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Cytosolic calcium homeostasis in fungi: Roles of plasma membrane transport and intracellular sequestration of calcium

(ion-selective microelectrodes/Ca^{2+}-ATPase/proton–calcium exchange/*Neurospora*)

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ABSTRACT **Cytosolic free calcium ($[Ca^{2+}]_c$) has been measured in the mycelial fungus *Neurospora crassa* with Ca^{2+}-selective microelectrodes. The mean value of $[Ca^{2+}]_c$ is 92 ± 15 nM and it is insensitive to external pH values between 5.8 and 8.4. Simultaneous measurement of membrane potential enables the electrochemical potential difference for Ca^{2+} across the plasma membrane to be estimated as about -60 kJ·mol^{-1}—a value that cannot be sustained either by a simple Ca^{2+}-ATPase, or, in alkaline conditions, by straightforward H^+/Ca^{2+} exchange with a stoichiometric ratio of <5 H^+/Ca^{2+}. We propose that the most likely alternative mechanism of Ca^{2+} efflux is ATP-driven H^+/Ca^{2+} exchange, with a stoichiometric ratio of at least 2 H^+/Ca^{2+}. In accord with this proposal, depletion of the ATP level from 2.5 to 0.5 mM by CN^- elicits an increase in $[Ca^{2+}]_c$, but only in alkaline conditions in which the putative H^+/Ca^{2+}-ATPase would be selectively stalled. The insensitivity of Ca^{2+} homeostasis to CN^- in more acid conditions implies that the K_m (ATP) of the transport system is 100 μM or less. The increase in $[Ca^{2+}]_c$ in the presence of CN^- at pH 8.4 (50 nM·min^{-1}) is compared with $^{45}Ca^{2+}$ influx (0.62 mM·min^{-1}) under the same conditions. The proportion of entering Ca^{2+} remaining free in the cytosol is only 8×10^{-5}, and since the concentration of available chelation sites on Ca^{2+}-binding proteins is unlikely to exceed 100 μM, a major role for the fungal vacuole in short-term Ca^{2+} homeostasis is indicated. This notion is supported by the observation that cytosolic Ca^{2+} homeostasis is disrupted by a protonophore, which rapidly abolishes the driving force (a transmembrane pH difference) for Ca^{2+} uptake into fungal vacuoles.**

Cytosolic free calcium ($[Ca^{2+}]_c$) plays a central role in signal transduction in animal and plant cells (1, 2). Modulation of the resting level of $[Ca^{2+}]_c$ by primary signals (hormones, membrane depolarization, growth regulators, or light) occurs via opening of ligand- or voltage-gated channels. A transmembrane electrochemical gradient for Ca^{2+} ($\Delta\bar{\mu}_{Ca^{2+}}$) is therefore a crucial prerequisite for signal transduction. Such gradients are sustained principally by transport systems that catalyze efflux of Ca^{2+} from the cytosol, with the result being that $[Ca^{2+}]_c$ is maintained at a low level (of the order of 0.1 μM) in the resting state.

A large body of evidence indicates that nutritional and developmental responses of fungi to environmental stimuli are similarly mediated by $[Ca^{2+}]_c$ (3). For example, Ca^{2+} ionophores induce branching in *Achlya* and *Neurospora* (4, 5) in a manner suggesting that apical dominance is abolished (6); Ca^{2+}-sensitive mutants of yeast are defective in budding (7); sporulation in *Penicillium* is dependent on extracellular Ca^{2+} (8); circadian rhythms in *Neurospora* are reset by the Ca^{2+} ionophore A23187 (9); the Ca^{2+}-binding protein calmodulin is present in *Neurospora* and yeast (10, 11); inositol 1,4,5-trisphosphate ($InsP_3$)—an established mediator of signal transduction in animal cells (12) and slime molds (13)—releases Ca^{2+} from vacuoles of *Neurospora* (14); and the precursor of $InsP_3$ (phosphatidylinositol 4,5-bisphosphate) plays an essential role in yeast cell proliferation (15). However, a detailed description of the role of Ca^{2+} in fungal signal transduction cannot proceed until methods are available for direct measurement of $[Ca^{2+}]_c$. One aim of the present study is therefore to investigate the possibility that $[Ca^{2+}]_c$ can be measured in fungal hyphae.

