Calcium-Induced Conidiation in *Penicillium cyclopium*: Calcium Triggers Cytosolic Alkalinization at the Hyphal Tip

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Addition of Ca^{2+} (1 to 10 mM) to submerged cultures of *Penicillium cyclopium* induces conidiation. Ca^{2+} induced an increase in cytosolic pH from ~ 7.00 to >7.60 in less than 10 min, as determined with the fluorescent pH probe fluorescein. Measurement of the H^+ -ATPase activity in total membrane fractions did not show any stable activation in vivo as a result of Ca^{2+} treatment. By fluorescence ratio imaging microscopy, it was observed that vegetative hyphae exhibit a tip-to-base pH gradient, with the tip being more acidic. Ca^{2+} caused this gradient to dissipate within 10 min. The effect of several agents that are supposed to cause internal acidification, by different means, on conidiation was tested. Concentrations of these agents that did not significantly affect growth but inhibited Ca^{2+} -induced conidiation also prevented the intracellular alkalinization observed after exposure to the cation. Calcium channel blockers (lanthanum, cobalt, verapamil, and nifedipine) were not able to inhibit Ca^{2+} -induced conidiation, although their effect on calcium uptake was not evaluated. However, the combined results point towards externally bound Ca^{2+} as the primary agent of conidiation induction, causing changes in plasma membrane function which disrupt the pH gradient observed during apical growth.

The presence of calcium ions in culture medium induces the start of morphogenetic and differentiative processes in several fungal species (including some penicillia) and slime molds (11, 16, 26, 29, 30).

Penicillium cyclopium undertakes conidiation in submerged culture upon addition of Ca^{2+} (1 to 10 mM) without undergoing nutrient starvation (52). Although the mechanism of induction is still unknown, previous studies showed that Ca^{2+} was associated with the plasma membrane, mitochondria, and vacuoles (53). Calcium uptake kinetics by hyphae and protoplasts is biphasic (54, 55); after a phase of binding to the mycelium follows a second phase of metabolism-dependent uptake. Moreover, Ca^{2+} exposures as short as 0.5 to 2 min, in which considerable Ca^{2+} binding to the plasma membrane but negligible uptake occurs, result in a conidiation pattern identical to that of cultures exposed continuously to the cation (54, 55). Finally, Ca^{2+} -induced conidiation can be inhibited within 2 h of the addition of Ca^{2+} by treatment with the Ca^{2+} chelator BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid], which withdraws up to 65 to 75% of the cell-associated calcium (55). The evidence supports the view that externally bound calcium is involved in the induction process.

Intracellular pH must be closely regulated by cells in order to maintain balanced growth and may be of great relevance in the spatial distribution of cellular functions. Changes in cytosolic pH have been observed to be associated with several microbial morphogenetic and differentiative processes, such as germ tube formation in *Candida albicans* (46), *Dictyostelium discoideum* differentiation (18), and light-induced conidiation in *Trichoderma viride* (15), as well as at the beginning of the cell cycle in yeasts, in which cytosolic alkalinization precedes DNA synthesis (12). Taking into account all these and other effects in microorganisms and animal and plant cells, it has been suggested that protons

could act as second messengers, triggering, mediating, or regulating a wide range of growth and developmental processes (for a review, see reference 22).

In this article, we show that addition of calcium ions to submerged cultures of *P. cyclopium* triggers a relatively fast cytosolic alkalinization that could be part of the mechanism underlying Ca^{2+} -induced conidiation.

MATERIALS AND METHODS

Organism and culture conditions. *P. cyclopium* Westling (IMI 229034) was cultured from conidia in aerated 2-liter fermentors, as described previously (52), containing 1 liter of F medium (16), containing (in grams per liter) sucrose, 20; NaNO_3 , 6; KH_2PO_4 , 1.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; and trace elements. This medium contains $<50 \mu\text{M}$ Ca^{2+} , as determined by atomic absorption.

Fluorescein loading. Hyphae grown for 24 h were filtered on a nylon mesh, washed with distilled water, and resuspended in F medium at a cell density of 0.75 mg (dry weight) of cells per ml. Samples (1 ml) from this suspension were incubated with 20 μM fluorescein diacetate (stock solution containing 20 mM in dimethyl sulfoxide) for 5 min at 25°C with gentle stirring. The final dimethyl sulfoxide concentration in the incubation medium (0.1%, vol/vol) did not affect fungal growth or conidiation. At this point, the ester diffuses through the fungal plasma membrane and is intracellularly hydrolyzed, releasing the active form (fluorescein) into the cytosol (43). Loaded hyphae were filtered on a Millipore HA filter (pore size, 0.45 μm), washed with distilled water, and resuspended in 5 ml of F medium.

