Plant Physiol. (1989) 91, 213–216
0032-0889/89/91/0213/04/$01.00/0

Received for publication November 30, 1988
and in revised form April 14, 1989

# Calcium Activates an Electrogenic Proton Pump in *Neurospora* Plasma Membrane[1]

**Roger R. Lew[2]**

*Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut 06510*

## ABSTRACT

**Calcium ionophoresis into coenocytic cells of *Neurospora crassa* activates the plasma membrane proton pump as measured by current-voltage analysis. This is direct evidence that intracellular calcium regulates the activity of a key transport enzyme found in higher plants and fungi.**

Calcium is a regulator of plant growth and development, usually through its role as a second messenger (20). It is required for maintenance of membrane permeability (4) and for high-affinity potassium uptake (7), so it is possible that it affects plant growth and development by regulating the transport properties of the plasma membrane. For example, inhibitors of calcium-calmodulin activation (phenothiazine derivatives) affect the electrical transmembrane potential across the plasma membrane of plant cells (2, 12, 14), which is generated by an electrogenic proton ATPase (25, 26). However, phenothiazines are accumulated by plants to high levels (5) and directly inhibit the plasma membrane proton ATPase (14), so their effect *in vivo* may be due to nonspecific actions rather than inhibition of calcium-calmodulin regulation of the proton pump. Using internal perfusion, calcium does not affect the plasma membrane proton ATPase of *Nitellopsis* or *Chara* at concentrations up to 0.1 mM; above this level, it inhibits their activity (16). The lack of any effect of calcium at physiological levels in Characean algae does not preclude possible calcium regulation of transport in other systems.

Calcium does regulate ion channels in the plant vacuolar membrane (9); the physiological significance of this regulation is still unclear. Inositol 1,4,5-trisphosphate triggers calcium release from vacuoles (23) (but not from endoplasmic reticulum [15]), and may act in concert with calcium activation of channels *in vivo*. Overall, these data are consistent with a model of calcium regulation of growth and development via release from internal stores induced by inositol 1,4,5-trisphosphate; but subsequent mechanisms of action and the universality of calcium regulatory functions are unknown.

To demonstrate that intracellular calcium directly affects electrogenic transport at the plasma membrane, I used ionophoretic injection of calcium to increase intracellular levels. The system I used, *Neurospora crassa* coenocytic cells, was ideal for such studies because the cells are small and spherical and lack a large central vacuole. Calcium plays a regulatory role in *N. crassa*: calmodulin is found in *Neurospora* (6), where it stimulates protein kinase (27) and adenylate cyclase (19). The membrane potential in *Neurospora* is generated by an electrogenic proton pump that is very similar to that found in higher plants (25). In this report, I present evidence that calcium stimulates the plasma membrane proton pump in *Neurospora*.

## MATERIALS AND METHODS

Cells of *Neurospora crassa* were grown from conidia in Vogel's minimal medium (28) plus 2% glucose and 15.5% ethylene glycol (1). After 3 d growth, the cells were washed in a fivefold excess of 9 mM Mes (pH adjusted to 5.7 with $Ca(OH)_2$) (Ca-Mes solution) plus 17.1% ethylene glycol, and diluted 10-fold with Ca-Mes solution over 4 h. Electrophysiology was done in 10 mM Bis-Tris propane/Mes solution (pH 6.0) containing 1 mM $CaCl_2$. Triple-barreled microelectrodes were used: the common filling solution was 100 mM K-acetate plus 10 mM KCl (the pH was about 7). Impaled cells had volumes ranging from 5 to 10 pL.

For calcium ionophoresis, the cation-ejecting microelectrode was filled with 25 mM $Ca^{2+}$. Currents were canceled with a return current (the clamping current) through one of the other microelectrodes in the assembly while the voltage was clamped to the resting potential as measured by the third microelectrode. The ionophoretic current was indirectly monitored by measuring the clamping current.