Furthermore, little is known about the mechanisms that serve to maintain $[Ca^{2+}]_c$ in fungi at the putatively low levels consistent with a role in signal transduction. Intracellular chelation and sequestration of Ca^{2+} are likely to serve important functions for short-term (minutes) homeostasis of $[Ca^{2+}]_c$. However, the precise mechanism of effective buffering is not clear, except that energized Ca^{2+} uptake into fungal vacuoles occurs via H^+/Ca^{2+} exchange (16).

Nevertheless, plasma membrane efflux must ultimately be responsible for sustaining intracellular Ca^{2+} within physiological limits. Two studies on fungal plasma membrane vesicles have suggested that the energized efflux of Ca^{2+} from the cytosol is accomplished by H^+/Ca^{2+} antiport, with the proton electrochemical gradient generated independently by the electrogenic plasma membrane H^+-ATPase (17, 18). These reports are perhaps surprising, since hitherto the plasma membranes of fungi have proved to be excellent models for the study of transport in plant cells (19); yet Ca^{2+} transport across plant membranes is mediated by a Ca^{2+}-ATPase (20). Furthermore, the identification of a yeast gene whose product is similar to plasma membrane Ca^{2+}-ATPases of animal cells (21) suggests that the mechanism of energized Ca^{2+} transport across fungal plasma membranes should be reexamined.

MATERIALS AND METHODS

Growth of Cells. *Neurospora crassa* (wild-type strain RL21a) was grown in mycelial culture on scratched cellophane as described (22). Segments of the hyphal culture were preincubated for 1–2 hr in recording medium and placed in a Plexiglas chamber (vol 0.75 ml). The preparation was viewed with a ×20 bright-field objective and was superfused continuously with medium (10 ml·min^{-1}) during microelectrode recordings.

Solutions. Standard recording media comprised either 20 mM dimethylglutaric acid (pH 5.8 medium) or 30 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (pH 8.4), plus 1 mM $CaCl_2$ and 1% (wt/vol) glucose, titrated

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Abbreviations: $[Ca^{2+}]_c$, cytosolic free calcium concentration; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; pH_c, cytosolic pH; pH_o, extracellular pH; pmf, proton-motive force; $InsP_3$, inositol 1,4,5-trisphosphate.

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to the appropriate pH with KOH (final K^+ concentration, 25–30 mM). Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added in ethanolic solution to a final concentration of 10 μM FCCP and 0.4% ethanol. Controls revealed no effect of 0.4% ethanol either on membrane potential or on $[Ca^{2+}]_c$. Experiments were performed at room temperature (22 ± 1°C).

Fabrication of Ca^{2+}-Selective Microelectrodes. Calcium-selective microelectrodes were constructed from silane-treated micropipettes back-filled with sensor mixture and calibrated as described (23, 24), except that ETH 129 (Fluka) replaced ETH 1001 as the neutral carrier ligand (25). Response over the range 0.1–1.0 μM Ca^{2+} varied from 23 mV to Nernstian (30 mV), with 90% response times between 10 and 30 s and detection limits of the order 3–30 nM.

Other Electrophysiological Techniques. Micropipettes of identical dimensions to Ca^{2+}-selective electrodes (tip diameter, ≈0.3 μm) were filled with 100 mM KCl for measurement of membrane potential. Individual "cells" (50–100 μm long; 12–15 μm diameter) were impaled first with the voltage-reporting electrode, and then, after the establishment of a stable membrane potential, with the Ca^{2+}-selective electrode from the opposite side and 10–30 μm distant from the first impalement. Output from the two electrodes was passed via a high-input impedance differential electrometer (FD-223, W-P Instruments, New Haven, CT) and an A/D converter at a sampling frequency of 10 Hz to a microcomputer.

Analysis of Microelectrode Data. Pre- and postimpalement calibration data from the Ca^{2+}-selective electrode were jointly fitted by a nonlinear least-squares routine (26) to a modified Nicolsky–Eisenman equation (23) as

$$E = E_o + S \cdot \log([Ca^{2+}] + K), \quad [1]$$

in which E is the measured output (mV) of the electrode, E_o is the constant electrode reference potential, S is a slope factor (ideally 29.6 mV per decade change in $[Ca^{2+}]$) and K is the effective detection limit (27). Free Ca^{2+} was then derived from the least-squares fit. Experiments in which the pre- and postimpalement calibration curves differed by >10 mV were rejected.