Intracellular pH measurement by fluorimetry. The intracellular pH of *P. cyclopium* was measured at 520 nm (emission wavelength) with the fluorescent pH probe fluorescein by the dual-excitation-wavelength (490 and 435 nm) ratio method (43). Fluorescence from hyphae was immediately measured after fluorescein loading at both excitation wavelengths (F_{490} and F_{435}) in a spectrofluorimeter. Cytosolic pH was deter-

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FIG. 1. Calibration curves of the F_{490}/F_{435} ratio versus pH for fluorescein. The in vitro calibration curve ($\circ$) was constructed by fluorimetry of 2 μM fluorescein solutions in buffers of various pHs. In situ calibration curves were constructed by fluorimetry ($\square$) and by fluorescence microscopy ratio imaging ($\triangle$) of fluorescein-loaded *P. cyclopius* hyphae; intracellular pH was held constant with nigericin in high- K^+ buffers of various pHs. Values are means $\pm$ SD for at least three experiments. For the in vitro curve, error bars (SD of $<5\%$) have been omitted for clarity.

mined from the F_{490}/F_{435} ratio, after subtraction of the background value (value for fluorophore-free hyphal suspensions at the same cell density) and corrections for fluorescein leakage into the extracellular medium, using a calibration curve constructed with pH-controlled hyphae (in situ calibration; see below).

When intracellular pH was measured after treatment with different compounds, the compounds were added to the culture 10 h after inoculation. At 24 h, cells were collected, washed, and resuspended in an identical medium before fluorescein loading, and intracellular pH measurement was carried out as above.

Intracellular pH measurement by fluorescence microscopy and digital imaging. *P. cyclopius* hyphae loaded with fluorescein as described above were resuspended in 1 ml of F medium and incubated for 3 min in order to reduce the cellular fluorescein content to levels suitable for photomicrographic recording. The cells were then filtered, washed, and resuspended in 2 ml of fresh F medium. Samples from this suspension were immediately viewed with a Nikon Optiphot microscope equipped with an episcopic fluorescence attachment, with CF DL 40 $\times$ (0.65 NA) and CF DL 100 $\times$ (1.25 NA; oil immersion) objectives. Samples were excited by light from a 100-W high-pressure mercury lamp, whose wavelength was alternatively selected by the use of 470–490 nm and 435 nm interference filters. Fluorescence passed through a dichroic mirror (barrier, 510 nm) and a 520-nm barrier filter. Excitation filters were changed manually. Photomicrographic recording was performed with Kodak TMAX 400 film. A pair of images were taken from each hypha in the fluorescence mode (at both excitation wavelengths).

Each pair of photomicrographs obtained by fluorescence microscopy were recorded with a Hitachi KP-140 video camera (averaging 10 frames to reduce noise) and digitalized and stored in an IBM PC/AT microcomputer. Digitalized images were analyzed with an application developed at the Facultad de Informática of the Universidad del País Vasco (San Sebastián, Spain) from a PCVISIONplus Frame Grab-

ber (Imaging Technology Inc., Woburn, Mass.) image processor. First, a threshold level was determined interactively for each cell to separate the cell from the background. Then both images were aligned by elimination of rotations and shifts, and when necessary, background was subtracted from cells (in general, the background was negligible). Finally, each pair of images were divided on a pixel-by-pixel basis to obtain the F_{490}/F_{435} ratio image. The ratio value in each pixel was transformed to a pH value by using a calibration curve constructed in situ by fluorescence microscopy and digital imaging (see below), and the resulting pH image was displayed on a Barco CDCT-6151 TV screen, giving a pseudocolor to each pH value.

Calibration. F_{490}/F_{435} ratio versus pH calibration curves were calculated in vitro and in situ (Fig. 1).

(i) **In vitro calibration (fluorimetry).** In vitro calibration was done with 2 μM sodium fluorescein solutions in 50 mM Tris-maleate buffer (pHs between 4.5 and 8.5).