## RESULTS AND DISCUSSION

Initial experiments were done without voltage clamping (13). This limited the magnitude of ionophoretic current that could be used (4 to 50 pA), and there was no effect of ionophoresis upon the current-voltage relations of the plasma membrane.

With voltage clamping to maintain the potential at its resting value, it was possible to increase the ionophoretic current up to 1.25 nA. For all cells in this experimental series, the initial membrane potential was $-225 \pm 27$ mV ($n = 21$).

In the sample experiment shown in Figure 1, control ionophoresis of potassium into the cell was without any large effect. Subsequent ionophoresis of calcium caused a hyper-

[1] Supported by National Institutes of Health training grant DK-7259 to Professor E. Boulpaep, and U.S. Department of Energy grant DE-FG02-85ER13359 to Professor C. L. Slayman.

[2] Present address: York University, Department of Biology, 4700 Keele St., North York, Ontario, Canada M3J 1P3.

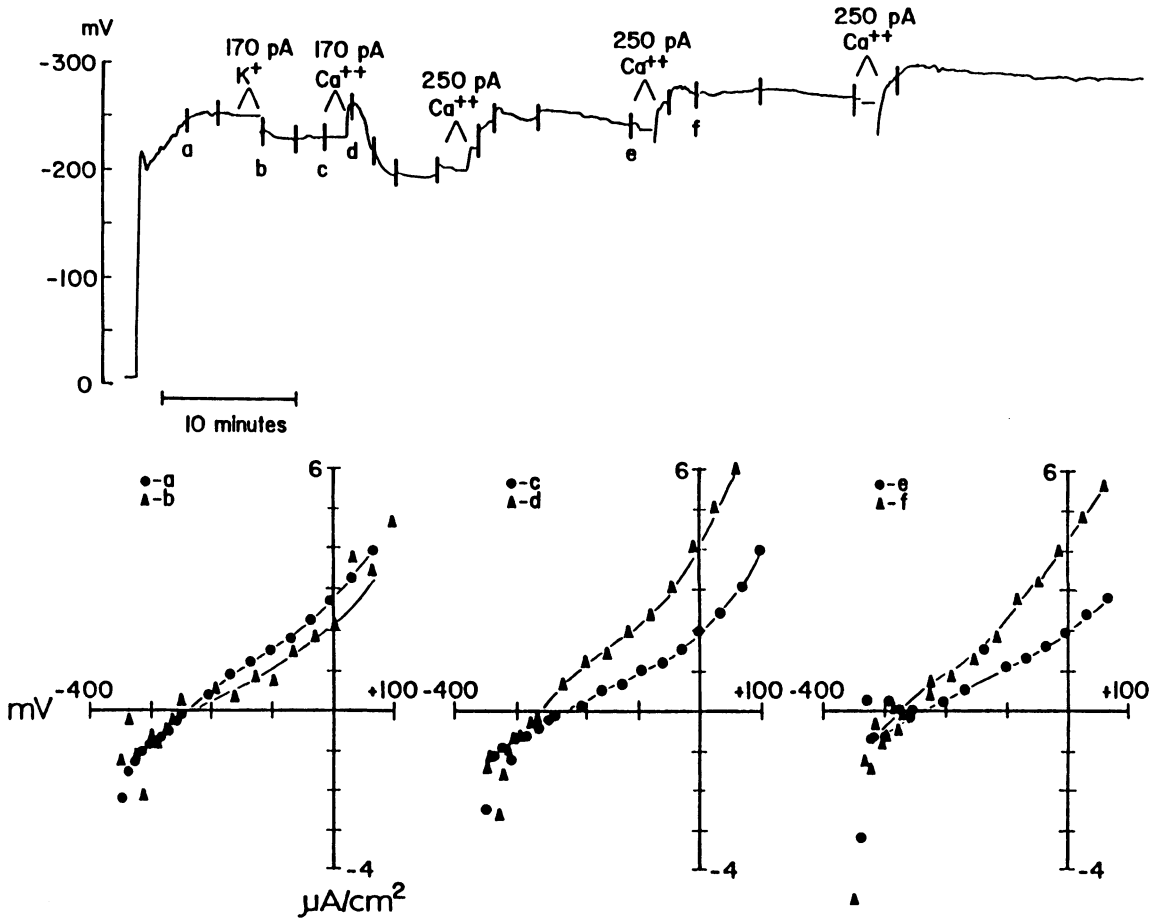


**Figure 1.** Calcium ionophoresis with voltage-clamping. The calcium-injecting microelectrode contained acetate (100 mM), $K^+$ (82.5 mM), $Cl^-$ (10 mM), and $Ca^{2+}$ (25 mM). The ionophoretic current was balanced by voltage-clamping the potential at the resting potential using the other two microelectrodes (both contained 100 mM K-acetate plus 10 mM KCl). By doing this, much larger ionophoretic currents could be passed than were previously possible (13). Current-voltage measurements (shown as vertical bars in the membrane potential trace) were measured with a conventional bipolar voltage clamp protocol. As a control, the initial ionophoresis was through one of the microelectrodes containing 100 mM K-acetate plus 10 mM KCl, so that $K^+$ was injected into the cell. The effect of this and subsequent calcium injections on the membrane potential is shown in the upper panel; their effects on the current-voltage relations are shown in the lower panel. Depolarizing spikes often seen at hyperpolarized potentials occurred during the experiment but are not shown in the potential trace. Only two examples of the effect of calcium ionophoresis on current-voltage relations are shown; the others were quite similar.