$^{45}Ca^{2+}$ Influx. Cells were grown in liquid culture as described (22). About 10 mg (dry weight) of cells was harvested and resuspended in 50 ml of pH 8.4 recording medium. After 15 min of incubation at 25°C with vigorous shaking, $^{45}CaCl_2$ was added to a final specific activity of ≈9 GBq·mol^{-1}. In experiments involving CN^-, the inhibitor was added 30 s before addition of the radioisotope to a final concentration of 1 mM from a stock solution of 100 mM NaCN. After mixing $^{45}Ca^{2+}$, 4 vol of ≈10 ml were sampled over the ensuing 2 min and filtered on prewetted filters. Cells were then rapidly washed with 5 ml of ice-cold recording medium containing 5 mM $LaCl_3$ [present to displace extracellular Ca^{2+} from binding sites in the cell wall (28)]. After drying overnight, cells were weighed and counted by liquid scintillation. Uptake of $^{45}Ca^{2+}$ was a linear function of time over the sampling period, although a small zero-time intercept (presumably extracellular binding) amounting to 0.32 ± 0.07 (9) mmol·liter (intracellular water)$^{-1}$ was invariably present. Calcium uptake was expressed on a cell water basis by the relationship 2.54 liter of cell water per kg (dry weight) (29). Provided that normalization is performed on the basis of cell volume (rather than surface area), previous measurements have shown that transport rates of both primary (H^+ translocating) and secondary (nutrient translocating) systems measured in mycelial and shaking culture cells are in good agreement (30–32).

RESULTS

The Free Energy Difference for Ca^{2+} Across the Plasma Membrane. The mean estimate of $[Ca^{2+}]_c$ in *Neurospora* was 92 ± 15 nM from 19 observations. Typically, membrane potential ($\Delta\psi$) was close to −200 mV. The free energy difference for Ca^{2+} across the plasma membrane can be calculated from the Nernst relationship

$$\Delta\bar{\mu}_{Ca^{2+}} = 2F\Delta\psi + RT\ln([Ca^{2+}]_c/[Ca^{2+}]_o), \quad [2]$$

where $[Ca^{2+}]_o$ is the extracellular free Ca^{2+} (1 mM), R = 8.31 J·mol^{-1}·K^{-1}, T = 295 K, and F = 96,500 coulomb·mol^{-1}. Thus, $\Delta\bar{\mu}_{Ca^{2+}}$ = −61.4 kJ·mol^{-1}. The mechanism of Ca^{2+} efflux against this large driving force is now considered.

The free energy of ATP hydrolysis in *Neurospora* is −44.2 kJ·mol^{-1}, calculated from the measured concentrations of adenine nucleotides ([ATP] = 2.5 mM, [ADP] = 0.5 mM; ref. 33), and orthophosphate ([P_i] = 10 mM; ref. 34), and taking the standard free energy for ATP hydrolysis as 29.0 kJ·mol^{-1} (35) at pH 7.2 (the cytosolic pH at extracellular pH 5.8; ref. 36). Thus, a simple Ca^{2+}-ATPase, with a stoichiometry of 1 Ca^{2+}/ATP, would not be thermodynamically competent to overcome the large electrochemical driving force for Ca^{2+}, since there remains an inwardly directed driving force on Ca^{2+} = 17.2 kJ·mol^{-1}. Only a stoichiometry of 1 Ca^{2+}/2 ATP would satisfy the criterion for an outwardly directed pump, and this seems mechanistically unlikely in light of studies on other ion-motive ATPases (37).

For Ca^{2+} efflux via H^+/Ca^{2+} antiport, the minimum required stoichiometry (H^+/Ca^{2+} = n) is given by the free energy relationship

