Electrical Phenotypes of Calcium Transport Mutant Strains of a Filamentous Fungus, *Neurospora crassa*

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We characterized the electrical phenotypes of calcium transporters—a mechanosensitive channel homolog (MscS), a Ca\(^{2+}/H^+\) exchange protein (cax), and Ca\(^{2+}\)-ATPases (nca-1, nca-2, nca-3)—as well as those of double mutants (the nca-2 cax, nca-2 nca-3, and nca-3 cax mutants). The electrical characterization used dual impedances to obtain cable-corrected current-voltage measurements. Only two types of mutants (the MscS mutant; the nca-2 mutant and nca-2-containing double mutants) exhibited lower resting potentials. For the nca-2 mutant, on the basis of unchanged conductance and cyanide-induced depolarization of the potential, the cause is attenuated H\(^+\)-ATPase activity. The growth of the nca-2 mutant-containing strains was inhibited by elevated extracellular Ca\(^{2+}\) levels, indicative of lesions in Ca\(^{2+}\) homeostasis. However, the net Ca\(^{2+}\) effluxes of the nca-2 mutant, measured noninvasively with a self-referencing Ca\(^{2+}\)-selective microelectrode, were similar to those of the wild type. All of the mutants exhibited osmosensitivity similar to that of the wild type (the turgor of the nca-2 mutant was also similar to that of the wild type), suggesting that Ca\(^{2+}\) signaling does not play a role in osmoregulation. The hyphal tip morphology and tip-localized mitochondria of the nca-2 mutant were similar to those of the wild type, even when the external [Ca\(^{2+}\)] was elevated. Thus, although Ca\(^{2+}\) homeostasis is perturbed in the nca-2 mutant (B. J. Bowman et al., Eurycaryot. Cell 10:654–661, 2011), the phenotype does not extend to tip growth or to osmoregulation but is revealed by lower H\(^+\)-ATPase activity.

*Neurospora crassa* has a relatively concise genome in which duplicate genes are few (11, 12). The ready availability of knock-out mutants for many of the known genes and open reading frames (ORFs) (9) simplifies the analysis of phenotypes associated with the absence of specific genes. This is of especial utility for studies of transport physiology, including explorations of the role of Ca\(^{2+}\) transport in hyphal morphogenesis. The central role of calcium in the life of the cell is well known (2, 7). In fungi, cytoplasmic Ca\(^{2+}\) is maintained at a very low level of 0.1 to 0.2 μM (29, 36). Ca\(^{2+}\) elevation induces signal transduction by Ca\(^{2+}\)-dependent kinases, Ca\(^{2+}\)/calmodulin, and calcineurin (45). Mutants of Ca\(^{2+}\) signaling components affect morphogenesis (15, 33), and hyphal tip growth requires a cytoplasmic tip-high Ca\(^{2+}\) gradient (39).

Calcium signaling in *N. crassa* is considered unique in that the genome sequence does not reveal the presence of gene families common in other clades—inositol-1,4,5-trisphosphate and ryanodine receptors and ADP ribosyl cyclase—that play central roles in calcium signaling via release from internal stores (4, 11). Overall, there are approximately 50 Ca\(^{2+}\) signaling proteins, 20 Ca\(^{2+}\) transport proteins, and about 20 Ca\(^{2+}\)/calmodulin-regulated proteins (45) in *N. crassa*. A larger number of calcium transporters have been reported in *Aspergillus* species (46 channels, 97 cotransporters, and 105 ATPases) (1). Bowman et al. (5, 6) undertook an extensive analysis of four of the major calcium transporters in *N. crassa*: cax, nca-1, nca-2, and nca-3. Briefly, cax encodes a calcium transporter located on the vacuolar membrane. nca-1, nca-2, and nca-3 encode Ca\(^{2+}\)-ATPases located on the endoplasmic reticulum (nca-1) and both the vacuolar and plasma membranes (nca-2 and nca-3) (5). Among these four calcium transporters, only the nca-2 mutant exhibits elevated intracellular calcium levels and growth inhibition by elevated extracellular calcium levels (6). Some of these transporters may affect transport at the plasma membrane, so we undertook an electrophysiological character-

**MATERIALS AND METHODS**

**Strains.** The genotypes of the strains used in the present study are shown in Table 1. Three double mutants were generously provided by Barry