polarization of the potential and an increase in the conductance, and thus an increase in the short circuit current (*i.e.* current at 0 mV) in the current-voltage relations. Both of these effects must be a consequence of calcium ionophoresis since neither is seen when calcium is absent from the electrode.

The hyperpolarization is a consequence of either positive charge movement out of the cell or negative charge movement into the cell; likely candidates are calcium, potassium, chloride, or protons. *Neurospora* has a $Ca^{2+}/nH^+$ antiporter (22), and energetically, calcium efflux would have to be either electrically silent or cause the potential to depolarize. Potassium outward movement is unlikely: no active efflux mechanism is known, and potassium ionophoresis (which would cause a more negative-inside Nernst potential for potassium) causes a slight depolarization (Fig. 1). This indicates that a potassium conductance does not dominate the electrical properties of the membrane. Finally, no chloride pump is known in *Neurospora*. By elimination, proton efflux is most probable.

The increase in conductance seen after calcium ionophoresis could be interpreted as an increased ionic "leak." But if this were the case, the potential would depolarize: the ionic leak potential is nearly 0 mV, as measured when the proton pump is inhibited by ATP depletion (cyanide or carbon monoxide treatment) or directly (vanadate ionophoresis) (data not shown). The concomitant hyperpolarization and increased conductance can be best explained by activation of the proton pump as follows: increased proton pump activity would cause the potential to hyperpolarize and would also increase the maximal pump current. This would be seen as increased current under no load conditions, *i.e.* at 0 mV, where passive leakage contributes virtually nothing to overall current (8), and thus increased conductance on the current-voltage relation.