(ii) **In situ calibration (fluorimetry).** In situ calibration was carried out with the H^+/K^+ exchanger nigericin and an extracellular potassium concentration similar to that found in the cell to keep the intracellular pH to a given level (49). *P. cyclopius* hyphae, loaded with fluorescein as described above, were suspended and incubated for 5 min in a medium containing 50 mM Tris-maleate buffer (pHs between 5.0 and 8.0), 20 μM nigericin, 10 mM sodium azide (for pHs below 6.8), and 190 mM KCl. This concentration is derived from the value for cellular potassium content of 190.7 mmol/kg of cell water measured by atomic absorption in *P. notatum* protoplasts prepared from hyphae grown under the same conditions as *P. cyclopius* (32) and that is assumed to be similar in both fungi. After incubation, the cells were filtered and then washed and resuspended in 5 volumes of the same medium but containing 10 μM nigericin. Fluorescence was measured immediately at both excitation wavelengths, and the resulting fluorescence ratio values, once corrected for background and dye leakage (see Results), were plotted versus pH. The calibration curve was adjusted by a nonlinear regression method.

(iii) **In situ calibration (microscopy and digital imaging).** Hyphae loaded with fluorescein and pH-controlled as described above were viewed by fluorescence microscopy. A pair of photomicrographs (for both excitation wavelengths) were taken at each pH, and the images were digitalized and analyzed by the method described above. Each fluorescence ratio value was obtained by averaging the values for all the pixels corresponding to each pH-controlled hypha. The calibration curve was adjusted as described above. The materials, procedure, and software used for digital image analysis are described in reference 1a.

Total membrane fraction preparation. *P. cyclopium* mycelium grown for 24 h was collected by filtration, and 0.5-g (wet weight) samples were suspended in 8 ml of an ice-cold medium containing 50 mM MOPS [3-(*N*-morpholino)propanesulfonic acid]-BTP (1,3-bis[tris(hydroxymethyl)-methylamino]propane) buffer (pH 7.5), 0.33 M sorbitol, 5 mM EDTA, 0.2% (wt/vol) bovine albumin, 0.2% (wt/vol) casein hydrolysate, and 5 mM dithioerythritol. The mycelium was disintegrated by being vortexed with 8 ml of glass beads for 30 s and then placed on ice for 30 s, repeated six times. The resulting suspension was diluted with 8 ml of the same medium and filtered through a nylon mesh, and 1 mM phenylmethylsulfonyl fluoride was added to the filtrate. This suspension was centrifuged for 5 min at $1,000 \times g$, and the supernatant was decanted and centrifuged for 40 min at $35,000 \times g$ in a Kontron A24.24 rotor. The pellet obtained (total membrane fraction) was suspended in a medium containing 0.33 M sucrose, 10 mM MOPS-BTP (pH 7.8), dithioerythritol, and EDTA as above.

ATPase assay. Vanadate-sensitive ATPase activity was calculated by subtracting the activity measured in the presence from that measured in the absence of 100 μ M sodium orthovanadate. The assay medium (120 μ l) contained 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid]-Tris buffer (pH 6.0), 0.33 M sucrose, 0.1 mM EDTA, 0.1 mM ammonium molybdate, 1 mM sodium azide, 50 mM sodium nitrate, 3 mM magnesium sulfate, 0.01% (wt/vol) Triton X-100, and 45 μ g of protein. The reaction was started with 3 mM MgATP and stopped after 30 min at 25°C with hydroquinone-2 N sulfuric acid. P_i release was measured by the method of Kitson and Mellon (19). ATPase activity is given as micromoles of P_i released per minute per milligram of protein.

Intracellular distribution of free fluorescein and subcellular localization of fluorescein diacetate esterase activity. Intracellular fluorescein localization was done with fluorescein diacetate-loaded cells by fluorimetric determination of the free dye present in subcellular fractions. These measurements were made with glass bead-disintegrated hyphae by differential centrifugation by the method of Ugalde et al. (51). Fluorescein trapped inside organelles was extracted by osmotic lysis in 50 mM MOPS-NaOH buffer (pH 7.0), and fluorescence was measured in the same medium.

Esterase activity in the fractions was measured at 25°C in a reaction mixture containing 50 mM MOPS-NaOH buffer (pH 7.0), 20 μ M fluorescein diacetate, and 50 μ g of protein from each fraction.