$$n > (\Delta\bar{\mu}_{Ca^{2+}}/\Delta\bar{\mu}_{H^+}), \quad [3]$$

where $\Delta\bar{\mu}_{H^+}$ is F·pmf (proton-motive force) (units, kJ·mol^{-1}). With a cytosolic pH (pH_c) of 7.2 at an external pH (pH_o) of 5.8 (36), $\Delta\bar{\mu}_{H^+}$ = −27.2 kJ·mol^{-1}. Thus, from the value of $\Delta\bar{\mu}_{Ca^{2+}}$ derived from Eq. 2, the minimum integral estimate of n is 3. However, we observed that $[Ca^{2+}]_c$ is insensitive to elevation of pH_o by as much as 2.6 units: a mean value of $[Ca^{2+}]_c$ = 79 ± 21 (5) nM at pH_o 8.4 is sustained for at least 2 hr at the higher pH. Taking into account the small (0.2 unit) increase in pH_c (36), $\Delta\bar{\mu}_{H^+}$ at pH_o 8.4 is −13.7 kJ·mol^{-1}. Thus, from Eq. 3, the minimum integral stoichiometry for antiport increases to 5 H^+/Ca^{2+}. While there are no *a priori* mechanistic reasons for rejecting the notion that $n > 4$, it must be noted that, if correct, this stoichiometry would significantly exceed any yet reported for H^+-coupled transport systems in plants and fungi: directly measured values of n are 1 or 2 for sugars (31, 38, 39) and amino acids (32, 39, 40), 1 for K^+ (41), 2 for monovalent inorganic anions (42, 43), and 3 for phosphate and sulfate (39).

Evidence for ATP-Driven H^+/Ca^{2+} Antiport at the Plasma Membrane. In view of the thermodynamic relationships explored above, we now consider a third possible mechanism for Ca^{2+} efflux, embodying energization both by the pmf and ATP hydrolysis. The free energy relationship for ATP-driven H^+/Ca^{2+} antiport, defined in the direction of Ca^{2+} efflux, is

$$\Delta G_{sys} = \Delta G_{ATP} + (n\Delta\bar{\mu}_{H^+}) - (\Delta\bar{\mu}_{Ca^{2+}}), \quad [4]$$

where ΔG_{ATP} is the apparent free energy for ATP hydrolysis, subsuming the mass action ratio, at the appropriate value of pH_c.

Neurospora is an obligate aerobe, and blockade of respiration with cyanide results in a significant decrease in ATP level, from ≈2.5 mM to 0.5 mM (33). Concomitantly, electrogenic proton pump activity at the plasma membrane is reduced (30), resulting in a depolarization of the membrane to about −80 mV. However, CN^- fails to increase the resting value of $[Ca^{2+}]_c$ (Fig. 1*B*). Thus, even though the membrane clearly depolarizes (Fig. 1*A*), $[Ca^{2+}]_c$ remains at its control value of 62 nM. {The small apparent increase in $[Ca^{2+}]_c$

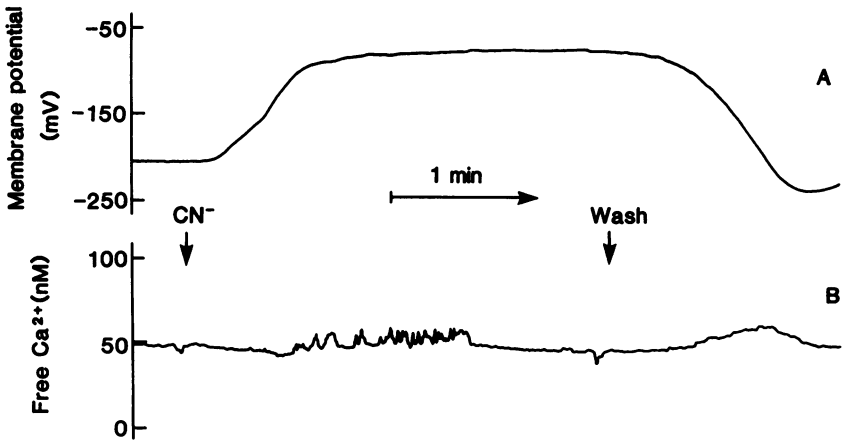

FIG. 1. Cyanide-induced membrane depolarization (*A*), but absence of effect of CN^- on $[Ca^{2+}]_c$ (*B*) at pH_o 5.8. Superfusion of the preparation with recording medium containing 1 mM NaCN was initiated as shown, with the return to CN^--free recording medium indicated (Wash). Ca^{2+}-selective electrode calibration data (see Eq. 1): E_o = 4 mV, S = 28.5 mV, K = 14.4 nM.

during CN^- washout is an artifact resulting from the differing electrical time constants of the two electrodes (36).}

Substituting a value of $n = 1$ into Eq. 4, the resultant values for ΔG_{sys} at pH_o 5.8 can be estimated (by using parameter values identical to those substituted into Eqs. 2 and 3) in the absence and presence of CN^-, respectively, as −10.0 kJ·mol^{-1} and −9.7 kJ·mol^{-1}. Thus, in thermodynamic terms, continued Ca^{2+} efflux via ATP-driven H^+/Ca^{2+} exchange is possible even after the dramatic depletion of ATP by respiratory inhibition. However, maintenance of a low resting $[Ca^{2+}]_c$ at pH_o 8.4 implies that n would have to be at least 2, since ΔG_{sys} in the absence of CN^- can be calculated for n = 1 or 2, respectively, as +2.8 kJ·mol^{-1} or −10.9 kJ·mol^{-1}.

