Ca\textsuperscript{2+} Influx Activated by Low pH in \textit{Chlamydomonas}

LYNNE M. QUARMBY

From the Department of Anatomy & Cell Biology, Emory University School of Medicine, Atlanta, Georgia 30322-3030

ABSTRACT Cytosolic acidification stimulates an influx of Ca\textsuperscript{2+} which results in shedding of the two flagella of \textit{Chlamydomonas}. Ca\textsuperscript{2+} influxes are also involved in the photoresponses of this alga, but it is not understood how the acidification-activated Ca\textsuperscript{2+} influx is distinguished from the Ca\textsuperscript{2+} influxes which mediate phototaxis and the photophobic response. The present study focuses on the deflagellation-inducing Ca\textsuperscript{2+} influx pathway. Influx occurs through an ion channel or transporter with low abundance or low permeability to Ca\textsuperscript{2+} (\textsim\textasciitilde 500 fmol/s/10\textsuperscript{6} cells in 50 \textmu M Ca\textsuperscript{2+}). Ca\textsuperscript{2+} influx was potently blocked by Gd\textsuperscript{3+} (EC\textsubscript{50} \textasciitilde 5 \textmu M), but was insensitive to Cd\textsuperscript{2+} (Quarmby, L.M., and H.C. Hartzell, 1994. \textit{J. Cell Biol.} 124:807) and organic blockers of Ca\textsuperscript{2+} channels including SKF-96365 (up to 100 \textmu M) and flufenamic acid (up to 1 mM). Experiments with a flagella-less mutant (\textit{bald-2}), isolated flagella, and a blocker of flagellar assembly (colchicine) indicated that the acidification-stimulated Ca\textsuperscript{2+} influx pathway is not localized to the flagellar membrane. The acid-stimulated influx pathway was transiently inactivated after cells shed their flagella. Inactivation did not occur in the deflagellation mutant, \textit{fa}-I, although acidification-stimulated Ca\textsuperscript{2+} influx was normal. This suggests that inactivation of this pathway in wild-type cells is probably not a direct consequence of acidification nor of Ca\textsuperscript{2+} influx, but may be related to deflagellation. Recovery of deflagellation-inducing Ca\textsuperscript{2+} influx occurred within 30 min after a 30 s exposure to acid and did not require flagellar assembly. The regulation, drug sensitivity, and subcellular localization identify acidification-stimulated Ca\textsuperscript{2+} influx as a specific Ca\textsuperscript{2+} entry pathway distinct from established Ca\textsuperscript{2+} channels.

KEY WORDS: acid \• calcium channels \• flagella \• gadolinium

INTRODUCTION

Temporal and spatial changes in [Ca\textsuperscript{2+}] are thought to transduce environmental signals into distinct and appropriate cellular responses (Berridge and Dupont, 1994; Clapham, 1995). A sophisticated interplay between the regulated release of internal stores of Ca\textsuperscript{2+} and the influx of extracellular Ca\textsuperscript{2+} across the plasma membrane contribute to the dynamic nature of Ca\textsuperscript{2+} signaling. There is also evidence that divergence in Ca\textsuperscript{2+} signaling pathways may come about by segregation of channels to different regions of the cell (Bixby et al., 1994; Haydon et al., 1994; Lenzi and Roberts, 1994). We are beginning to understand the different mechanisms through which spatiotemporal patterns of Ca\textsuperscript{2+} arise (for examples, see Amundson and Clapham, 1993; Dolmetsch and Lewis, 1994; Stehno-Bittel et al., 1995; Toescu and Petersen, 1995). The next challenge is to understand how the cell responds specifically to the different signals. The unicellular protist, \textit{Chlamydomonas}, is a model system to study how a single cell uses Ca\textsuperscript{2+} in distinct signaling pathways (Quarmby and Hartzell, 1994a).

\textit{Chlamydomonas} propels itself by the ciliary-type beating of two apical flagella. Ca\textsuperscript{2+} influxes play important roles in the regulation of the motile responses to light (Harz et al., 1992; Witman, 1993; Pazour et al., 1995). Ca\textsuperscript{2+} influx also mediates deflagellation, the rapid shedding of both flagella in response to a drop in pH (Quarmby and Hartzell, 1994b). The cells rapidly regenerate new flagella, but this too depends on Ca\textsuperscript{2+} (Cheshire and Keller, 1991). Ca\textsuperscript{2+}, therefore, mediates both the dramatic severing of flagella as well as more subtle changes in flagellar beat or waveform, which determine phototaxis and the photophobic response. These behaviors can be directly observed; consequently, Ca\textsuperscript{2+} signaling mutants are easy to identify in \textit{Chlamydomonas}.

Delflagellation has been extensively characterized ultrastructurally. It is a highly specific event wherein outer doublet microtubules are severed, and the flagellar membrane pinches off just distal to the transition zone between the basal body and the beginning of the flagellar shaft (Satir et al., 1976; Lewin and Lee, 1985; Sanders and Salisbury, 1989; Jarvik and Suhan, 1991). Severing of the outer doublet microtubules may be homologous to the microtubule severing observed in mitotic cells (Vale, 1991; Shiina et al., 1992; McNally and Vale, 1993; Shiina et al., 1994). In addition to microtubule severing, Ca\textsuperscript{2+}-mediated contraction of a stellate array of centrin fibers in the transition zone provides a mechanical force that facilitates deflagellation (Sanders and Salisbury, 1994). A variety of stimuli, including ethanol, mastoparan, and dibucaine, can cause deflag-
ellation (Minz and Lewin, 1954; Thompson et al., 1974; Lewin et al., 1980; Witman, 1986; Quarmby et al., 1992). In detergent-permeabilized cells, Ca$^{2+}$ is necessary and sufficient to activate the machinery of deflagellation (Sanders and Salisbury, 1994). Presumably, agents which cause deflagellation in living cells do so by elevating cytosolic [Ca$^{2+}$].