Other methods. Protein content was determined by the method of Bearden (2). Percent conidiation (percentage of conidiating tips) and dry weights were determined as previously described (52).

Reagents. Sodium fluorescein, fluorescein diacetate, nigericin, CCCP (carbonyl cyanide *m*-chlorophenylhydrazine), verapamil, and nifedipine were purchased from Sigma. Prochloraz, tridemorph, and propiconazole were obtained from Promochem Ltd. (St. Albans, United Kingdom).

TABLE 1. Subcellular localization of fluorescein diacetate esterase activity and free fluorescein in *P. cyclopium* hyphae^a

Subcellular fraction	Mean % $\pm$ SD ($n = 3$)	
	Esterase activity ^a	Fluorescein content ^b
Mitochondrial	1.1 $\pm$ 0.05	0.7 $\pm$ 0.06
Microsomal	1.2 $\pm$ 0.06	0.6 $\pm$ 0.04
Cytosolic	91.3 $\pm$ 2.0	98.7 $\pm$ 2.3

^a Percent of total in each fraction. Total fluorescein diacetate esterase activity, measured in the crude homogenate, corresponds to 31 ± 1.5 nmol of fluorescein released per min.

RESULTS

Intracellular pH measurement validation. One of the main advantages in the use of fluorescein as an intracellular pH probe is its lack of adverse effects on the organism being studied. Fluorescein diacetate has been used regularly as a vital stain to determine cell viability (4). In *P. cyclopium*, we confirmed that intracellularly trapped fluorescein did not interfere with growth or with Ca^{2+} -induced conidiation (results not shown), at least at those concentrations found inside the cell.

Various lines of evidence indicate that fluorescein was located in the cytosol, and therefore, cytosolic pH was reported. A study of the free fluorescein content of several subcellular fractions obtained from fluorescein-loaded hyphae indicated that almost all the free dye was in the cytosol (Table 1); more than 98% of the indicator was found in the supernatant, while the proportion included in the mitochondrial and microsomal fractions represented less than 1% in both cases.

Esterase activity in the fractions was confined mainly (more than 90%) to the cytosol (Table 1), and only minor activity levels were recorded in the mitochondrial and microsomal fractions. Membrane fractions contained large quantities of unhydrolyzed ester which, after heat-promoted hydrolysis at neutral pH, represented 25 to 35% of total cellular dye. This finding further confirms the absence of esterase activity in those fractions.

Microscopic observation of fluorescein-loaded hyphae (5 min) showed no dye accumulation in subcellular organelles, as can be seen from the general distribution pattern of the fluorescence (see Fig. 3A and B). The indicator could not be detected in vacuoles, which are the largest subcellular organelle, and this finding is in agreement with previous observations with *P. cyclopium* (39) and other fungi (4). Only by incubation of the hyphae with the ester for longer than 10 to 15 min could fluorescein accumulation inside longitudinally aligned tubular structures be observed. These structures were probably mitochondria, as they were identical to structures that stained with rhodamine (results not shown), a known mitochondrial stain (4).

Consequently, we conclude that the combination of the method and the dye used for intracellular pH quantification gave the cytosolic pH with little interference from intracellular organelles.

A practical drawback of this procedure, however, was dye leakage into the medium. In *P. cyclopium* hyphae, more than 50% of the trapped dye leaked within 5 min after loading. To avoid this problem, we tried a fluorescein derivative, 6-carboxyfluorescein, that is reported to leak out more slowly (49). However, the dye accumulated in vacuoles (results not shown), as reported for other systems (4, 37).

Therefore, in order to validate the use of fluorescein for

FIG. 2. Variations in the cytosolic pH of *P. cyclopium* hyphae over time after exposure to calcium. Calcium (5 mM) was added at time zero to hyphae growing in F medium. Cytosolic pH was measured by fluorimetry of fluorescein-loaded hyphae, as described in the text. Results are shown as means $\pm$ SD for at least three experiments.

intracellular pH measurement, it was necessary to make corrections for fluorescence resulting from dye released into the medium. Thus, immediately after fluorescence measurement, cell suspensions were rapidly filtered under vacuum, and the signal from the medium, measured at the same pair of excitation wavelengths, was then subtracted. These corrections were relevant for fluorimetric measurements and negligible for those performed by fluorescence microscopy.

