Inhibition of Ca\textsuperscript{2+} uptake in Neurospora crassa by La\textsuperscript{3+}: a mechanistic study

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Addition of 1 mM-LaCl\textsubscript{3} to Neurospora crassa 30 s prior to the initiation of \textsuperscript{45}Ca\textsuperscript{2+} uptake resulted in a dramatic inhibition of Ca\textsuperscript{2+} influx to 7% of the control value. The lanthanide Gd\textsuperscript{3+} and several other recognized Ca\textsuperscript{2+} channel blockers (ruthenium red, nifedipine, methoxyverapamil) failed to inhibit Ca\textsuperscript{2+} influx. Direct measurement of membrane potential (\(\Delta\Psi\)) with micro-electrodes revealed a La\textsuperscript{3+}-induced depolarization of about 80 mV for 1 mM-La\textsuperscript{3+} in the presence of 1 mM-Ca\textsuperscript{2+}. The depolarization is rapid and partially reversible on La\textsuperscript{3+} washout. The concentration-dependence of the depolarization can be described by a rectangular hyperbola with a \(K_{0.5}\) for La\textsuperscript{3+} = 0.11 mM. The La\textsuperscript{3+}-induced depolarization is Ca\textsuperscript{2+}-sensitive, decreasing as external Ca\textsuperscript{2+} increases. The inhibitory effect of Ca\textsuperscript{2+} also exhibits a hyperbolic concentration-dependence, with a \(K_{0.5}\) for Ca\textsuperscript{2+} = 2.5 mM for depolarization induced by 1 mM-La\textsuperscript{3+}. While the flux data suggest a direct effect of La\textsuperscript{3+} on Ca\textsuperscript{2+} uptake, the electrophysiological data imply additional effects of La\textsuperscript{3+} on the membrane. Three hypotheses were considered: (1) La\textsuperscript{3+} interacts with K\textsuperscript{+} channels; (2) La\textsuperscript{3+} entry into cells carries a large depolarizing current; (3) La\textsuperscript{3+} inhibits the electrogenic H\textsuperscript{+}-pump. Hypothesis (1) was eliminated by experiments showing that depolarization occurs regardless of whether the equilibrium potential for K\textsuperscript{+} is positive or negative of the resting value of \(\Delta\Psi\). Hypotheses (2) and (3) remain possible, although La\textsuperscript{3+} influx of the magnitude required to generate the observed depolarization seems very unlikely. We conclude that La\textsuperscript{3+} should be deployed only with considerable caution as a blocker of plasma-membrane Ca\textsuperscript{2+} influx in fungi.

Introduction

Cytosolic free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]) plays a ubiquitous role as a second messenger in animal and plant cells (Williamson & Monck, 1989; Johannes et al., 1991). A similar function for [Ca\textsuperscript{2+}] in eukaryotic micro-organisms is indicated by the presence of the calcium-binding protein calmodulin (Cox et al., 1982; Davis et al., 1986) and by the capacity of Ca\textsuperscript{2+} ionophores to induce a range of physiological responses (Reissig & Kinney, 1983; Nakashima, 1984; Harold & Harold, 1986).

In resting fungal cells, [Ca\textsuperscript{2+}]\textsubscript{c} resides in the range 100–300 nm (Halachmi & Eilam, 1989; Miller et al., 1990). This low level is sustained by energized Ca\textsuperscript{2+} transport at both the plasma and vacuolar membranes. Efflux of Ca\textsuperscript{2+} to the external medium is probably mediated by an ATPase exchanging Ca\textsuperscript{2+} for (at least two) H\textsuperscript{+} (Miller et al., 1990), while Ca\textsuperscript{2+} accumulation in vacuoles is energized by simple Ca\textsuperscript{2+}/H\textsuperscript{+} exchange (Ohsumi & Anraku, 1983). However, by analogy with animal cells, an increase in [Ca\textsuperscript{2+}], during signal transduction is likely to be generated by the opening of discrete Ca\textsuperscript{2+} channels which mediate passive, downhill movement of Ca\textsuperscript{2+} into the cytosol.

Little is known about the nature of Ca\textsuperscript{2+} channels in fungi. Inositol 1,4,5-trisphosphate (InsP\textsubscript{3}), which in animal cells is known to be generated by phospholipaseC-mediated hydrolysis of the trace plasma-membrane lipid phosphatidylinositol 4,5-bisphosphate (Berridge & Irvine, 1989), releases Ca\textsuperscript{2+} from vacuolar membrane vesicles of Neurospora (Cornelius et al., 1989). In this respect fungi resemble plants rather than animals, where the major site of InsP\textsubscript{3} action is likely to be the endoplasmic reticulum (Berridge & Irvine, 1989). Nevertheless, fungal InsP\textsubscript{3}-elicited Ca\textsuperscript{2+} release is distinguished from that in both animals and plants in being insensitive to heparin (Ghosh et al., 1988; Brosnan & Sanders, 1990). In yeast, Ca\textsuperscript{2+} entry across the plasma membrane is thought to be sensitive to membrane

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Abbreviation: \(\Delta\Psi\), membrane potential.
voltage ($\Delta \psi$; Eilam & Chernichovsky, 1987), although in the absence of methods for direct determination of $\Delta \psi$ in yeast, the quantitative significance of this finding might be questioned.

