flagellar gene induction. However, this induction was submaximal. Because this study and previous studies implicated a requirement for \( \text{Ca}^{2+} \) in controlling flagellar excision and outgrowth (Cheshire and Keller, 1991; Lefebvre et al., 1978; Quader et al., 1978; Quarby and Hartzell, 1994; Salisbury et al., 1987; Yueh and Crain, 1993), we designed the following experiment to test the role of \( \text{Ca}^{2+} \) in the flagellar gene induction response.

We examined the responses of flagellar excision, gene induction and outgrowth in cells stimulated by pH shock in 10 mM PIPES buffer to which increasing concentrations of \( \text{Ca}^{2+} \) were added. Cultures of cells were divided into several samples, washed, and resuspended in 10 mM PIPES buffer. One sample was pH shocked in PIPES buffer alone, as described in the preceding section. Other samples were supplemented with 10-200 \( \mu \text{M} \) \( \text{CaCl}_2 \) and then stimulated by pH shock. As shown in Fig. 4 and summarized in Table 1, cells pH shocked in PIPES buffer alone, or in PIPES buffer plus 10 \( \mu \text{M} \) \( \text{CaCl}_2 \) did not excise their flagella, but exhibited a 3- to 4-fold increase in \( \beta\)-tubulin and Rsp3 mRNA abundance. Approximately 90% of cells pH shocked in PIPES buffer plus 25 \( \mu \text{M} \) \( \text{CaCl}_2 \) excised their flagella, and the abundance of \( \beta\)-tubulin and Rsp3 mRNAs increased ~7- to 8-fold. In these conditions, a 15 minute lag occurred before flagellar outgrowth was initiated. Cells pH shocked in PIPES buffer plus 50-100 \( \mu \text{M} \) \( \text{CaCl}_2 \) excised their flagella, exhibited an 8.5- to 11.5-fold increase in \( \beta\)-tubulin and Rsp3 mRNA abundances, and regrew their flagella with normal kinetics. Inclusion of additional \( \text{Ca}^{2+} \) up to 200 \( \mu \text{M} \) caused no further increase in flagellar mRNA abundance in response to pH shock.

Together with our previous studies (Cheshire and Keller, 1991), these results demonstrate that flagellar excision and outgrowth, and the level of flagellar gene induction after stimulation by pH shock, are sensitive to the extracellular free \( \text{Ca}^{2+} \) concentration. The results summarized in Table 1 show that flagellar excision is blocked after pH shock in media containing 10 mM free \( \text{Ca}^{2+} \), while 90-100% of cells excise their flagella at extracellular free \( \text{Ca}^{2+} \) concentrations of 25 \( \mu \text{M} \) and above. Flagellar outgrowth was delayed in cells stimulated by pH shock in PIPES plus 25 \( \mu \text{M} \) \( \text{Ca}^{2+} \), but occurred normally in higher concentrations of 50-100 \( \mu \text{M} \) \( \text{Ca}^{2+} \). The concentration of \( \text{Ca}^{2+} \) required for flagellar outgrowth after pH shock depends on the means of cellular stimulation, and apparently is lower in cells stimulated by mechanical shear than in cells stimulated by pH shock. For flagellar gene induction, our previous studies showed that flagellar gene induction was blocked after mechanical shear in 10^{-7} M free \( \text{Ca}^{2+} \) (in EGTA-buffered medium). Here, as the extracellular free \( \text{Ca}^{2+} \) concentration is increased from 10^{-7} M (Cheshire and Keller, 1991) to low levels in the nominally \( \text{Ca}^{2+} \)-free conditions, and finally up to ~100 \( \mu \text{M} \), the level of flagellar gene induction gradually increases (Table 1). An extracellular free \( \text{Ca}^{2+} \) concentration of ~50 to 100 \( \mu \text{M} \) is required for full flagellar gene induction after pH shock. Thus, the concentration of extracellular \( \text{Ca}^{2+} \) apparently controls not only flagellar excision and outgrowth, but also the magnitude of the flagellar gene induction response.

DISCUSSION

Flagellar gene induction does not require flagellar excision or flagellar outgrowth

Our results demonstrate that the flagellar genes can be induced in the absence of flagellar excision and flagellar outgrowth. In these studies, we examined gene induction in response to stimuli such as pH shock in cells where flagellar excision and outgrowth were blocked. We found that flagellar gene induction occurs in cells lacking flagella, such as cells of the bald-2 mutant strain or flagella-less wild-type cells. Induction also occurs in flagellated cells without flagellar loss, such as cells of the fa-1 mutant strain, neomycin-treated cells, or cells pH shocked in nominally \( \text{Ca}^{2+} \)-free medium. These results demonstrate that flagellar excision and outgrowth are not required stimuli for flagellar gene induction. We conclude from these studies that flagellar gene induction is one of several responses to a common stimulus, along with flagellar excision and flagellar outgrowth.