Effect of Ca^{2+} on conidiation, growth, and cytosolic pH of *P. cyclopium*. Conidiation levels, reported as the percentage of conidiating tips, varied from $35\% \pm 7\%$ ($n = 6$) for mycelium exposed to calcium for 8 h to $8\% \pm 2\%$ ($n = 6$) for mycelium growing in Ca^{2+} -free medium (less than $50 \mu\text{M}$ contaminating calcium). In both cases, growth measured on a dry-weight basis was similar, 3.8 to 4.0 mg/ml ($n = 6$).

The addition of calcium ions (5 mM) to submerged cultures of *P. cyclopium* caused cytosolic alkalization, as determined with fluorescein. The mean cytosolic pH $\pm$ standard deviation (SD), measured by fluorimetry of cell suspensions, increased from 7.07 ± 0.10 ($n = 20$) for vegetative hyphae growing in F medium to 7.63 ± 0.24 ($n = 18$) for conidiating hyphae.

The time course variation in intracellular pH after calcium addition is shown in Fig. 2. Cytosolic alkalization took place early after calcium addition; alkalization could be detected within 2 min, and cytosolic pH reached its maximum value (~ 7.6) after 10 min and remained unchanged for the following 2 h. Furthermore, this value was maintained throughout even in conidiating hyphae for 8 h after Ca^{2+} induction.

Intracellular pH mapping in *P. cyclopium* hyphae. Growing *P. cyclopium* hyphae showed a tip-to-base pH gradient, with the tip more acidic (by 0.5 to 1.0 pH unit) than the distal regions (Fig. 3C). This acidic area generally occupied the apical region (10 to 15 μm), although some hyphae with more extended acidic areas (up to 40 to 50 μm) were also observed (two of eight hyphae). The observed acidic area did not correspond to that occupied by apical vesicles, which are restricted to the extreme apex, where they constitute up to 42% of the intracellular volume at 1 μm , dropping sharply

thereafter (8). Moreover, the observed pH gradient did not coincide with the spatial distribution of other subcellular structures.

The cytoplasmic layer near the hyphal plasma membrane appeared to be more acidic than the cytosolic central region. Although the pH measurement at the cell surface is less accurate than that in the hyphal bulk because of its reduced width and, therefore, its smaller signal-to-noise ratio, the observation of an acidic peripheral area whose pH value was close to the extracellular pH agrees with previous results obtained with *P. cyclopium* protoplasts (39).

Addition of calcium ions caused this longitudinal pH gradient to dissipate within 10 to 15 min ($n = 9$) by triggering cytosolic alkalization, mainly at the hyphal tip (Fig. 4C). Thus, the cytosolic pH became more uniform along the hyphae and reached a value close to that found in the distal region of untreated cells.

H^+ -ATPase activity in vegetative and Ca^{2+} -induced *P. cyclopium* hyphae. One possibility to explain the observed cytosolic alkalization was activation of proton pumping by the plasma membrane H^+ ATPase. Measurement of the vanadate-sensitive ATPase activity in total membrane fractions from vegetative hyphae ($0.121 \pm 0.015 \mu\text{mol}$ of P_i min^{-1} mg of protein $^{-1}$, $n = 4$) and hyphae exposed to Ca^{2+} for different periods of time (between 0.114 and $0.134 \mu\text{mol}$ of P_i min^{-1} mg of protein $^{-1}$ for exposures of between 2 min and 1 h, $n = 4$) did not show any kind of stable activation of the enzyme.

Effect of Ca^{2+} channel blockers on Ca^{2+} -induced conidiation. As in a great diversity of cellular processes, the mechanism by which calcium induces conidiation might involve an increase in its free concentration in the cytosol as a consequence of the entry of exogenously added calcium, perhaps through putative Ca^{2+} channels. If this were the case, then Ca^{2+} channel blockers could probably prevent the process. In general, none of the four Ca^{2+} channel blockers tested (La^{3+} , Co^{2+} , verapamil, and nifedipine) were able to inhibit Ca^{2+} -induced conidiation (Table 2). Only lanthanum and cobalt ions at the highest concentrations assayed caused inhibition; however, they also severely impaired growth. This means that inhibition at these high concentrations could be due mainly to interference with general cellular processes and/or direct competition with calcium rather than to channel blockage. For instance, addition of 5 mM lanthanum ions to F medium caused the appearance of a lanthanum phosphate precipitate, resulting in phosphate depletion.