A critical test of the hypothesis for ATP-driven H^+/Ca^{2+} antiport is attained by application of CN^- at pH_o 8.4. Under these conditions, ΔG_{ATP} is −37.2 kJ·mol^{-1}, and $-\Delta\bar{\mu}_{Ca^{2+}}$ is +37.8 kJ·mol^{-1} after membrane depolarization to −75 mV (Fig. 2*A*), but with $[Ca^{2+}]_c$ still at the resting level. Crucially, after a 0.8-unit CN^--induced decline in pH_c to pH 6.6 (44), $\Delta\bar{\mu}_{H^+}$ is poised very close to equilibrium (+2.6 kJ·mol^{-1}). Thus, regardless of the value of n, Eq. 4 predicts that ATP-driven H^+/Ca^{2+} exchange will not be competent to execute net Ca^{2+} efflux under these conditions, and $[Ca^{2+}]_c$ will be expected to increase accordingly. Typical results are shown in Fig. 2*B*: in dramatic contrast to the effects of CN^- at the lower pH_o, $[Ca^{2+}]_c$ increases when CN^- is applied at pH_o 8.4. The rate of increase of $[Ca^{2+}]_c$ varied in three recordings between 40 and 60 nM·min^{-1}. These results are therefore in accord with the existence of ATP-driven H^+/Ca^{2+} exchange.

Intracellular Buffering of Ca^{2+}. With energized Ca^{2+} efflux putatively inhibited by CN^- at pH_o 8.4, a general indication of effective cytosolic buffering of Ca^{2+} is obtained by comparing the increase in $[Ca^{2+}]_c$ with unidirectional influx. In the absence of CN^-, influx of $^{45}Ca^{2+}$ is 0.47 mmol·liter (intracellular water)$^{-1}$·min^{-1}. Influx increases slightly in the presence of CN^- to 0.624 ± 0.049 (9) mmol·liter (intracellular water)$^{-1}$·min^{-1}. Thus, of the Ca^{2+} that enters cells in the presence of CN^- at pH 8.4, only $(50 \times 10^{-9})/(0.62 \times 10^{-3})$ = 1 part in 1.2×10^4 appears in the pool of free Ca^{2+}. The remaining Ca^{2+} must be either chelated in the cytosol or sequestered in intracellular compartments that are at least partially competent in energization of Ca^{2+} transport during the first few minutes of inhibition. As discussed below, Ca^{2+} chelation sites in the cytosol are not present in sufficient concentration to provide buffering to this extent. On the other hand, fungal vacuoles have a potentially very large capacity for Ca^{2+} sequestration.

Elevation of $[Ca^{2+}]_c$ by a Protonophore. The protonophoric uncoupler FCCP, applied at 10 μM at pH_o 5.8 to *Neurospora*, has quantitatively similar effects to CN^- on ATP level (decreases to 0.5 mM), pH_c (decreases to 6.4), and $\Delta\psi$ (depolarizes to around −80 mV) (A.J.M. and D.S., unpublished observations). However, analogous experiments on yeast have also shown that the pH difference between vacuole and cytoplasm is collapsed more rapidly by uncouplers than by other metabolic or respiratory inhibitors (45), since the mode of action of uncouplers on proton fluxes is direct. We therefore reasoned that if simple H^+/Ca^{2+} antiport at the vacuolar membrane is indeed a major factor in cytosolic Ca^{2+} homeostasis, then $[Ca^{2+}]_c$ should increase after rapid collapse of the vacuole/cytosol pH difference.

Fig. 3 shows that 10 μM FCCP does indeed result in elevation of $[Ca^{2+}]_c$ at a rate of ≈50 nM·min^{-1}. The increase in $[Ca^{2+}]_c$ is slowly reversible on FCCP washout. Identical results were obtained in two replicate experiments. Since the effects of CN^- and FCCP on ATP level, pH_c, and transplasma membrane electrical potential are so similar, we take this differential response of $[Ca^{2+}]_c$ to the two inhibitors at pH_o 5.8 to indicate a major role of uncoupler-sensitive vacuolar Ca^{2+} uptake in cytosolic Ca^{2+} homeostasis.