As a result of these studies, our perspective of the Chlamydomonas flagellar regeneration system has changed. Flagellar excision is no longer considered the stimulus for flagellar gene induction and flagellar outgrowth in a dependent pathway. We now recognize flagellar excision, gene induction and outgrowth as independently regulated responses to cellular stimulation by extracellular cues.

 Extracellular \( \text{Ca}^{2+} \) is required for flagellar gene induction

Although flagellar excision and outgrowth are not required, extracellular free \( \text{Ca}^{2+} \) is required for flagellar gene induction after stimulation. More specifically, extracellular \( \text{Ca}^{2+} \) apparently acts as a rheostat to control the magnitude of the flagellar gene induction response. Our previous studies, in which flagellar gene induction was blocked after mechanical shear in EGTA-buffered medium containing 10^{-7} M free \( \text{Ca}^{2+} \) (Cheshire and Keller, 1991), demonstrated that flagellar gene induction requires micromolar concentrations of free \( \text{Ca}^{2+} \). In the present study, the flagellar genes are induced submaximally in cells stimulated by pH shock or mechanical shear in PIPES buffer without added \( \text{Ca}^{2+} \) (nominally \( \text{Ca}^{2+} \)-free medium). As the extracellular \( \text{Ca}^{2+} \) concentration is gradually increased, the level of flagellar gene induction in response to pH shock increases gradually also. Extracellular free \( \text{Ca}^{2+} \) concentrations of ~50-100 \( \mu \text{M} \) are required for induction of the flagellar genes to maximal levels after stimulation by pH shock (Fig. 4 and Table 1).

The molecular mechanisms by which \( \text{Ca}^{2+} \) regulates the Chlamydomonas flagellar genes are at present unknown. In several systems, \( \text{Ca}^{2+} \) regulates gene induction at a transcriptional level by affecting the activity of specific transcription factors (Bading et al., 1993; Ginty et al., 1992; Sheng et al., 1990; Resendez et al., 1985). \( \text{Ca}^{2+} \) is also known to regulate gene expression by altering mRNA stability (White and Bancroft, 1987). Changes in both transcription rates and
Table 1. Summary of Ca\(^{2+}\)-sensitive responses to stimulation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conditions</th>
<th>Excision (%)</th>
<th>Induction (%)</th>
<th>Outgrowth</th>
</tr>
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<tbody>
<tr>
<td>Mechanical shear</td>
<td>10(^{-7}) M Ca(^{2+})†</td>
<td>100</td>
<td>0</td>
<td>Blocked</td>
</tr>
<tr>
<td>Mechanical shear</td>
<td>Nom. Ca(^{2+})-free</td>
<td>100</td>
<td>–42</td>
<td>Normal</td>
</tr>
<tr>
<td>pH shock</td>
<td>Nom. Ca(^{2+})-free</td>
<td>0</td>
<td>–28</td>
<td>NA‡</td>
</tr>
<tr>
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<td>+ 10 (\mu)M Ca(^{2+})</td>
<td>0</td>
<td>–36</td>
<td>NA‡</td>
</tr>
<tr>
<td>pH shock</td>
<td>+ 25 (\mu)M Ca(^{2+})</td>
<td>90</td>
<td>–76</td>
<td>Delayed</td>
</tr>
<tr>
<td>pH shock</td>
<td>+ 50 (\mu)M Ca(^{2+})</td>
<td>100</td>
<td>–93</td>
<td>Normal</td>
</tr>
<tr>
<td>pH shock</td>
<td>+ 75 (\mu)M Ca(^{2+})</td>
<td>100</td>
<td>–96</td>
<td>Normal</td>
</tr>
<tr>
<td>pH shock</td>
<td>+ 100 (\mu)M Ca(^{2+})</td>
<td>100</td>
<td>–100</td>
<td>Normal</td>
</tr>
</tbody>
</table>

*Induction is the average fold induction of β2-tubulin and Rsp3, determined relative to maximal induction measured in cells pH shocked in PIPES buffer + 100 nM Ca\(^{2+}\) (from Fig. 5).
†Cells were stimulated by mechanical shear in growth medium buffered to contain 10\(^{-7}\) M Ca\(^{2+}\) by addition of EGTA (Cheshire and Keller, 1991). Nom., nominally.
‡NA, not applicable. Since flagellar excision does not occur, neither does flagellar outgrowth occur.

mRNA stability contribute to the mRNA abundance change during Chlamydomonas flagellar regeneration (Keller et al., 1984; Baker et al., 1984). Preliminary evidence using isolated nuclei suggests that Ca\(^{2+}\) may effect both flagellar gene transcription and flagellar transcript stability (J. Cheshire, unpublished observations).