The focus of this paper is on the signaling pathway by which the "pH shock" protocol causes deflagellation (Witman, 1986). A brief treatment with acid causes deflagellation only if the solution provides a sufficient concentration of protonated, membrane-permeant acid (Hartzell et al., 1993). Acidification triggers deflagellation only if it is accompanied by an influx of extracellular Ca$^{2+}$ (Quarmby and Hartzell, 1994b). Two components of acidification-activated Ca$^{2+}$ influx can be distinguished on the basis of Cd$^{2+}$ sensitivity. Flux of Ca$^{2+}$ through the Cd$^{2+}$-insensitive component is necessary and sufficient for deflagellation in response to acidification (Quarmby and Hartzell, 1994b). The goal of the present study was to characterize the Cd$^{2+}$-insensitive, deflagellation-inducing component of acidification-stimulated Ca$^{2+}$ influx.

MATERIALS AND METHODS

Cells and Culture Conditions

Wild-type (137c), bald-2, fa-1, and yf82 strains of Chlamydomonas reinhardii were obtained from the Chlamydomonas Culture Center (Duke University). Cells were maintained under constant light on TAP-Agar plates (see Harris, 1989 for formulation of media). For most experiments 3–4 d old plates were flooded with 10 ml of TAP media for 2 h. Cells which had swum up from the agar were then transferred to sterile 50 ml conical tubes, pelleted (2 min; 2,500 g; 4°C), resuspended in appropriate buffer, counted, pelleted again, and resuspended in the assay buffer. Cells were then counted, and the volume was adjusted to yield the specified cell densities.

Calcium Influx Assay

$^{45}$Ca influx was measured as described previously (Quarmby and Hartzell, 1994b). Briefly, cells were washed into H$_{10}$M$_{Ca}$ (10 mM Na-HEPES, pH 7.0; 1 mM MgCl$_2$, 50 μM CaCl$_2$), and at timed intervals, 250 μl of cells (10$^7$ cells/ml) were added to 250 μl of test solution containing 5–25 μM $^{45}$Ca (≈20 mCi/mg) with CaCl$_2$ added to achieve a total [Ca$^{2+}$] of 50 μM. The test solution was usually 40 mM Na-acetate, pH 4.5, with 1 mM MgCl$_2$. In some experiments 100 mM Na-benzoate, pH 6.0, was used in place of the acetate. The concentration of protonated acid in each of these solutions is similar, and both acids were equally effective (Hartzell et al., 1993; Quarmby and Hartzell, 1994b). Ca$^{2+}$ influx was quenched by the addition of 2 ml of ice-cold wash buffer (10 mM Tris HCl, pH 7.0, 1 mM MgCl$_2$, 25 mM CaCl$_2$, 10 μM LaCl$_3$) followed immediately (within 1 s) by aspiration onto glass fiber filters using a Cell Harvester (Brandel, Gaithersburg, MD). The filters were washed twice with 2 ml of cold wash buffer, dried, immersed in 3 ml of Bio-Safe II counting cocktail (Research Products International, Mt. Prospect, IL), and radioactivity was detected by a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA).

pH Shock

Cells which were subjected to a pH shock before assay for acidification-induced Ca$^{2+}$ influx were treated as follows: 20 ml of cells (10$^7$ cells/ml) in H$_{10}$M$_{Ca}$, pH 7, were stirred gently with a magnetic stir bar. At time (t) = 0, 1 N acetic acid was added dropwise to bring the pH from ≈6.8 to ≈4.5 within 10 s. After 30 s at the reduced pH value, the culture was neutralized by the dropwise addition of 0.5 N NaOH or 0.5 N KOH. Mock-treated cells were handled in the same way, except that buffer was added in place of the dropwise additions of acid and base. Both pH-shocked and mock-treated cells were then washed into H$_{10}$M$_{Ca}$ and assayed for $^{45}$Ca influx in response to acid.

Measurement of Flagellar Length

Aliquots of cells were fixed in 2% glutaraldehyde at times during flagellar regeneration. Cells were observed by dark-field microscopy and recorded by a silicon-intensified target camera (66 SIT; Dage-MTI, Inc., Michigan City, IN) onto videotape by a video cassette recorder. Flagellar lengths were analyzed from the videotapes using measurements made manually from calibrated video screens at a final magnification of 2,500.

Reagents and Sources

$[^45]$Ca-CaCl$_2$ was from Dupont NEN (Boston, MA). SKF-96365 was from Calbiochem Corp. (La Jolla, CA) prepared as a 100 mM stock solution in DMSO. Mastoparan from Peninsula Laboratories (Belmont, CA) was prepared as a 10 mM stock solution in 10 mM Na-HEPES, pH 7.0. GdCl$_3$(H$_2$O)$_6$. 5-flufenamic acid, and colchicine were from Sigma Chemical Co. (St. Louis, MO). Gd$^{3+}$ was prepared as a 10 mM stock solution in water which was bubbled with N$_2$(g) before the addition of GdCl$_3$(H$_2$O)$_6$. Colchicine was dissolved in 95% ethanol to yield a 0.4 mg/μl stock solution which was stored at −20°C. Flufenamic acid was prepared as a 100 mM stock solution in 95% ethanol.