DISCUSSION

The Resting Level of $[Ca^{2+}]_c$ in *Neurospora*. $[Ca^{2+}]_c$ in animal and plant cells is generally observed to be in the region of 100 nM (1, 27, 46)—a level sufficiently low to permit significant amplification of primary signals via severalfold

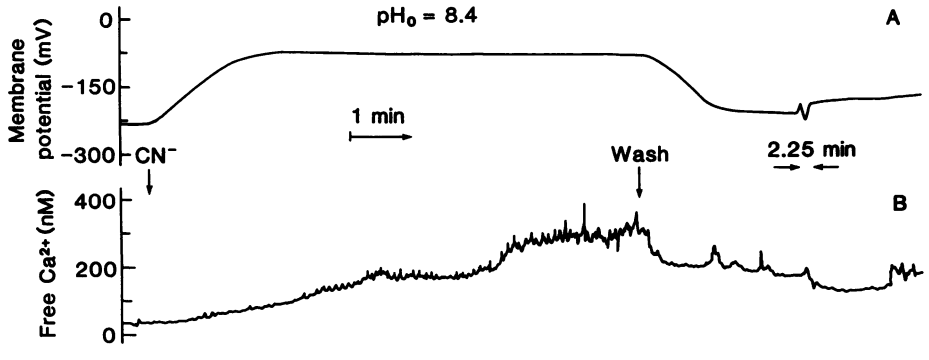

FIG. 2. Cyanide-elicited membrane depolarization (*A*) and increase in $[Ca^{2+}]_c$ (*B*) at pH_o 8.4. Cyanide (1 mM) was applied (CN^-) and removed (Wash) as indicated. Ca^{2+}-selective electrode calibration data (see Eq. 1): E_o = 103 mV, S = 23.9 mV, K = 3.1 nM.

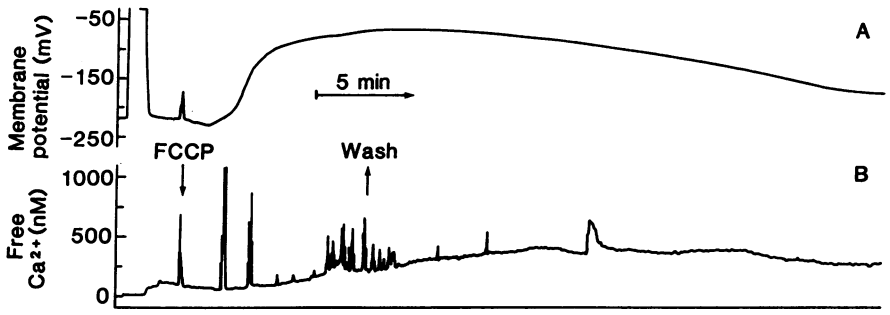

FIG. 3. Protonophore-induced depolarization of membrane (*A*) and elevation of $[Ca^{2+}]_c$ (*B*). FCCP (10 μM) was added (FCCP) and removed (Wash) as shown. pH 5.8 recording medium. Ca^{2+}-selective electrode calibration data (see Eq. 1): $E_0' = 167$ mV, $S = 30.9$ mV, $K = 25.0$ nM.

elevation of the resting value of $[Ca^{2+}]_c$. Recent studies on yeast in which the fluorescent dye indo-1 was used have yielded an estimate for $[Ca^{2+}]_c$ of 346 nM (47). The present estimate of 92 ± 15 nM in *Neurospora* is some 3-fold lower than the value derived for yeast. Although microelectrode determination of $[Ca^{2+}]_c$ suffers from restricted temporal and spatial resolution in comparison with fluorescent dye methods, the electrophysiological approach is not subject to calibration problems associated with dye loading, intracellular localization of dye, and high autofluorescence of fungal cells. The present studies therefore open the way for detailed work on the role of $[Ca^{2+}]_c$ as a second messenger in the hyphae of ascomycete fungi.