Recently, we have demonstrated that Ca$^{2+}$ influx into *Neurospora crassa* can be determined if, as in yeast, wall-bound Ca$^{2+}$ is removed by washing cells in ice-cold La$^{3+}$ (Miller et al., 1990). The objectives of this study were to determine (a) whether Ca$^{2+}$ uptake in *Neurospora* is sensitive to any of the pharmacological agents which have been used to define various classes of Ca$^{2+}$ channels in animals (Bean, 1989) and plants (Johannes et al., 1991); (b) whether the inhibitory effects of Ca$^{2+}$ channel antagonists on Ca$^{2+}$ transport are specific; and (c) to what extent such effects can be considered direct, or are mediated indirectly, for example through changes in $\Delta \psi$.

**Methods**

**Fungal culture and flux measurements.** *Neurospora crassa* (wild-type strain RL21a) was grown on Vogel's (1956) medium for flux and electrophysiological experiments basically as described by Slayman & Slayman (1979). Briefly, for the flux experiments, liquid cultures (160 ml) were inoculated with about 10$^6$ conidia ml$^{-1}$ and shaken vigorously for 12 h at 25 °C. Cells were then collected by filtration (Whatman no. 1) and resuspended in 50 ml of Standard Buffer (SB) comprising (concentrations in mM): MES/KOH (25), CaCl$_2$ (1), KCl (15), glucose (56), pH 5-8. The final K$^+$ concentration was 25 mM. After a further 15 min incubation with vigorous shaking at 25 °C, unidirectional Ca$^{2+}$ influx was measured by the addition of $^{45}$Ca$^{2+}$ (final specific activity, 28 GBq mol$^{-1}$). At various times thereafter, samples (8 ml) were removed with a syringe, split into two aliquots and filtered separately (Whatman GF/A). Cells were then rapidly washed with 10 ml of ice-cold SB $+$ 10 mM-LaCl$_3$, to displace Ca$^{2+}$ from binding sites in the cell wall. After drying overnight, cells were weighed and radioactivity determined after addition of 3 ml of xylene-based scintillation fluid. Ca$^{2+}$ uptake is expressed on a cell-water basis using the relationships 2-54 litre cell water per kg dry wt (Slayman & Tatum, 1964).

**Electrical measurements.** Cells for micro-electrode impalement were grown for 24 h on scratched cellophane placed over agar containing Vogel's medium. Hyphae were continuously perfused with SB solution throughout recordings. Electrical measurements were made with glass capillary micro-electrodes (tip diameter about 0.3 μm) filled with 100 mM-KCl. Micromanipulation was performed with Huxley–Goodfellow micromanipulators (Goodfellow Metals, Cambridge, UK) and the preparation was viewed under bright field illumination with a Nikon Diaphot microscope. Output from the micro-electrode was passed via a high input impedance differential electrometer (FD-223, WPI, New Haven, CT, USA) and recorded on a chart recorder. The circuit was completed with an KCl-agar bridge placed in the external medium.

**Results**

Addition of 1 mM-La$^{3+}$ 30 s prior to the initiation of $^{45}$Ca$^{2+}$ uptake resulted in a dramatic inhibition of Ca$^{2+}$ influx (Fig. 1). Thus, the absolute value for Ca$^{2+}$ influx declined from 0:32 to only 0:02 mmol (litre intracellular water)$^{-1}$, or 7% of the control value.

It might be argued that this effect of La$^{3+}$ relates simply to competition for extracellular Ca$^{2+}$-binding sites, which are not efficiently depleted of Ca$^{2+}$ during the filtration/wash. However, several lines of evidence suggest that extracellular binding of Ca$^{2+}$ is very small. First, cell-associated Ca$^{2+}$ rises as a linear function of time for at least the first 90 s, with the ‘zero-time’ intercept comprising less than 0-1 mmol (litre intracellular water)$^{-1}$. Second, preliminary experiments with the lanthanide Gd$^{3+}$ (1 mM) failed to show the inhibition of influx which might have been anticipated if La$^{3+}$ and Ca$^{2+}$ were simply competing for non-specific binding sites in the cell wall. We therefore conclude that La$^{3+}$ inhibits Ca$^{2+}$ transport into the cell. Several other compounds known to be Ca$^{2+}$ channel antagonists in animal cells were tested – ruthenium red (50 μM), nifedipine (50 μM) and methoxyverapamil (80 μM) – but none elicited any inhibited effects on Ca$^{2+}$ influx (data not shown).