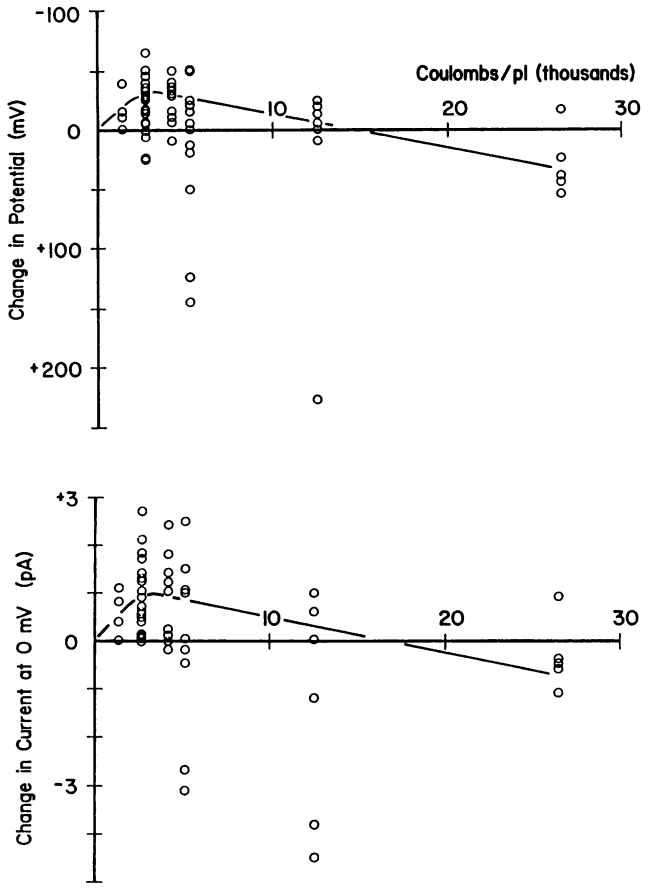


**Figure 2.** Effects of calcium ionophoresis on the membrane potential and short circuit current. The *x* axis shows the ionophoretic current normalized for duration and cellular volume, *i.e.* coulombs of charge per picoliter of cell volume. The actual concentrations are unknown because the efficiency of current passage by calcium relative to the other ions present is unknown. The upper panel shows the change in membrane potential after calcium ionophoresis; the upward direction is hyperpolarization. The lower panel shows the change in short circuit current (*i.e.* current at 0 mV) after calcium ionophoresis. Outlier data points are not shown; these were present at higher ionophoretic currents that caused inhibition of pump activity and possibly cellular damage.

The dependence of changes in potential and short circuit current on the amount of ionophoretic current is shown in Figure 2. At lower currents (105–230 pA, 2,500 coulombs/pL of cell volume ionophoresed into the cell), calcium injection caused membrane potential hyperpolarization and increased short circuit current. At higher ionophoretic currents (800–1250 pA, 12,600 coulombs/pL of cell volume), the data are more scattered, but in general, calcium injection caused depolarization and decreased short circuit current. The depolarization and decreased conductance on the current-voltage relations is also seen when the proton pump is inhibited by cyanide, carbon monoxide, or vanadate ionophoresis (data not shown). Thus, as intracellular calcium is increased by ionophoresis, there is stimulation of proton pump activity, but at some threshold, calcium levels are so high that they inhibit pump activity and may have other deleterious effects.

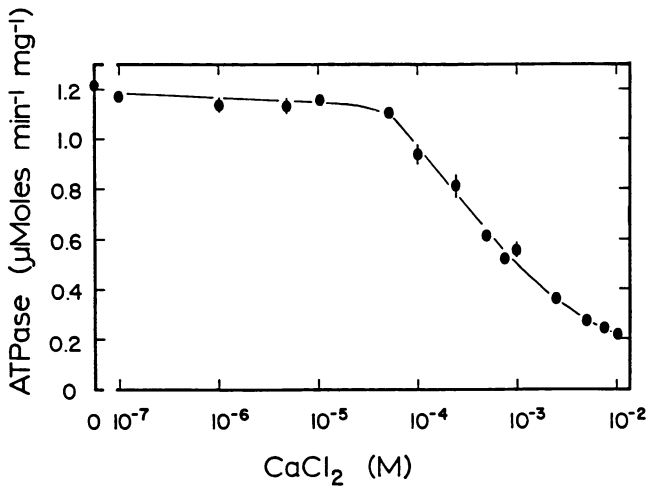


**Figure 3.** Calcium inhibition of proton ATPase in isolated plasma membranes. The calcium is shown as total concentration. Free concentrations were approximately 20% to 30% lower due to the presence of 5 mM ATP and 5 mM phospho*enol*pyruvate in the reaction medium. The plasma membrane isolation and ATPase measurements were according to Bowman et al. (3).