Mechanism of Ca^{2+} Efflux in Fungi: Comparison with Studies on Plasma Membrane Vesicles. Our hypothesis that Ca^{2+} efflux across the plasma membrane of *Neurospora* is driven by a H^+/Ca^{2+}-ATPase is strongly supported by experiments in which the pmf is poised close to equilibrium with CN^- at high pH_o. In these conditions, thermodynamic constraints would not permit a H^+/Ca^{2+}-ATPase to catalyze outward Ca^{2+} transport, and $[Ca^{2+}]_c$ increases accordingly. A trivial alternative interpretation for the elevation of $[Ca^{2+}]_c$—that respiratory inhibition with CN^- releases Ca^{2+} from intracellular stores—can be eliminated because a CN^--induced increase in $[Ca^{2+}]_c$ does not occur at lower pH_o where the trans-plasma membrane pmf is inwardly directed.

Our conclusions are at variance with two previous proposals (17, 18) that Ca^{2+} efflux across fungal plasma membranes is energized by simple H^+/Ca^{2+} antiport. In the previous studies on *Neurospora* (17) and *Phytophthora* (18), the protonophore carbonylcyanide *m*-chlorophenylhydrazone was shown to inhibit, but not to eliminate completely, ATP-dependent Ca^{2+} uptake into plasma membrane vesicles. This result is compatible with the present proposal if the kinetics of ATP-driven H^+/Ca^{2+} exchange are dependent on lowering of intravesicular pH by the primary H^+-ATPase. Giannini *et al.* (18) also demonstrated that uncoupler-sensitive Ca^{2+} accumulation into plasma membrane vesicles could be driven in the absence of ATP via an artificially imposed inside acid pH gradient, albeit at a rate equivalent to only 10% of the ATP-driven rate. However, in view of the significant H^+ permeability of fungal plasma membrane vesicles (48), it seems likely that an inside-negative $\Delta\psi$ forms in these conditions (49), thus explaining both the ability of the vesicles to accumulate Ca^{2+}, and the significantly reduced rate of transport. In addition, it should be noted that the vesicle studies were performed at a $[Ca^{2+}]$ of 50–100 μM—a concentration between 500- and 1000-fold higher than the free Ca^{2+} pertaining *in vivo*.

ATP-Driven Ca^{2+} Efflux Across the Plasma Membranes of Eukaryotic Cells. Although final confirmation of our proposal for an H^+/Ca^{2+}-ATPase at the fungal plasma membrane must await purification and reconstitution studies, some general comparisons with the mechanisms of ATP-driven Ca^{2+} efflux at the plasma membranes of animal and plant cells strengthen the proposal. Thus, the purified animal and plant enzymes are immunologically cross-reactive (50) and both appear to catalyze H^+/Ca^{2+} exchange (51, 52). Early estimates of the H^+/Ca^{2+} stoichiometric ratio of the erythrocyte enzyme suggested electroneutral operation (i.e., a value of 2; ref. 51), and the Ca^{2+}-ATPase in the basolateral membrane from kidney cortex appears to exhibit similar properties (53). However, more recent studies on intact erythrocytes have implied a stoichiometric ratio of 1 (54). Plant cells, like fungi but in contrast to animal cells, exhibit highly negative membrane potentials of the order of −200 mV (55). Thus, thermodynamic constraints, as in *Neurospora*, suggest a minimum stoichiometric ratio of 2 H^+/Ca^{2+} for the Ca^{2+}-ATPase of plants (52).

The present work shows that lowering of ATP to 0.5 mM with CN^- at pH_o 5.8 has no effect on $[Ca^{2+}]_c$. This suggests that 0.5 mM ATP is a V_{max} concentration for ATP-driven Ca^{2+} transport and, therefore, that the K_m (ATP) must be considerably less than this value (i.e., less than ≈100 μM). By contrast, the operational K_m of the dominant H^+-ATPase of *Neurospora* plasma membrane is 0.7–3.8 mM (66) and that of the related Na^+,K^+-ATPase of animal plasma membranes is 0.1–2 mM, depending on the conditions (67). Nevertheless, the K_m values of the Ca^{2+}-ATPases of both animal and plant plasma membranes are also low: 2.5 and 145 μM for the Ca^{2+}-ATPase of erythrocytes (58); 15–20 μM for the Ca^{2+}-ATPase of radish (59). Thus, similarities in the mode of Ca^{2+} efflux across the plasma membranes of animal, plant, and fungal cells with respect to ionic substrates, coupled with the low K_m for ATP, might indicate that eukaryotes share a common class of enzyme.