Flagellar excision, flagellar gene induction, and flagellar outgrowth are all related by a requirement for extracellular Ca\(^{2+}\)

Flagellar excision, outgrowth and gene induction are experimentally separable processes that are related by their requirement for extracellular Ca\(^{2+}\). Apparently, different threshold concentrations of Ca\(^{2+}\) are required for flagellar excision, flagellar gene induction and, possibly, flagellar outgrowth. The present study shows that a threshold extracellular Ca\(^{2+}\) concentration of between 10 and 25 \(\mu\)M is required for flagellar excision after pH shock, and extends several other studies demonstrating a requirement for Ca\(^{2+}\) in flagellar excision (Huber et al., 1986; Quarmby and Hartzell, 1994; Salisbury et al., 1987; Sanders and Salisbury, 1989; Yueh and Crain, 1993). A threshold concentration of 25 to 50 \(\mu\)M Ca\(^{2+}\) is required for normal flagellar outgrowth after pH shock; apparently, this threshold is lower in cells stimulated by mechanical shear than in pH-shocked cells. The results presented here and in our previous work demonstrate that flagellar gene induction is sensitive to a wider range of Ca\(^{2+}\) concentrations, since partial induction occurs in nominally Ca\(^{2+}\)-free media, but full induction after pH shock requires higher Ca\(^{2+}\) concentrations than are required for either flagellar excision or flagellar outgrowth (see Table 1 and Fig. 4). Thus, the Ca\(^{2+}\) requirements for flagellar excision and flagellar gene induction clearly differ.

Presumably, the extracellular Ca\(^{2+}\) concentration effectively correlates with an intracellular Ca\(^{2+}\) flux. Recently, Quarmby and Hartzell (1994) have measured an influx of radiolabeled \(^{45}\)Ca\(^{2+}\) following stimulation of wild-type cells by pH shock or mastoparan. Application of this technique to our future studies will correlate intracellular Ca\(^{2+}\) fluxes with the level of flagellar gene induction and extracellular Ca\(^{2+}\) concentrations. Such correlations may provide important clues about the molecules involved in transmitting to the nucleus the signals from external stimuli.

The signaling pathways for flagellar excision, gene induction and outgrowth

A question raised by these and other studies concerns whether the intracellular signaling pathways involved in flagellar excision, flagellar gene induction and flagellar outgrowth are distinct. The pathways either overlap or are superimposed, because the responses are tightly temporally synchronized and they all require Ca\(^{2+}\) in normal regenerating cells. Evidence that the pathways are distinct comes from our ability to stimulate the events independently in different concentrations of extracellular Ca\(^{2+}\) (Table 1 and Fig. 4). Evidence that the pathways are distinct also comes from experiments in which neomycin-treated cells were stimulated by either pH shock or mechanical shear. Stimulation of neomycin-treated wild-type cells by mechanical shear results in flagellar loss, gene induction and outgrowth, with kinetics similar to wild-type cells not treated with neomycin. Neomycin-treated wild-type cells do not excise their flagella in response to pH shock, but nonetheless induce their flagellar genes. This result suggests that flagellar excision and production of the intracellular messengers IP\(_3\) and diacylglycerol from hydrolysis of PIP\(_2\) by phospholipase C are not required for flagellar gene induction. Thus, at least one signaling pathway found in higher eukaryotes may function independently in the flagellar excision response but apparently not in the flagellar gene induction response to stimulation. Other evidence that distinct signaling pathways are utilized for flagellar excision and flagellar gene induction comes from our studies using pharmacological agents that affect protein kinase activity (to be published elsewhere), in which flagellar excision is stimulated in the absence of flagellar gene induction. A consideration of whether flagellar excision, gene induction and outgrowth are independently regulated raises the question of how Ca\(^{2+}\) fluxes associated with a multitude of other cellular activities are silenced in normally swimming and growing cells (Goodenough et al., 1993).

A related question raised by these studies concerns whether the different means of cell stimulation used affect flagellar gene induction through the same, different or overlapping signaling pathways. The wide range of Ca\(^{2+}\) concentrations that stimulate flagellar gene induction provides strong evidence that multiple signaling pathways are involved. The existence of different pathways signaling flagellar gene induction in vegetative and gametic cells has been suggested (Lefebvre et al., 1988). Recently, evidence for the existence of two different pathways signaling flagellar excision has been presented by Quarmby and Hartzell (1994). Thus, flagellar excision, gene induction and outgrowth may each be stimulated through multiple pathways.

General conclusions

Chlamydomonas flagellar regeneration is a complex process in which at least three cellular and molecular responses, including flagellar excision, flagellar gene induction and flagellar outgrowth, are stimulated by extracellular environmental cues or stress. Our studies have defined flagellar gene induction as...
a molecular response to stimulation that is distinct from the cellular responses of flagellar excision and flagellar outgrowth, and have demonstrated a critical role for Ca²⁺ in regulating the magnitude of the flagellar gene induction response. Clues about how the *Chlamydomonas* flagellar regeneration system works as an integrated whole should be obtained by understanding the signaling mechanisms that regulate each response.

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