RESULTS

The Flagella-less Mutant, Bald-2, Expresses Acid-stimulated Ca$^{2+}$ Influx

A voltage-gated Ca$^{2+}$ channel, implicated in the photoresponses, is localized on the flagellar membrane of Chlamydomonas (Beck and Uhl, 1994). Yoshimura (1996) recently reported that a Ca$^{2+}$-permeant mechanoreceptor current is also expressed on Chlamydomonas flagellar membranes. To test the hypothesis that the acidification-stimulated Ca$^{2+}$ influx pathways are also localized to the flagella, I used the flagella-less mutant, bald-2 (Goodenough and St. Clair, 1975). If the acid-stimulated Ca$^{2+}$ influx is localized to the flagella, bald-2 should not respond to acidification with an influx of Ca$^{2+}$, however, bald-2 shows robust influx of Ca$^{2+}$ in response to acidification (Fig. 1). Treatment with Cd$^{2+}$ blocked a large component of the Ca$^{2+}$ influx, but did not affect Ca$^{2+}$ influx during the first 3 s after stimulation (Fig. 1). We have previously demonstrated that in wild-type flagellated cells this initial Ca$^{2+}$ influx (through the Cd$^{2+}$-
The acid-stimulated influx pathways are localized to the flagella, but in bald-2 the channels are inserted in the plasma membrane and remain associated with the cell body simply because there are no flagella.

### Isolated Flagella Do Not Show Acid-stimulated ⁴⁵Ca Accumulation

To test directly whether the acid-stimulated Ca²⁺ influx pathways are localized on flagellar membranes, I compared Ca²⁺ influx in isolated wild-type cell bodies and flagella. In our earlier experiments (Quarmby & Hartzell, 1994b) deflagellation occurred within the first second of ⁴⁵Ca assay, and both cell bodies and flagella were trapped on the filter and contributed to ⁴⁵Ca uptake during the 30-s time course of the assay. To distinguish flagellar Ca²⁺ uptake, cells were deflagellated, as described above, and the flagella and cell bodies separated by centrifugation (2 min; 4°C; 4,000 g). Cell bodies and flagella were handled in parallel. To achieve pure preparations of each, the fractions were centrifuged three times. After each centrifugation, flagella (in the supernate) were transferred to a clean tube, and cell bodies (in the pellet) were resuspended in fresh buffer. Measurement of acid-stimulated Ca²⁺ influx was made 30 min after the pH shock that was used to induce flagellar shedding. At this time, under these conditions, the cells have regrown short flagella (<20% of full length, see below). Acid-stimulated ⁴⁵Ca influx into the cell bodies with short flagella equaled influx into whole cells with long flagella, but no measurable ⁴⁵Ca influx was observed in the isolated full-length flagella (Fig. 2 A). Fig. 2 B illustrates that another stimulus, mastoparan, induced ⁴⁵Ca influx into flagella, whereas acid did not, therefore indicating that the flagella are capable of accumulating ⁴⁵Ca in this assay. The low level of accumulation in response to masto-

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**FIGURE 1.** Bald-2 cells show acid-stimulated Ca²⁺ influx. 250-µl aliquots of flagella-less cells (bald-2) in H₁₀M₆Ca₇₀M₅ were added to 250 µl aliquots of the ⁴⁵Ca influx cocktail. The cocktail contained 1 mM MgCl₂, 45 µM CaCl₂, 5 µM ⁴⁵CaCl₂, and: (●) 100 mM Na-benzoate, pH 6.0, (▲) 100 mM Na-benzoate, pH 6.0, cells pretreated for 1 min with 100 µM GdCl₃, or (□) 10 mM Na-HEPES, pH 7.0. Data points are the mean of duplicate samples; the data is representative of three independent experiments. Error bars in this and subsequent figures represent the standard deviation.

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**FIGURE 2.** Acid does not stimulate Ca²⁺ influx in isolated flagella. Flagella were removed from cell bodies by pH shock (see MATERIALS AND METHODS). Three cycles of centrifugation were used to prepare homogeneous samples of flagella and cell bodies. (A) Acid-stimulated Ca²⁺ influx was assayed at 30 min after pH shock. The stimulating cocktail contained 40 mM Na-acetate, pH 4.5, 1 mM MgCl₂, 25 µM CaCl₂, and 25 µM ⁴⁵CaCl₂. (●) Cells with very short flagella, 30 min after pH shock, (▲) isolated flagella, and (□) cells with long flagella, 100 min after pH shock. (B) Flagellar preparations were assayed for ⁴⁵Ca influx in response to (▲) 10 µM mastoparan in H₁₀M₆Ca₇₀M₅, (●) acetate (as in A), or (□) H₁₀M₆Ca₇₀M₅, the same buffer in which the flagella were resuspended. Data points are the mean of duplicate samples; the data is representative of three independent experiments.
toparan is probably a consequence of the relatively small volume of the flagella. The experiment in Fig. 2 A was done with “cell-equivalents” of bodies and flagella, rather than equal volumes, in order to account for the influx observed in experiments with whole cells. The results in Fig. 2 indicate that, unlike a voltage-gated Ca\(^{2+}\) channel (Beck and Uhl, 1994), or the Ca\(^{2+}\)-permeant mechanoreceptor current (Yoshimura, 1996), the acidification-stimulated Ca\(^{2+}\) influx pathways are not uniformly distributed along the length of the flagella. The lack of acid-stimulated influx in isolated flagella suggests that most or all of the acid-stimulated Ca\(^{2+}\) influx pathway might be excluded from the flagellar membrane.