Effect of several cytosolic acidification inducers on Ca^{2+} -induced conidiation. In order to test whether Ca^{2+} -induced cytosolic alkalization was necessary to trigger conidiation, several compounds supposed to cause intracellular acidification by different means were assayed. Some of them are well-known antifungal agents and can be classified into four groups: alcohols (ethanol and *n*-propanol); ergosterol biosynthesis inhibitors (prochloraz, tridemorph, and propiconazole); weak acids (sorbic acid); and protonophores (CCCP). The effects of these agents on intracellular pH, conidiation, and growth are shown in Table 3. At the concentrations tested, all of them inhibited Ca^{2+} -induced cytosolic alkalization and conidiation without significantly affecting growth (on a dry-weight basis). These compounds, used at the same concentrations and in the absence of calcium, significantly impaired fungal growth (results not shown), suggesting a protective effect of calcium against their antifungal action.

FIG. 3. Cytosolic pH map of a *P. cyclopium* hypha in F medium. Fluorescein loading and microscopic observation were done as described in the text. The three images correspond to the same fluorescein-loaded hypha. (A) Fluorescence at 490 nm (F_{490}); (B) fluorescence at 435 nm (F_{435}); (C) F_{490}/F_{435} ratio image. Cytosolic pHs in panel C (pH map) are represented as pseudocolors. Inset: Twofold magnification of the hyphal tip. Bars, 10 μ m.

DISCUSSION

Two questions may be addressed in the light of the results obtained in this study. (i) What are the site and mode of action of Ca^{2+} in the process of conidiation induction? (ii) Is the cytosolic alkalization observed after Ca^{2+} addition mediating the mechanism of conidiation induction?

The first question has been the subject of previous investigations, and various hypotheses have been studied. The possibility that extracellularly added calcium may interfere with intracellular second-messenger regulation systems involving this cation has been considered (35), although little

and controversial evidence has so far emerged to substantiate this option; trifluoperazine and ophiobolin have been reported to inhibit Ca^{2+} -induced conidiation in *P. notatum* without reference to inhibition of general growth processes (35). Conversely, no rises in the intracellular free calcium concentration in *P. notatum* could be measured after Ca^{2+} addition (35), and Ca^{2+} channel blockers do not appear to inhibit Ca^{2+} -induced conidiation in *P. cyclopium* (see Results).

The existence in *Penicillium* spp. of Ca^{2+} channels that could be inhibited by the blockers tested remains to be

FIG. 4. Cytosolic pH map of a *P. cyclopium* hypha in F medium, 10 min after addition of 5 mM calcium. Fluorescein loading and microscopic observation were done as described in the text. The three images correspond to the same fluorescein-loaded hypha. (A) Fluorescence image at 490 nm (F_{490}); (B) fluorescence image at 435 nm (F_{435}); (C) F_{490}/F_{435} ratio image. Cytosolic pHs in panel C (pH map) are represented as pseudocolors. Inset: Twofold magnification of the hyphal tip. Bars, 10 μ m.

TABLE 2. Effects of several Ca^{2+} channel blockers on Ca^{2+} -induced conidiation and growth of *P. cyclopium*^a

Treatment	% Conidiation ^b	Dry wt (mg/ml)
Control (no addition)	8 ± 2	3.8 ± 0.2
Ca^{2+}	36 ± 7	4.1 ± 0.3
Ca^{2+} + La^{3+} (100 μM)	37 ± 9	3.7 ± 0.3
Ca^{2+} + La^{3+} (1 mM)	41 ± 9	3.8 ± 0.6
Ca^{2+} + La^{3+} (5 mM)	0	2.1 ± 0.1
Ca^{2+} + Co^{2+} (10 μM)	37 ± 9	3.9 ± 0.2
Ca^{2+} + Co^{2+} (100 μM)	35 ± 4	3.4 ± 0.4
Ca^{2+} + Co^{2+} (1 mM)	0	2.0 ± 0.1
Ca^{2+} + verapamil (100 μM)	35 ± 7	4.2 ± 0.2
Ca^{2+} + verapamil (1 mM)	34 ± 5	4.3 ± 0.4
Ca^{2+} + nifedipine (100 μM)	33 ± 3	4.4 ± 0.2
Ca^{2+} + nifedipine (1 mM)	35 ± 4	4.4 ± 0.3

^a Calcium (5 mM) and Ca^{2+} channel blockers were added as indicated to cultures 24 h after inoculation, and the percent conidiation and dry weight were measured 8 to 10 h later. Values are means ± SD for at least three experiments.