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**TABLE 1 List of strains used in this study**

| Genotype | Locus tag | Strain designation | Mating type | Function of mutated protein |
|----------|-----------|--------------------|-------------|-----------------------------|
| MscS     | NCU09595.2| FGSC 14504         | a           | Mechanosensitive channel    |
| cax      | NCU07075.2| FGSC 11249         | A           | Ca\(^{2+}\)/H\(^{+}\) exchange protein |
| nca-1    | NCU03305.2| FGSC 13287         | a           | Ca\(^{2+}\)-ATPase            |
| nca-2    | NCU04736.2| FGSC 13071         | A           | Ca\(^{2+}\)-ATPase            |
| nca-3    | NCU05154.2| FGSC 13037         | A           | Ca\(^{2+}\)-ATPase            |
| cax nca-2|           |                    |             |                             |
| nca-2    |           |                    |             |                             |
| nca-3    |           |                    |             |                             |
| Wild type |           | FGSC 2489 (74-OR23-1VA) | A |                             |

Bowman (University of California, Santa Cruz): the cax nca-2, nca-2 nca-3, and cax nca-3 strains. Single knockout mutants (9) were obtained from the Fungal Genetic Stock Center (School of Biological Sciences, University of Missouri, Kansas City) (28). The knockout mutants were the cax (NCU07075.2; FGSC 11249), MscS (NCU09595.2; FGSC 14504), nca-1 (NCU03305.2; FGSC 13287), nca-2 (NCU04736.2; FGSC 13071), and nca-3 (NCU05154.2; FGSC 13037) mutants. The wild-type strain used for comparisons with the mutants was strain 74-OR23-1VA (FGSC 2489).

Stock cultures were grown and maintained on Vogel’s minimal medium (VM) (44) plus 1.5% sucrose and 2.0% agar.

**Growth measurements.** The wild type and the various mutant strains were grown on the following media: YPD (1% [wt/vol] yeast extract, 2% [wt/vol] glucose, and 2% [wt/vol] agar) as a control, containing NaCl (0.1 to 1.2 M). A plug of agar containing mycelium from the stock cultures was placed in the center of the agar dishes and was grown in the dark at 28°C. Growth measurements were taken every 1 to 2 h, and the growth rates were calculated as colony diameter increases (in centimeters per hour).

**Electrical measurements.** Single-barrel micropipettes were fabricated from borosilicate glass capillaries (outside diameter [i.d.], 1.0 mm; inside diameter [o.d.], 0.58 mm, with filament; Friedrich and Dimmock Inc., Millville, NJ) using a model P-30 micropipette puller (Sutter Instrument Co., Novato, CA). Both micropipettes were filled with a 3 M KCl solution and were inserted into microelectrode holders containing a Ag/AgCl electrode. The microelectrodes were connected to a 251A electrometers (input impedance, 10\(^{12}\) Ω; Warner Instruments). The microelectrodes had tip resistances of about 20 to 30 MΩ. The circuit was completed with a salt bridge containing 3 M KCl in 2% (wt/vol) agar and a Ag/AgCl electrode. Large hypae (15 to 20 μm in diameter) with visible cytoplasmic flow were selected for electrophysiological measurements. Dual impalements were conducted with a separation distance of 10 to 20 μm (Fig. 1). After the second impalement, a voltage clamp protocol was performed using an operational amplifier configured for voltage clamp that was controlled by a data acquisition board (Scientific Solutions, Solon, OH) (22). The clamp protocol was a bipolar stepwise staircase of alternating positive and negative voltage clamps and resting potential with a clamp duration of 200 ms (the clamp duration ensured that clamping currents were at steady state). During the last 10 ms of the voltage clamp, both the clamping current and the clamped voltage (to ensure clamping fidelity) were sampled. For current-voltage measurements, sample sizes ranged from 10 to 15 separate preparations.

To obtain the current density (in milliamperes per square meter), the cable properties (32) of the hypha must be taken into account (Fig. 1).
FIG 1  Hyphal impalement and equivalent circuit. (Top) Dual impalements of a hyphal segment. (A) Before impalement; (B) first impalement; (C) second impalement; (D) after second impalement. Arrows in the sequence from A through D show the movement of a spherical particle through the hypha due to mass flow (21). Bar, 10 μm. (Bottom) The equivalent electrical circuit of the hyphal segment. Due to the long cylindrical shape of the cell, the cell membrane resistances are in series. Thus, clamping currents are increasingly attenuated with increasing distance from the site of impalements as current passes through the plasma membrane into the external, grounded medium (22). Corrections for the cable properties of the hypha are described in Materials and Methods.