The Cd\(^{2+}\)-insensitive Component of Ca\(^{2+}\) Influx

Inactivates after pH Shock

There is evidence in Fig. 1 and our earlier work (Quarmby and Hartzell, 1994b) to suggest that the rapid, Cd\(^{2+}\)-insensitive, Ca\(^{2+}\) influx inactivates after acidification. The initial rapid rate of Ca\(^{2+}\) influx, which is carried primarily by the Cd\(^{2+}\)-insensitive com-

![Figure 3](image-url)

**Figure 3.** pH shock attenuates acid-stimulated Ca\(^{2+}\) influx. 20 ml of cells in HgmC\(_{10}\)M, were stirred during the dropwise addition of 1 N acetic acid. The pH was lowered from ~6.8 to ~4.5 within 10 to 15 s. Cells were held at the low pH value for 30 s and then neutralized by the dropwise addition of 0.5 N NaOH. This treatment, which causes deflagellation, was followed immediately by 2 cycles of centrifugation and resuspension in HgmC\(_{10}\)M in preparation for assay of \(^{45}\)Ca influx. Assay cocktail contained 40 mM NaAcetate, pH 4.5, 1 mM MgCl\(_2\), 25 \(\mu\)M CaCl\(_2\), and 25 \(\mu\)M \(^{45}\)CaCl\(_2\). (A) Wild type (137c) cells assayed at (1) 15, (O) 30, (V) 45, (V) 60, and (4) 90 min after pH shock. Data points are individual measurements from a single experiment. In some experiments, full recovery was observed as early as 30 min, whereas, in other experiments such as the one illustrated, full recovery did not occur until 60 min. In three experiments a second flask of cells was mock-treated (by the addition of buffer or, in one case, the addition of a neutral mix of acetic acid and NaOH). In each of these experiments, mock treated cells showed no inactivation of \(^{45}\)Ca influx and the recovery of pH shocked cells was to full mock-treated levels. (B) In this experiment with wild type (137c) cells, colchicine was added to a final concentration of 3 mg/ml immediately after neutralization of the pH shock. Colchicine (3 mg/ml) was also added to the HgmC\(_{10}\)M, solution in which cells were resuspended. Colchicine-treated cells were assayed for acid-stimulated \(^{45}\)Ca influx at (1) 15, (O) 30, (V) 45, (V) 60, and (4) 90 min after deflagellation. Data points are individual measurements from a single experiment. The experiment illustrated is representative of four independent experiments. (C) Data from the experiments in A and B replotted to facilitate comparison of the recovery of acid-stimulated \(^{45}\)Ca influx in control and colchicine-treated cells. (D) Flagellar regeneration was quantified during the recovery from pH shock for the experiments presented in A and B. Aliquots of cells were fixed at 15, 30, 45, 60, and 90 min after pH shock. At least 20 flagella were measured, and the lengths averaged for each data point. The data plotted is from a single experiment. Quantitatively indistinguishable growth rates were measured in an independent experiment. Flagellar regeneration (or lack of regeneration) was assessed qualitatively in all seven of the control experiments (A) and in all four of the experiments with colchicine (B). (1) Control cells; (O) colchicine-treated cells.
component, is sustained for ≤10 s (see Fig. 1). The lower rate of accumulation of 45Ca beyond 10 s of stimulation is not due to a turn on of Ca2+ efflux (unpublished observations), suggesting that the rate of Ca2+ influx is decreasing. To study directly this phenomenon, cells were pH shocked (i.e., challenged with low pH for 30 s; see MATERIALS AND METHODS) before they were assayed for acid-stimulated Ca2+ influx. The rate of acid-stimulated Ca2+ influx was much lower in cells assayed 15 min after pH shock than it was in cells assayed after 30-90 min of recovery from the pH shock (Fig. 3 A). In particular, the early phase (within ~3 s of stimulation) was greatly diminished. The Cd2+-insensitive component, which is necessary for the deflagellation response, is predominant in this early phase (see Fig. 1). Recovery of the deflagellation-inducing Ca2+ influx pathway is indicated by (a) recovery of the early rapid component of acidification-activated Ca2+ influx and (b) the short flagella present on cells 30 min after pH shock were shed in response to a second pH shock. In addition, cells assayed for 45Ca influx in the presence of Cd2+ at early times after pH shock expressed no acid-stimulated Ca2+ influx (data not shown). The data presented in Figs. 1 and 2 support the conclusion that the acid-stimulated Ca2+ influx pathway is not expressed on the flagella; however, the data in Fig. 3 A indicate that the pathway was lost when the flagella were shed and was re-established when the flagella began to regenerate.

Recovery of the Ca2+ Influx Does Not Require Flagellar Regeneration

One possible explanation is that the acidification-stimulated Ca2+ influx pathway is localized to the flagellar transition zone, which is not shed with the flagella, but undergoes dramatic structural changes during deflagellation. This hypothesis predicts that recovery of the Ca2+ influx should not require flagellar assembly. When Chlamydomonas cells are deflagellated in the presence of the microtubule-disrupting agent, colchicine, they do not regenerate their flagella (Rosenbaum et al., 1969); therefore, colchicine treatment allows one to distinguish whether recovery of Ca2+ influx is dependent upon flagellar regeneration. Cells deflagellated by pH shock, as described above, show a loss and recovery of Ca2+ influx in the presence of 5 mg/ml colchicine (Fig. 3 B). Although the time course of recovery was slower in the colchicine-treated cells than in control cells (compare A and B, Fig. 3), the colchicine-treated cells recovered to control levels by 90 min (Fig. 3 C). During the course of this experiment, control cells grew new flagella while no flagellar growth occurred in the colchicine-treated cells (Fig. 3 D). I conclude that recovery of acidification-stimulated Ca2+ influx is independent of flagellar regeneration.