^b Percent conidiating tips.

clarified, and therefore, the results obtained in this study do not indicate whether calcium entered the cell to induce alkalization and conidiation. The mechanism of calcium entry into fungal cells is presently a confusing issue. As far as we know, Ca^{2+} channels in fungi have been demonstrated only in *Blastocladiella emersonii*; La^{3+} and Co^{2+} (but not verapamil) blocked the Ca^{2+} channels in this fungus (5). Furthermore, the great diversity of effects of the assayed Ca^{2+} channel blockers reported for different fungal species, ranging from reduction in hyphal extension rate and increase in branching (38) to complete lack of effects (47), casts great ambiguity over this issue. Lanthanum ions have been reported to inhibit Ca^{2+} uptake and depolarize the plasma membrane in *Neurospora crassa* (9) but have also been found not to have appreciable effects on either hyphal extension or branching (47).

In conclusion, apart from the reasonable doubts concerning the experiments with Ca^{2+} channel blockers, the experimental evidence suggests that the primary site of the action of calcium may not necessarily be intracellular. In addition, the fact that strontium and barium may partially replace calcium as conidiation inducers (16) further discredits the possibility of interference with intracellular Ca^{2+} signal transduction systems, given their high specificity for this cation.

Alternatively, calcium ions may bind to the plasma mem-

brane, changing its functional properties and resulting in conidiation induction (54, 55). Indirect evidence pointing to a specific effect on plasma membrane permeability to protons has been presented in this study and may be inferred for *N. crassa*, in which Ca^{2+} causes hyperpolarization of the plasma membrane (44). Observations to this effect have also been reported elsewhere (3, 17). The conidiation response of different fungi to Ca^{2+} addition may depend on the binding characteristics of each strain surpassing a threshold level, as in the case of the differential response to calcium by strains of *P. notatum* with different binding capacities (34).

A decrease in plasma membrane permeability through calcium binding would be expected to cause a hyperpolarization (44), with direct consequences on proton gradient-dependent active transport, such as an increase in solute accumulation, which have been observed (1, 6, 33). On the other hand, the resulting changes in surface potential derived from Ca^{2+} binding could increase the apparent K_m of membrane transporters for charged substrates (23), such as nutrient- H^+ symports, which has also been reported (33). Finally, the alkalization effect of Ca^{2+} addition described in this study could be prevented by compounds which are known to increase plasma membrane permeability to protons, such as alcohols (24, 31), ergosterol biosynthesis inhibitors (28, 56), and a protonophore, and furthermore, calcium had a protective effect on growth in the presence of these compounds.

The above evidence stands in favor of a direct effect of calcium ions on plasma membrane function, with consequences for the induction of conidiation. Further research with purified plasma membrane vesicles (51) should provide a more detailed view of the precise nature of this effect and its biochemical implications.

The second question, dealing with the role of the observed alkalization on conidiation induction, would be solved by triggering a cytosolic pH increase in the absence of calcium. A widely used method to increase intracellular pH is incubation of cells with a weak base. However, in our case, this procedure proved unsuitable. In order to accumulate intracellularly, the pK_a of the weak base must be between the intracellular and extracellular pH values, and the extracellular pH must be greater than the cytosolic pH. In our system, the extracellular pH (~5.0) is below the cytosolic pH (~7.0), so that weak base accumulation inside the cells would be negligible and unable to induce alkalization (46).

At least two mechanisms may explain the Ca^{2+} -induced cytosolic alkalization: (i) activation of proton pumping

TABLE 3. Effects of cytosolic acidification inducers on cytosolic pH, conidiation, and growth of *P. cyclopium* in the presence of 5 mM Ca^{2+} ^a