(22). At increasing distances from the site of current injection, the voltage change is attenuated exponentially, as expressed by the equation $V/V_o = e^{-x/\lambda}$, where $V$ is the voltage at distance $x$, $V_o$ is the voltage at the current injection site, $x$ is the distance, and $\lambda$ is the length constant. For the cylindrical geometry of the hypha, the current density ($I_m$) can be calculated as $I/(2\pi d\lambda)$, where $I$ is the clamping current, $d$ is the diameter of the impaled hypha, and $\lambda$ is the length constant of the hyphae. Based on direct measurements of voltage attenuation along hyphae under the same preparation and measurement conditions (22), a length constant of 407 μm was used to calculate the current density. Clamping currents at the extreme negative and positive voltages were occasionally clipped (so that the voltage could not be clamped with fidelity). The clipped clamping currents were discarded prior to estimating conductances (in millisiemens per square meter) from the slopes of linear regression fits of the current-voltage relations.

One concern that arises in quantifying current densities is the effect of multiple impalements on membrane resistance. That is, impalements may cause shunt resistances at the sites of impalement that attenuate voltage measurements because of significant voltage dividing. When dual impalements are performed, there can be a noticeable decrease in the measured potential. The effect was quantified as the ratio of the final potential (after the second impalement) to the initial potential (after the first impalement). The average ratio was 0.90 ± 0.03 ($n = 26$). The effect of the impalements will differ depending on the care taken during the impalement and the degree of robustness of recovery by the impaled hyphae, but the effect is fairly small, so no attempt was made to correct for shunt resistance. It does, however, lead to higher clamping currents and thus to an overestimate of current density by approximately 10%.

Turgor measurements. Cultures for turgor measurements (23, 24) were prepared similarly to those for electrical measurements. Sections of mycelium grown overnight on cellophane dialysis tubing were cut, transferred to the lid of a 30-mm culture dish, and flooded with 3 ml of BS. Micropipettes were prepared with a large aperture tip and were filled with a silicone oil of low viscosity (polydimethylsiloxane, 1.5 centistokes; Dow Corning, Midland, WI). The micropipette was connected to a holder that, in turn, was connected to a micrometer-driven piston with thick-wall Tellon tubing. A pressure transducer (XT-190-300G; Kulite Semiconductor, Leonia, NJ) was mounted on the holder. Upon impalement of the hypha with the micropipette, the silicon oil/cell sap meniscus moved back from the tip. The pressure required to move the meniscus back to the micropipette tip was used as a measure of turgor. Hyphal mass flow (21) normally continued during the measurements, indicative of hyphal health. About 6 to 8 measurements (over 6 min) were made on a single hypha by drawing the meniscus back and then returning it to the micropipette tip (to ensure that the tip had not become plugged). Measurements for the wild type and the nca-2 mutant were normally interspersed during the experimental runs.

Ca$^{2+}$ flux measurements. Cultures for Ca$^{2+}$ flux measurements were prepared similarly to those for electrical and turgor measurements. However, the mycelium was grown between two sheets of cellophane dialysis tubing overlaying YPD agar plates. This ensured that fluxes were being measured from a flat planar source. YPD contains about 0.13 mM Ca$^{2+}$ (3); thus, the mycelia would be Ca$^{2+}$ replete prior to the flux measurements so as to enhance our ability to measure the net efflux of Ca$^{2+}$ directly. Sections of mycelium grown overnight were cut, gently lifted with a small spatula, transferred to the lid of a 30-mm culture dish, taped down with masking tape, and flooded with 3 ml of OM. OM contains lower levels of Ca$^{2+}$ than YPD (increasing the signal-to-noise ratio for flux measurements) and has been used for previous Ca$^{2+}$ flux measurements (20).

The technique for measuring net ion fluxes (the scanning ion-selective electrode technique [SIET]) was recently described by Nguyen and Donini (31). The external steady-state diffusive gradient created by solute transport across the plasma membrane is measured by sampling the solute concentrations at two positions, one near and one far from the cell, with the ion-selective microelectrode. The flux can be calculated from the difference in concentration (10, 17, 22, 42).