Inactivation of Ca2+ Influx Correlates with Deflagellation

Although recovery of Ca2+ influx did not require flagellar regeneration, attenuation of Ca2+ influx correlated with the shedding of the flagella. One possibility is that the pathway is inactivated, directly or indirectly, by the acidification or by the influx of Ca2+. This possibility can be directly tested using the Chlamydomonas mutant, fa-1, which does not deflagellate in response to acidification but does show a pronounced acidification-stimulated 45Ca influx (Lewin and Burrascano, 1983; Quarmby and Hartzell, 1994b). Fig. 4 A shows that fa-1 cells express both components of acid-stimulated Ca2+ influx, but influx does not inactivate after pH shock. (A) WT (137c) and fa-1 mutant cells were assayed for 45Ca influx stimulated by the acetate cocktail (Fig. 3) in the absence or presence of 100 μM CdCl2. (B) fa-1 cells without Cd2+, (A) WT cells without Cd2+, (●) fa-1 cells with Cd2+, ( ○) WT cells with Cd2+. In addition, (○) basal 45Ca influx in fa-1 cells was assayed by adding the 45Ca in the same buffer in which the cells were resuspended (H10M, Ca10). There is variability in the magnitude of acid-stimulated influx in both WT and fa-1 cultures. The experiment illustrated is representative of at least 12 similar experiments with wild type cells and 3 with fa-1 cells, with the exception that it is coincidental that the two different cultures had almost identical fluxes on this particular day. (B) Fa-1 and 137c cells in H10M, Ca1 were pH shocked as described in the legend to Fig. 3. Cells were washed into H10M, Ca10 and assayed for 45Ca influx using the acetate cocktail at 16 (○ fa-1; ∆ WT) and 45 (▲ fa-1; ■ WT) min after pH shock. Data points are individual measurements from a single experiment. The absence of inactivation of 45Ca influx in fa-1 cells was observed in three independent experiments, with the earliest assay of influx done 7 min after pH shock.
hibit both Cd\textsuperscript{2+}-sensitive and Cd\textsuperscript{2+}-insensitive components of acid-stimulated Ca\textsuperscript{2+} influx. Although flux of Ca\textsuperscript{2+} through the Cd\textsuperscript{2+}-insensitive pathway triggers deflagellation in wild-type cells (Quarmby and Hartzell, 1994b), the \textit{fa-1} cells are defective in their response to this Ca\textsuperscript{2+} signal. This mutant provided, therefore, a situation where acidification and the consequent Ca\textsuperscript{2+} influx was normal, but the flagella were not shed. Acid-stimulated Ca\textsuperscript{2+} influx did not inactivate after pH shock of \textit{fa-1} cells (Fig. 4 B). This suggests that attenuation of Ca\textsuperscript{2+} influx is not coupled to pH shock or Ca\textsuperscript{2+} influx, but rather is tightly linked to the occurrence of deflagellation.

\textit{Centrin Contraction Does Not Mediate Inactivation of Acid-induced Ca\textsuperscript{2+} Influx}

The observation that attenuation did not appear to be linked to acidification or Ca\textsuperscript{2+} influx (Fig. 4) suggested that inactivation of acidification-stimulated Ca\textsuperscript{2+} influx might be related to changes which occur in the transition zone during deflagellation.

A contraction of the transition zone, mediated by a stellate array of centrin-containing fibers, is tightly coupled to deflagellation in wild-type cells (Lewin and Lee, 1985; Sanders and Salisbury, 1989). The \textit{vfl-2} mutant strain carries a missense mutation in the centrin gene, and the transition zone of these cells is disorganized (Jarvik and Suhan, 1991; Taillon et al., 1992). \textit{Vfl-2} cells will deflagellate in response to acid, provided that an external shear force (stirring or mixing) is applied, but contraction of the transition zone is greatly reduced relative to wild-type cells (Jarvik and Suhan, 1991; Sanders and Salisbury, 1994). \textit{Vfl-2} cells were used, therefore, to test the hypothesis that inactivation of Ca\textsuperscript{2+} influx is related to the mechanical stress of contraction that normally accompanies deflagellation. Fig. 5 shows that \textit{vfl-2} cells can express a robust \textit{45Ca} influx in response to acidification, but the response is attenuated after pH shock. The \textit{vfl-2} cells deflagellated due to the mixing provided in the pH shock protocol, therefore, attenuation remained correlated with deflagellation, but wild-type centrin contraction was not required. I conclude that contraction of the stellate fibers of the transition zone is not the cause of inactivation of Ca\textsuperscript{2+} influx after pH shock.