Treatment	Cytosolic pH (no. of measurements)	% Conidiation ^b	Dry wt (mg/ml)
Control (no added Ca^{2+})	7.07 ± 0.10 (20)	8 ± 2	3.8 ± 0.2
Ca^{2+}	7.63 ± 0.24 (18)	35 ± 7	4.0 ± 0.3
Ca^{2+} + ethanol (1.5%, vol/vol)	7.04 ± 0.10 (20)	4 ± 1	3.4 ± 0.2
Ca^{2+} + <i>n</i> -propanol (0.5%, vol/vol)	6.91 ± 0.09 (21)	8 ± 2	3.6 ± 0.2
Ca^{2+} + prochloraz (0.05 $\mu\text{g/ml}$)	7.03 ± 0.11 (20)	0	4.2 ± 0.4
Ca^{2+} + tridemorph (0.1 $\mu\text{g/ml}$)	6.92 ± 0.11 (20)	3 ± 0.4	3.6 ± 0.2
Ca^{2+} + propiconazole (0.1 $\mu\text{g/ml}$)	6.76 ± 0.08 (23)	7 ± 1	3.6 ± 0.3
Ca^{2+} + sorbic acid (80 μM)	6.78 ± 0.09 (21)	9 ± 2.5	3.4 ± 0.2
Ca^{2+} + CCCP (3 μM)	5.95 ± 0.15 (18)	8 ± 2	3.6 ± 0.4

^a Compounds were added as indicated to cultures (in F medium) 10 h after inoculation. Cytosolic pH was measured at 24 h and percent conidiation and dry weight were measured at 32 to 34 h after inoculation. Values are means ± SD for at least three experiments.

^b Percent conidiating tips.

through the plasma membrane, or (ii) a decrease in the proton influx inside the cell as a result of a decrease in plasma membrane permeability to protons.

In *Saccharomyces cerevisiae*, a stable in vivo activation of the plasma membrane H^+ -ATPase has been observed after the addition of glucose (42) or ethanol (40), and such a glucose-induced activation was shown to be accompanied by enzyme phosphorylation (7). On the other hand, in *N. crassa*, an ionophoresis-induced increase in the cytosolic Ca^{2+} concentration causes hyperpolarization and a conductance increase in the plasma membrane, which has been interpreted as a presumptive in vivo activation of the proton pump, despite contrary observations in vitro (25). Experiments constructed to show a similar activation in *P. cyclopium* could not demonstrate any stable activation of the H^+ -ATPase in total membrane fractions. However, considering the postulated gradient of ATPase activity along fungal hyphae (41), this activation may have occurred locally at the tip and may have been masked by the background of distal H^+ -ATPase activity. Therefore, it cannot be ruled out.

The experiments in which intracellular pH was controlled through careful manipulation of the concentrations of acidification inducers of different types in the presence of calcium indicate that this cation triggers an intracellular alkalization which may be prevented without significantly affecting growth. When this occurs, no conidiation can be observed in the presence of calcium. The mechanism of induction of cytosolic acidification varies with different agents, but the final result is similar, ruling out the possibility that secondary effects lessen the validity of the results.

In view of these results and the fact that the Ca^{2+} -induced cytosolic alkalization takes place immediately after calcium addition, we consider that the observed alkalization plays an active part in the mechanism of Ca^{2+} -induced conidiation.

The results and conclusions obtained in this study may be placed in the general context of apical growth and morphogenesis in filamentous organisms. Filamentous fungi have been reported to drive electric currents through themselves so that a positive charge enters the apex and leaves distally (13, 17). This effect has been interpreted as an uneven distribution of proton-driving pumps and proton-coupled transport systems in apically growing cells (14, 20). Apical proton influx would result in local acidification (27, 50) and depolarization (36, 45) at the tip. Moreover, new hyphal tips (branches) emerge at new points of proton influx (21). Under certain conditions, some hyphae present differing current flow characteristics (41, 48), which disfavors the idea that electric currents are responsible for polarized growth. However, it is generally accepted that proton influx takes place apically. The local acidification observed at the hyphal apex of vegetatively growing hyphae of *P. cyclopium* (this study) and other fungi (27, 50) agrees with this idea.

The morphogenetic process leading to conidiation is normally preceded by an arrest in hyphal extension and concludes with conidium formation (52). Therefore, apical extension growth and conidiation could in principle be related and controlled by the same kinds of mechanisms.

Calcium ions may be instrumental in decreasing the proton permeability of the plasma membrane at the hyphal apex, resulting in apical alkalization. This stimulus may be related to the more common emergence of hyphae from the growth medium to the air, presumably also resulting in interruption of the proton influx at the apex and conidiation. Thus, the localized entry of protons at the hyphal tip, and the resulting apical acidification, may represent a natural signal-

ing system indicating the nature of the medium surrounding the cell and controlling the responses accordingly.

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