The final concentrations of calcium in the cell cannot be directly measured. Theoretical evaluations of the amount of calcium ionophoresed into the cell depend upon how much of the ionic current is passed by calcium (18); this value is uncertain, especially since intermittent blockage of the microelectrode was occasionally observed during the ionophoresis of calcium as a decline in the clamping current (data not shown). To determine the probable real increase in the calcium concentration, I measured the effect of calcium on ATPase activity of isolated plasma membrane (Fig. 3). ATPase activity is inhibited at calcium concentrations higher than 100 $\mu$M. This can be matched to inhibition by calcium ionophoresis (Fig. 2), which occurs at a value of 10,000 coulombs/pL of cell volume. Thus, the actual increase in intracellular calcium required to stimulate proton pump activity is lower than 100 $\mu$M, probably in the range of 10 to 50 $\mu$M. The fact that calcium does not activate the proton ATPase *in vitro* suggests that cytosolic factors required for activation *in vivo* are lost during isolation of the plasma membranes.

There is a general consensus that cytoplasmic calcium in higher plants is regulated at a level of about $10^{-7}$ M (17, 29). Transient increases to the micromolar level may trigger a variety of cellular processes (20); these are often responses to environmental stimuli (10, 11, 24, 30). The data presented here are direct evidence that elevation of calcium has a specific effect upon the activity of an electrogenic proton pump known to play a central role in plant growth and development (25, 26). The activation requires cytosolic factors and may be via a calcium-stimulated phosphorylation of the pump (21).

## LITERATURE CITED

1. **Bates JK, Wilson JF** (1974) Ethylene glycol-induced alteration of conidial germination in *Neurospora crassa*. J Bacteriol **117**: 560–567
2. **Beilby MJ, MacRobbie EAC** (1984) Is calmodulin involved in electrophysiology of *Chara corallina*? J Exp Bot **35**: 468–480
3. **Bowman EJ, Bowman BJ, Slayman CW** (1981) Isolation and