\textit{Inhibition of Deflagellation and Acid-stimulated \textit{45Ca} Influx}

La\textsuperscript{3+} completely blocks acidification-activated Ca\textsuperscript{2+} influx (EC\textsubscript{50} ~2 \mu M in 50 \mu M Ca\textsuperscript{2+}; Quarmby and Hartzell, 1994b). This concentration of La\textsuperscript{3+}, however, also affects cell motility and viability. To test the hypothesis that acidification-stimulated Ca\textsuperscript{2+} influx is through a specific channel or transporter, I screened for a specific blocker of acid-stimulated, deflagellation-inducing Ca\textsuperscript{2+} influx. Pharmacological agents from a number of different classes were tested for their ability to inhibit acid-induced deflagellation by blocking the Cd\textsuperscript{2+}-insensitive Ca\textsuperscript{2+} influx. Flufenamic acid inhibited a large component of acid-induced Ca\textsuperscript{2+} influx, but had only a modest effect on the early phase of influx (Fig. 6 A). There was no further inhibition of Ca\textsuperscript{2+} influx when Ca\textsuperscript{2+} was applied with flufenamic acid, suggesting that flufenamic acid was blocking the Cd\textsuperscript{2+}-sensitive component of acidification-stimulated Ca\textsuperscript{2+} influx (Fig. 6 A). This inference was confirmed by the observation that flufenamic acid did not block acid-induced deflagellation, which requires flux of Ca\textsuperscript{2+} through the Cd\textsuperscript{2+}-insensitive pathway (Fig. 6 D). A similar pattern of partial block of acidification-stimulated Ca\textsuperscript{2+} influx, with little or no effect on acidification-induced deflagellation, was also observed with D-600, nifedipine, nicardipine, and SKF-96365 (Ca\textsuperscript{2+} influx data not shown; deflagellation data presented in Fig. 6 D). Cobalt, nickel, and \omega-conotoxin did not block acid-induced deflagellation (Fig. 6 D) and, therefore, were not assayed for blockade of the Cd\textsuperscript{2+}-insensitive component of acidification-stimulated Ca\textsuperscript{2+} influx.

In contrast, Gd\textsuperscript{3+} was a potent inhibitor of acid-stimulated deflagellation (IC\textsubscript{50} ~5 \mu M; Fig. 6 C), as well as a potent blocker of acid-stimulated \textit{45Ca} influx (shown for 10 \mu M Gd\textsuperscript{3+} in Fig. 6 B). Importantly, 10 \mu M Gd\textsuperscript{3+}...
had no discernible effect on cell motility or motile responses to light (data not shown).

**DISCUSSION**

We have previously shown that the classical "pH shock" protocol (e.g., Witman, 1986) triggers deflagellation via acidification of the cytosol (Hartzell et al., 1993). Acidification causes deflagellation only if it is accompanied by an influx of Ca$^{2+}$ (Quarmby and Hartzell, 1994b). A Ca$^{2+}$-permeant ion channel or transporter was postulated to provide the route of entry for Ca$^{2+}$. My working model for this signaling pathway is presented in Fig. 7. In the present work I show that the regulation, drug sensitivity, and subcellular localization identify acidification-stimulated Ca$^{2+}$ influx as a specific Ca$^{2+}$ entry pathway distinct from established Ca$^{2+}$ channels.

Total Ca$^{2+}$ influx through this pathway is small. The Ca$^{2+}$ influx of 500 f mol/s/10$^6$ cells in 50 μM Ca$^{2+}$ is roughly equivalent to 7 pS conductance per cell (assuming a membrane potential of −70 mV, a reversal potential for Ca$^{2+}$ of +70 mV, and an inward current carried exclusively by Ca$^{2+}$; see Hille, 1992). This low rate of Ca$^{2+}$ influx is not due to a coincident efflux of Ca$^{2+}$ because acidification does not stimulate $^{45}$Ca efflux (unpublished observations). The transient and small influx of Ca$^{2+}$ suggests, therefore, either a trans-

**FIGURE 6.** Effects of Ca$^{2+}$ channel blockers on acid-stimulated deflagellation and Ca$^{2+}$ influx. (A) 1.5 ml of 137c cells in H$_2$O/MgCl$_2$ were incubated with (▲) 100 μM flufenamic acid, (♦) 100 μM flufenamic acid and 100 μM CdCl$_2$, (▼) 0.095% ethanol, which is equivalent to the amount of ethanol added as vehicle with the flufenamic acid, or (■) nothing, for 1 min before assay for $^{45}$Ca influx in response to the acetate cocktail which included the same concentrations of ethanol, flufenamic acid, and CdCl$_2$, as appropriate. (B) 1.5 ml of 137c cells in H$_2$O/MgCl$_2$ were incubated with (▲) 10 μM GdCl$_3$, (♦) 10 μM GdCl$_3$, and 100 μM CdCl$_2$, (▼) 100 μM CdCl$_2$, or (■) nothing, for 1 min before assay for $^{45}$Ca influx in response to the acetate cocktail (which included the same concentrations of GdCl$_3$ and CdCl$_2$, as appropriate). Data points are the means of three trials in a single experiment. The results illustrated in A and B are representative of two and three independent experiments, respectively. (C) 100 μl of cells (137c) in H$_2$O/MgCl$_2$ were incubated with the specified [GdCl$_3$] for 1 min before the addition of 100 μl of 40 mM Na-acetate, pH 4.5, with 1 mM CaCl$_2$. After 30 s of exposure to the acid, cells were fixed by the addition of 200 μl of 4% glutaraldehyde. Cells were scored for the loss of flagella by phase-contrast microscopy. At least 100 cells were scored for each data point. (D) Cells (137c) were incubated with the specified Ca$^{2+}$ channel blocker for 1 min before the addition of an equal volume of 40 mM Na-acetate, pH 4.5, with 1 mM CaCl$_2$ and the appropriate concentration of blocker. At least 100 cells in each of four to nine independent trials were scored for deflagellation. The concentrations of blocker used were: flufenamic acid, up to 1 mM; SKF-96365, up to 100 μM; gadolinium, up to 50 μM (see C; w-conotoxin (up to 5 μM with a 3-h pretreatment); cobalt, 1 mM; nickel, 1 mM; nicardipine, up to 100 μM; nifedipine, 20 μM. 