Interrelationship Between pH_c and $[Ca^{2+}]_c$. The insensitivity of $[Ca^{2+}]_c$ to CN^- at pH_o 5.8 is, perhaps, surprising given the decline in pH_c (from 7.2 to 6.4) that accompanies respiratory inhibition (36). Thus, cytosolic Ca^{2+} chelation (for example, by calmodulin) can be anticipated to decrease as pH_c is lowered (68). However, we have independently confirmed that cytosolic Ca^{2+} homeostasis in *Neurospora* is indeed insensitive to moderate reduction in pH_c: butyric acid at 15 mM (which elicits a decrease in pH_c to 6.3; ref. 36) fails to elevate $[Ca^{2+}]_c$ (A.J.M. and D.S., unpublished data). By contrast, reduction of pH_c by ≈0.7 unit with a weak acid results in a complementary increase in $[Ca^{2+}]_c$ from 120 nM to ≈200 nM in higher plants (46). The disparate effects of pH_c on $[Ca^{2+}]_c$ in fungi and plants might be interpreted simply as indicative of differential sensitivity of the plasma membrane H^+/Ca^{2+}-ATPase to pH_c. This explanation is compatible with the finding that the affinity of the erythrocyte Ca^{2+}-ATPase for Ca^{2+} decreases significantly for shifts in pH_c as small as 0.3 unit (54).

Intracellular Buffering of Ca^{2+}: Role of the Vacuole. With energized Ca^{2+} efflux putatively inhibited by CN^- at high pH_o, the observed increase in $[Ca^{2+}]_c$ of 40–60 nM·min^{-1} in *Neurospora* is comparable with that of 20 nM·min^{-1} observed in sheep ventricular muscle when Na^+/Ca^{2+} antiport is inhibited after restriction of the Na^+,K^+-ATPase (56). Nevertheless, in *Neurospora* only 0.008% of incoming Ca^{2+} appears in the pool of cytosolic free Ca^{2+} during the first few minutes of inhibition. Ca^{2+}-binding proteins in the cytosol

can be anticipated to make only a minor contribution to this high buffer capacity. For example, if the calmodulin concentration in *Neurospora* is 10 μM (57), the 40 μM concentration of available Ca^{2+}-binding sites will rapidly be exhausted. Furthermore, studies on neuronal tissue indicate that as much as 0.1–1% of imposed Ca^{2+} loads remain free (61, 62). The presence of vacuoles in fungal and plant cells, but not in animal cells, suggests that this organelle might provide the major intracellular source of very effective Ca^{2+} buffering in *Neurospora*. Ca^{2+} uptake by fungal and plant vacuoles is driven by straightforward H^+/Ca^{2+} exchange (16, 49), and although in the presence of CN^- the reaction ultimately energizing uptake (ATP-driven H^+ pumping into the vacuole) is probably inhibited, a considerable reservoir of H^+ will nevertheless be sustained within the vacuoles for the duration of our experiments (45).

The increase in $[Ca^{2+}]_c$ elicited by FCCP supports the interpretation that vacuoles, by energizing Ca^{2+} sequestration with a large intracellular store of protons, can represent a major site of short-term Ca^{2+} homeostasis. FCCP can be anticipated to catalyze direct and rapid dissipation of the pmf across the vacuolar membrane, thereby eliminating the driving force for H^+-coupled transport into the vacuole. Thus, uncompensated Ca^{2+} efflux from the vacuole would play a major role in elevating $[Ca^{2+}]_c$ under these conditions.

This view of the effect of FCCP on $[Ca^{2+}]_c$ also provides an interpretation for a long-standing, parallel observation on squid axons. There, FCCP is also a far more potent inhibitor of cytosolic Ca^{2+} homeostasis than CN^- (60, 61). It is now known that Ca^{2+} uptake into the major endoplasmic reticulum pool involved in signal transduction in animal cells (i.e., that pool that is releasable by InsP_3) is dependent on energization by a pmf (63). Thus, uncompensated Ca^{2+} efflux from InsP_3-sensitive Ca^{2+} stores in the presence of FCCP is likely to elevate $[Ca^{2+}]_c$, regardless of whether these stores are located in fungal or plant vacuoles (14, 64) or in the smaller compartment of endoplasmic reticulum in animal cells. We conclude, therefore, that the fungal vacuole is likely to provide an effective intracellular buffer against short-term perturbation of cytosolic Ca^{2+} homeostasis in *Neurospora*. This proposal is in accord with studies on yeast (65), which have indicated that Ca^{2+}-sensitive mutants are defective in vacuolar uptake of Ca^{2+}.

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