Do the effects of La$^{3+}$ on Ca$^{2+}$ influx result from direct interaction of La$^{3+}$ with the Ca$^{2+}$-permeable pathway, or...
Inhibition of Ca\(^{2+}\) uptake by La\(^{3+}\) in *Neurospora*

...are the effects indirect? One possible mechanism for inhibition of Ca\(^{2+}\) influx might arise, for example, if La\(^{3+}\) perturbs \(\Delta\psi\). Fig. 2 shows that 1 mM-La\(^{3+}\), applied in identical conditions to those used in the \(^{45}\)Ca\(^{2+}\) flux determinations, generates a rapid, extensive and reproducible membrane depolarization amounting to about 80 mV (Fig. 2). This depolarization was succeeded by a spontaneous, but slower partial repolarization which accounted for about 40\% of the initial depolarization in Fig. 2, but was more variable between experiments. The effect of La\(^{3+}\) on \(\Delta\psi\) was only partially reversible, with a sustained 20 mV depolarization remaining after La\(^{3+}\) washout.

La\(^{3+}\)-induced depolarization is concentration-dependent (Fig. 3). Fitting the data to a rectangular hyperbola yields an estimated maximum depolarization amounting to 87 mV and an apparent \(K_{0.5}\) for La\(^{3+}\) = 110 \(\mu\)M.

The major portion of the La\(^{3+}\)-induced depolarization is Ca\(^{2+}\)-sensitive, being reduced as the [Ca\(^{2+}\)] is raised above the control value of 1 mM and increased to as much as 100 mM as the [Ca\(^{2+}\)] is lowered close to zero (Fig. 4). This Ca\(^{2+}\)-sensitive inhibition saturates as a function of Ca\(^{2+}\) concentration, and has a maximum \((I_{\text{max}}) = 86\) mV and an apparent \(K_I\) for Ca\(^{2+}\) = 2.5 mM.

If the sole action of La\(^{3+}\) were to block Ca\(^{2+}\) channels, then membrane hyperpolarization might be anticipated because the equilibrium potential for Ca\(^{2+}\) is positive of zero. The marked membrane depolarization elicited by La\(^{3+}\) demonstrates a more complex action of La\(^{3+}\). In plant cells, K\(^{+}\) channels are blocked both by extracellular Ca\(^{2+}\) (Tester, 1990) and by La\(^{3+}\) (Keifer & Spanswick, 1978; Tester & MacRobbie, 1990). Since the La\(^{3+}\)-induced depolarization in *Neurospora* is largely inhibited by extracellular Ca\(^{2+}\), one possible secondary effect of La\(^{3+}\) might be interaction with Ca\(^{2+}\)-sensitive K\(^{+}\) channels. This possibility is most simple tested by comparing the polarity and magnitude of \(\Delta\psi\) changes in response to La\(^{3+}\) for conditions in which the K\(^{+}\) equilibrium potential \((E_K)\) is first one side, then the other, of the resting \(\Delta\psi\) just before La\(^{3+}\) addition.

Fig. 5 shows the results of one such experiment. In order to bring \(\Delta\psi\) within the range of accessible values of \(E_K\), the membrane was first depolarized with 1 mM-CN\(^{-}\). In these conditions, electrogenic H\(^{+}\) pumping is inhibited considerably (Gradmann et al., 1978). For the control K\(^{+}\) concentration (25 mM), \(E_K\) was \(-36\) mV (assuming cytoplasmic K\(^{+}\) activity is 100 mM) and a small depolarization from \(-70\) mV to \(-35\) mV resulted on La\(^{3+}\) application (Fig. 5a). The mean resting \(\Delta\psi\) in the presence of CN\(^{-}\) and La\(^{3+}\) was \(-43 \pm 7\) mV (six observations). Thus, La\(^{3+}\)-induced opening of K\(^{+}\) channels remains a possible explanation for the depolarization. However, K\(^{+}\) channel involvement is excluded by the results of experiments in which the external K\(^{+}\) concentration is reduced to 2.5 mM and \(E_K\) therefore shifts to \(-94\) mV. Application of La\(^{3+}\) in the presence of CN\(^{-}\) still induces a small depolarization (Fig. 5b) to a mean resting value of \(\Delta\psi = -36 \pm 6\) mV (three
Fig. 5. (a) Effect of La$^{3+}$ on $\Delta \psi$ in presence of 1 mM-NaCN at an external K+ concentration ([K$^+$]$_o$) of 25 mM (experiment representative of four performed). (b), as (a) but with [K$^+$]$_o$ = 2.5 mM (experiment representative of three performed).

observations), despite the considerably more negative value of $E_K$.