**TABLE 1.** The effects of Ca$^{2+}$ channel blockers on acid-stimulated deflagellation and Ca$^{2+}$ influx. The Ca$^{2+}$ influx of 500 f mol/s/10$^6$ cells in 50 μM Ca$^{2+}$ is roughly equivalent to 7 pS conductance per cell (assuming a membrane potential of −70 mV, a reversal potential for Ca$^{2+}$ of +70 mV, and an inward current carried exclusively by Ca$^{2+}$; see Hille, 1992). This low rate of Ca$^{2+}$ influx is not due to a coincident efflux of Ca$^{2+}$ because acidification does not stimulate $^{45}$Ca influx (unpublished observations). The transient and small influx of Ca$^{2+}$ suggests, therefore, either a trans-

**FIGURE 7.** The working model for the signaling pathway is presented in this diagram. The model suggests that acidification of the cytosol triggers deflagellation via an influx of Ca$^{2+}$, which is mediated by a Ca$^{2+}$ channel or transporter. The influx of Ca$^{2+}$ is small, as indicated by the low rate of Ca$^{2+}$ influx (500 f mol/s/10$^6$ cells in 50 μM Ca$^{2+}$). The pathway is distinct from established Ca$^{2+}$ channels, as evidenced by the lack of drug sensitivity and subcellular localization. The low rate of Ca$^{2+}$ influx suggests a transient and small influx of Ca$^{2+}$, which is consistent with the absence of any effect on cell motility or motile responses to light (data not shown).

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porter, a sparsely expressed ion channel, or a channel with low permeability to Ca$^{2+}$ (for example, either nonspecific cation channels, or low-conductance Ca$^{2+}$ channels such as \( I_{\text{Ca}} \) (Hoth and Penner, 1992; Lewis and Cahalan, 1993; Zweifach and Lewis, 1993). The possibility that the Ca$^{2+}$ influx might be mediated by a transporter is particularly intriguing. In general, Ca$^{2+}$ signals are generated when ion channels open and allow Ca$^{2+}$ to flow down its electrochemical gradient into the cytosol; however, a strong electrochemical gradient for Ca$^{2+}$ cannot be assumed when the cell is a free-living unicellular organism such as \textit{Chlamydomonas}. The [Ca$^{2+}$] in forest soils, where \textit{Chlamydomonas} is often found, can be in the low \( \mu \)M range (Lawrence et al., 1995); therefore, it is possible that the acidification-stimulated Ca$^{2+}$ influx is mediated by a transporter.

La$^{3+}$ is a potent blocker of acidification-stimulated Ca$^{2+}$ influx (Quarmby and Hartzell, 1994b); however, treatment with La$^{3+}$ also affects cell motility and viability. In the present study, Gd$^{3+}$ potently blocked both the acid-induced Ca$^{2+}$ influx and acid-induced deflagellation (IC$_{50}$ $\sim$ 5 \( \mu \)M) with no apparent effect on cell motility or motile responses to light, even after 18 h of incubation with 10 \( \mu \)M Gd$^{3+}$. Gadolinium blocks mechano-sensitive and receptor-activated nonspecific cation channels (Zhou et al., 1991; Hajduczok et al., 1994; Liu et al., 1994; Musgrave et al., 1994; Schumann et al., 1994; Matsumoto et al., 1995) as well as a subset of voltage-sensitive Ca$^{2+}$ channels and the low-conductance voltage-insensitive Ca$^{2+}$ channel, \( I_{\text{Ca}} \) (Roman-Silva et al., 1994; R. Penner, personal communication). The nonspecific cation channels and the \( I_{\text{gmc}} \) type channels are both Ca$^{2+}$ influx pathways that are not blocked by the classic organic Ca$^{2+}$ channel blockers, but are sensitive to Gd$^{3+}$. The deflagellation-inducing Ca$^{2+}$ influx was not blocked by Ni$^{2+}$, Co$^{2+}$, SKF-96365, flufenamic acid, o-conotoxin, nifedipine, nicardipine, diltiazem, and D-600 (Fig. 6D; Quarmby and Hartzell, 1994b). The array of agents which did not block the Cd$^{2+}$-insensitive component of acidification-stimulated Ca$^{2+}$ influx includes potent blockers of many Ca$^{2+}$ channels including voltage-gated channels and "receptor-activated" channels (Merritt et al., 1990; Hille, 1992). Although some of the blockers reduced total $^{45}$Ca influx in response to acidification, none of them blocked the deflagellation-inducing influx. Other Ca$^{2+}$-permeant channels, including the stretch-activated nonspecific cation channels and a class of neuronal Ca$^{2+}$ channel, are also refractory to the agents tested here but are potently blocked by gadolinium (Zhou et al., 1991; Roman-Silva et al., 1994).

The acid-stimulated Ca$^{2+}$ influx of wild-type cells was attenuated after pH shock (Fig. 3), although attenuation was not observed in \( f/a-1 \), a mutant that shows acid-stimulated Ca$^{2+}$ influx but does not deflagellate (Fig. 4). Attenuation of the Ca$^{2+}$ influx pathway, therefore, correlated with the deflagellation event and did not appear to be a consequence of acidification and Ca$^{2+}$ influx. Recovery of Ca$^{2+}$ influx, furthermore, occurred in the absence of flagellar regeneration (Fig. 3). It is not known whether inactivation observed 15 min after pH shock of wild-type cells is the same phenomenon as the decreasing rate of Ca$^{2+}$ influx which occurs during the first few seconds of exposure to acid (e.g., Fig. 1).