characterization of plasma membranes from wild-type *Neurospora crassa.* J Biol Chem **256:** 12336–12342
4. **Brummer B, Warncke J, Slayman CL** (1985) Control of membrane permeability by extracellular calcium: pump and leak behavior of the *Neurospora* plasma membrane (Abstract No. 785). Plant Physiol **77:** S-143
5. **Cerana R, Bonetti A, Lado P** (1983) Uptake and accumulation at membrane level of the calmodulin antagonist chlorpromazine in higher plants. Plant Sci Lett **30:** 267–277
6. **Cox JA, Ferraz C, Demaille JG, Perez RO, van Tuinen D, Marme D** (1982) Calmodulin from *Neurospora crassa.* General properties and conformational changes. J Biol Chem **257:** 10694–10700
7. **Epstein EE** (1966) Dual patterns of ion absorption by plant cells and by plants. Nature **212:** 1324–1327
8. **Gradmann D, Hansen U-P, Long WS, Slayman CL, Warncke J** (1978) Current-voltage relationships for the plasma membrane and its principal electrogenic pump in *Neurospora crassa*: I. Steady-state conditions. J Membr Biol **39:** 333–367
9. **Hedrich R, Neher E** (1987) Cytoplasmic calcium regulates voltage-dependent ion channels in plant vacuoles. Nature **329:** 833–835
10. **Kauss H** (1983) Volume regulation in *Poterioochromas.* Involvement of calmodulin in the $Ca^{2+}$-stimulated activation of isofloridoside-phosphate synthase. Plant Physiol **71:** 169–172
11. **Kohle H, Jeblick W, Poten F, Blaschek W, Kauss H** (1985) Chitosan-elicited callose synthesis in soybean cells as a $Ca^{2+}$-dependent process. Plant Physiol **77:** 544–551
12. **Lado P, Cerana R, Bonetti A, Marre MT, Marre E** (1981) Effects of calmodulin inhibitors in plants. I. Synergism with fusicoccin in the stimulation of growth and $H^+$ secretion and in the hyperpolarization of the transmembrane electrical potential. Plant Sci Lett **23:** 253–262
13. **Lew RR** (1989) Using ionophoresis, I find no evidence that intracellular calcium modulates the electrical properties of *Neurospora crassa* plasma membrane. *In* MJ Beilby, JR Smith, NA Walker, eds, Proceedings of the Seventh International Workshop on Plant Membrane Transport, Sydney, 1986, Australian Academy of Science, in press
14. **Lew RR, Spanswick RM** (1983) Chlorpromazine induces membrane potential depolarization and ATP loss, and inhibits microsomal ATPase in soybean (*Glycine max* L.) roots. Biochim Biophys Acta **731:** 421–427
15. **Lew RR, Briskin DP, Wyse RE** (1986) $Ca^{2+}$ uptake by endoplasmic reticulum from zucchini hypocotyls. The use of chlorotetracycline as a probe for $Ca^{2+}$ uptake. Plant Physiol **82:** 47–53
16. **Luhring H, Tazawa M** (1985) Effect of cytoplasmic $Ca^{2+}$ on the membrane potential and membrane resistance of *Chara* plasmalemma. Plant Cell Physiol **26:** 634–646
17. **Miller AJ, Sanders D** (1987) Depletion of cytosolic free calcium induced by photosynthesis. Nature **326:** 397–400
18. **Purves RD** (1981) Microelectrode Methods for Intracellular Recording and Ionophoresis. Academic Press, New York
19. **Reig JA, Tellez-Inon MT, Flawia MM, Torres HN** (1984) Activation of *Neurospora crassa* soluble adenylate cyclase by calmodulin. Biochem J **221:** 541–543
20. **Roux SJ, Slocum RD** (1982) Role of calcium in mediating cellular functions important for growth and development of higher plants. *In* W Y Cheung, ed, Calcium and Cell Function Vol. 3. Academic Press, New York, pp 409–453
21. **Schaller GE, Sussman MR** (1988) Phosphorylation of the plasma membrane $H^+$-ATPase of oat roots by a calcium-stimulated protein kinase. Planta **173:** 509–518
22. **Stroobant P, Scarborough GA** (1979) Active transport of calcium in *Neurospora* plasma membrane vesicles. Proc Natl Acad Sci USA **76:** 3102–3106
23. **Schumaker KS, Sze H** (1987) Inositol 1,4,5-trisphosphate releases $Ca^{2+}$ from vacuolar membrane vesicles of oat roots. J Biol Chem **262:** 3944–3946
24. **Serlin BS, Roux SJ** (1984) Modulation of chloroplast movement in the green alga *Mougeotia* by the $Ca^{2+}$ ionophore A23187 and by calmodulin antagonists. Proc Natl Acad Sci USA **81:** 6368–6372
25. **Serrano R** (1984) Plasma membrane ATPase of fungi and plants as a novel type of proton pump. Curr Top Cell Regul **23:** 87–126
26. **Spanswick RM** (1981) Electrogenic ion pumps. Annu Rev Plant Physiol **32:** 267–289
27. **van Tuinen D, Perez RO, Marme D, Turian G** (1984) Calcium, calmodulin-dependent protein phosphorylation in *Neurospora crassa.* FEBS Lett **176:** 317–320
28. **Vogel HJ** (1956) A convenient growth medium for *Neurospora.* Microb Genet Bull **13:** 42–46
29. **Williamson RE** (1981) Free $Ca^{2+}$ concentration in the cytoplasm: A regulator of cell function. What's New Plant Physiol **12:** 45–48
30. **Woods CM, Polito VS, Reid MS** (1984) Response to chilling stress in plant cells. II. Redistribution of intracellular calcium. Protoplasma **121:** 17–24