**Discussion**

Our radiometric studies with $^{45}$Ca$^{2+}$ confirm that La$^{3+}$ inhibits Ca$^{2+}$ influx in *Neurospora*, as is the case for other fungi (Borbolla & Peña, 1980; Eilam & Chernichovsky, 1987). Ruthenium red (an inhibitor of endomembrane Ca$^{2+}$ channels), nifedipine (a dihydropyridine) and methoxyverapamil (a phenylalkylamine) - all inhibitors of L-type Ca$^{2+}$ channels in animal cells - were ineffective as inhibitors of Ca$^{2+}$ influx in *Neurospora*.

These results appear to indicate inhibition of a Ca$^{2+}$ channel by La$^{3+}$. This interpretation is supported by recordings of the effects of La$^{3+}$ on $\Delta \psi$. Previous estimates of Ca$^{2+}$ influx in *Neurospora* (Miller et al., 1990) revealed only a slight stimulation of flux by CN$^-$, which strongly depolarizes the membrane, and therefore the potent inhibition of Ca$^{2+}$ influx by La$^{3+}$ appears unlikely to have its origins in any decrease in inward driving force on Ca$^{2+}$ associated with La$^{3+}$-induced depolarization. However, if La$^{3+}$ does indeed interact directly with a plasma-membrane Ca$^{2+}$ channel, then some explanation must be sought for the La$^{3+}$-induced depolarization itself. Thus, the equilibrium potential for Ca$^{2+}$ is of the order +100 mV, and simple blockage of a Ca$^{2+}$ channel might be expected to hyperpolarize, rather than depolarize, the membrane.

Three potential alternative modes of action of La$^{3+}$ on membrane electrical properties might be considered. First, by analogy with plant cells (Keifer & Spanswick, 1978; Tester & MacRobbie, 1990), La$^{3+}$ might interact with K+ channels - perhaps mimicking inhibition by external Ca$^{2+}$, or possibly antagonizing an inhibitory effect of Ca$^{2+}$. Clearly, the capacity of Ca$^{2+}$ to antagonize the La$^{3+}$-induced depolarization (Fig. 4) indicates some form of interaction between the two ions. Nevertheless, an involvement of K+-selective channels can be eliminated by the observation that La$^{3+}$ depolarizes the membrane regardless of whether the equilibrium potential for K+ is positive or negative of the resting $\Delta \psi$.

A second possibility is that the depolarization is generated by an in-going La$^{3+}$ current. However, simple calculations based on the near-linear current-voltage characteristic of *Neurospora* do not support this notion either. Thus, the membrane conductance of *Neurospora* lies between 100 and 500 pS cm$^{-2}$ (Gradmann et al., 1978), so a membrane depolarization of 100 mV (Fig. 4) indicates the presence of an inward current of 10 to 50 $\mu$A cm$^{-2}$. This value converts to a charge flow of between 25 and 120 meq (litre intracellular water)$^{-1}$ min$^{-1}$ (Sanders et al., 1983), which for a trivalent ion is 8 to 40 mmol (litre intracellular water)$^{-1}$ min$^{-1}$. These values are one or two orders of magnitude higher than the
measured influx of Ca\(^{2+}\) [0.32 mmol (litre intracellular water)\(^{-1}\) min\(^{-1}\); Fig. 1]. For the depolarization to result solely from an inward La\(^{3+}\) current, the membrane would therefore have to be considerably more permeable to La\(^{3+}\) than to Ca\(^{2+}\), which is unlikely. For example, semi-quantitative studies on a number of plant tissues have indicated that lanthanides enter cells only slowly, or not at all (Campbell et al., 1974; Cambell & Thomson, 1977; Thomson et al., 1973; Quiquampoix et al., 1990).

A third possible mechanism for La\(^{3+}\)-induced depolarization invokes inhibition of the primary plasma membrane H\(^+\)-ATPase. Previous work with charophyte algae (Tsutsui et al., 1987) has suggested that La\(^{3+}\) is a potent inhibitor of the plasma-membrane electrogenic pump of plants, and a similar effect appears, by default, to comprise the most likely explanation for the present results. Our data give no indication concerning the location (extracellular or intracellular) at which La\(^{3+}\) interacts with transport. However, the clear antagonism by Ca\(^{2+}\) of the La\(^{3+}\)-induced depolarization suggest that, whatever the cause of the depolarization, La\(^{3+}\) interferes with some Ca\(^{2+}\)-dependent event.

We conclude that although La\(^{3+}\) is likely to interact directly with Ca\(^{2+}\)-permeable pathway(s) in the plasma membrane of Neurospora, the ion also has other effects on the membrane, which result in a marked membrane depolarization. Considerable caution should therefore be exercised in the interpretation of investigations which rely on La\(^{3+}\) to block plasma-membrane Ca\(^{2+}\) influx in fungi (for example, during signal transduction).

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### References