In addition to deflagellation, Ca$^{2+}$ mediates other behaviors of \textit{Chlamydomonas}. Most notable are the light-triggered changes in flagellar motility which regulate phototaxis and the photoshock response. Positive and negative phototaxis, swimming toward or away from light, is mediated by a change in flagellar dominance and is dependent on genetic, environmental, and physiological factors (Witman, 1993). In contrast, a temporary switch from the ciliary-type beat that normally pulls the cell forward to a flagellar wave-form that propels the cell in the reverse direction is responsible for the photoshock response to a sudden step-up in light intensity. Deflagellation is a dramatically different behavior than a change in flagellar dominance, beat frequency, or waveform. How are these Ca$^{2+}$ signals distinguished? The phenomenon of Ca$^{2+}$ signal discrimination is well known but its mechanisms are not understood, although the route of Ca$^{2+}$ entry clearly plays a role in some systems (Lenzi and Roberts, 1994; Ghosh and Greenberg, 1995). One goal of the present study was to
determine whether the Ca\textsuperscript{2+} entry pathway of deflagellation is distinct from the light-activated Ca\textsuperscript{2+} channels involved in phototaxis and the photoshock response.

A distinct subcellular localization might explain how the Ca\textsuperscript{2+} signal of deflagellation is distinguished from the Ca\textsuperscript{2+} signals involved in other flagellar behaviors. A voltage-gated Ca\textsuperscript{2+} channel involved in the photoshock response and a mechanoreceptor Ca\textsuperscript{2+} current are both expressed exclusively along the length of the flagellar membrane (Beck and Uhl, 1994; Yoshimura, 1996). In contrast, acidification-activated Ca\textsuperscript{2+} influx was (a) expressed in a flagella-less mutant, balb-2 (Fig. 1), (b) not expressed in isolated flagella (Fig. 2), and (c) expressed in cells lacking flagella as a consequence of colchicine treatment (Fig. 3 C). Together, these observations indicate that the acidification-stimulated Ca\textsuperscript{2+} influx is expressed primarily on non-flagellar membrane. On the basis of subcellular localization, therefore, this Ca\textsuperscript{2+} influx pathway is distinct from both the voltage-gated Ca\textsuperscript{2+} channel of the photoshock response and the mechanoreceptor Ca\textsuperscript{2+} current.

Although the acid-stimulated Ca\textsuperscript{2+} influx pathway did not localize to the flagella, it was lost when the flagella were shed (Fig. 3). One possible explanation is that the acidification-stimulated Ca\textsuperscript{2+} influx pathway is localized to the site of deflagellation (the transition zone) and is disrupted in some way by the excision of flagella. The flagellar transition zone is a highly specialized region serving many functions including partitioning of the flagellosome from the cytoplasm and the plasma membrane from the flagellar membrane (Musgrave et al., 1986; Besharse and Horst, 1990; Kaneshiro, 1990). The simplest explanation for tight linkage between inactivation of the Ca\textsuperscript{2+} influx and deflagellation is that the acidification-stimulated Ca\textsuperscript{2+} influx pathway localizes to the transition zone. A direct test of this hypothesis will require Ca\textsuperscript{2+} imaging or immunofluorescent localization of proteins in the pathway.

Ca\textsuperscript{2+} influx at the transition zone has also been postulated to play a role in phototaxis. It has been hypothesized that Ca\textsuperscript{2+} channels along the flagella are important for “reprogramming” responses, such as the switch from ciliary to flagellar beat in the photophobic response of Chlamydomonas, whereas changes in beat frequency (“on-off” responses), such as occurs during the turning of phototactic cells, are mediated by Ca\textsuperscript{2+} sensors at the base of the flagella (Tamm, 1994). Consistent with Tamm’s hypothesis, the voltage-gated Ca\textsuperscript{2+} channels which mediate the photophobic response of Chlamydomonas are evenly distributed along the length of the flagella (Beck and Uhl, 1994). Tamm’s hypothesis predicts that the Ca\textsuperscript{2+} channels involved in phototaxis are localized at the flagellar transition zone, but this is yet to be demonstrated. Nevertheless, the Ca\textsuperscript{2+} channels of phototaxis are distinct from the acid-stimulated pathway. First, phototaxis is inhibited by the N-type Ca\textsuperscript{2+} channel blocker, ω-conotoxin (Hegemann et al., 1990) whereas ω-conotoxin does not block deflagellation (Fig. 6 D). Second, the adf-1 mutant, which does not express acid-stimulated Ca\textsuperscript{2+} influx, appears to phototax normally (Quarmby and Hartzell, 1994b; unpublished observations).

The characteristics of the Ca\textsuperscript{2+} influx that triggers deflagellation distinguish it from known Ca\textsuperscript{2+} influx pathways, including those that mediate other behaviors in Chlamydomonas. The results of this study support the idea that the route of Ca\textsuperscript{2+} entry plays an important role in Ca\textsuperscript{2+} signal segregation. Reception of the Ca\textsuperscript{2+} signal may, however, also play an important role. The Ca\textsuperscript{2+}-binding protein in the fa pathway (Fig. 7) may, for example, have a low affinity for Ca\textsuperscript{2+} or slow binding kinetics, which would make it “blind” to fast or small Ca\textsuperscript{2+} signals. Sophisticated timing is required to communicate a light signal, received by the eyespot region of the cell body, to the flagella in order to mediate a turn towards the light source. It is reasonable to expect the temporal component of the light-induced Ca\textsuperscript{2+} signal to be important for phototaxis. I hypothesize, therefore, that the Ca\textsuperscript{2+}-binding properties of a protein in the fa pathway help to isolate these Ca\textsuperscript{2+} signaling pathways. We are currently defining the deflagellation pathway genetically and cloning genes for the acid-activated Ca\textsuperscript{2+} channel and a putative Ca\textsuperscript{2+}-binding protein in the fa pathway.
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