The micropipettes were pulled from borosilicate tubes (catalog no. TW150-4; World Precision Instruments, Sarasota, FL) to form a tip aperture of 5 to 8 μm. The micropipettes were silanized by exposure to N,N-dimethyltrimethylsilylamine vapors after heating to 300°C. After cooling, the micropipette was filled with 100 mM CaCl$_2$, and the tip was dipped in a Ca$^{2+}$-selective cocktail (Ca$^{2+}$ ionophore I cocktail A; catalog no. 21048; Sigma-Aldrich) to form a column of 150 to 300 μm. The microelectrode was mounted on the headstage of an ion/polarographic amplifier (IPA-2; Applicable Electronics, Forestdale, MA) using an AgCl/Ag half-cell. The reference electrode was 500 mM KCl in 2% agar connected to ground with a AgCl/Ag half-cell. Calibrations were performed with CaCl$_2$ with [Ca$^{2+}$] of 0.01, 0.1, and 1.0 mM. Slopes were 29.6 ± 0.9 mV per decade difference in [Ca$^{2+}$] ($n = 4$).

The ion-selective electrode was positioned as close as possible to the...
mycelium and 50 μm away to measure the diffusive gradient of Ca$^{2+}$ caused by ion flux at the surface of the mycelium. The differences in ion concentration were converted into a flux by assuming steady-state conditions and a planar source. Fluxes were corrected for background by measuring regions of the cellophane dialysis tubing into which the mycelium had not yet grown. To correct the area so as to account for spaces between hyphae, a threshold was applied to digital images of the mycelium in order to distinguish interhyphal space from the hyphae, and the hyphal area was measured using ImageJ (34). Measurements for the wild type and the nca-2 mutant (6) showed that the hyphal area was smaller for the nca-2 mutant than for the wild type (11 ± 8 μM) (n = 3), possibly due to the reported elevated Ca$^{2+}$ concentration in the nca-2 mutant (6).

Imaging of mitochondria. Tip-localized mitochondria were imaged using MitoFluor Red 589 (18). Overnight cultures grown on YPD plus agar were flooded with 2 ml of OM (containing the dye), and growing hyphae at the colony edge were imaged with a 40× water immersion objective on a Zeiss Axioskop 2 microscope. Fluorescence was viewed with Zeiss filter set 15 (excitation band-pass filter, 546 nm; emission long-pass filter, 590 nm). Images were taken every 10 s with an Orca C-4742-95 camera (Hamamatsu Photonics KK, Japan) using OpenLab software (version 3.1.7; Improvision, Coventry, United Kingdom). The growing hyphae were treated with 125 mM CaCl$_2$ in OM by perfusing 2 ml of the solution through the dish, and imaging was recommenced as soon as within 45 s of the beginning of perfusion.

Statistical analysis. Statistics are shown as means ± standard deviations (SD) (with the sample size in parentheses) (SD was used as an estimator of population variance) unless stated otherwise. Independent two-tailed or one-tailed t tests were performed in Excel (Microsoft). Nonlinear regressions were performed in KaleidaGraph (Synergy Software, Reading, PA).

RESULTS

Much of our phenotypic analysis focused on electrical characterization of the calcium transport mutants, but we extended our characterization to include some aspects of growth and morphology. In general, all of the knockout mutants exhibited normal morphology both for leading hyphae at the colony edge and for the hyphal network within the mycelium. No differences in cell size or in hyphal cytolgy were observed.

Growth of calcium transport mutants. The effects of Ca$^{2+}$ depletion (by addition of the Ca$^{2+}$ chelator BAPTA at 1 mM) and excess Ca$^{2+}$ on colony growth were examined for all single and double mutants (Fig. 2A). Under normal conditions (YPD), the nca-2 nca-3 and nca-2 cax double mutants grew more slowly than other mutant strains and the wild type. Ca$^{2+}$ depletion with BAPTA caused slight growth inhibition for all strains (including the wild type). Growth recovered when 1.1 mM CaCl$_2$ was added to ensure that the effect was due to Ca$^{2+}$ depletion rather than to some other effect of BAPTA addition. The growth of the nca-2 mutant and of all double mutants that included the nca-2 deletion was strongly inhibited by elevated Ca$^{2+}$ concentrations, confirming the results of Bowman et al. (5).

To determine if the inhibition of growth at elevated Ca$^{2+}$ concentrations was an osmotic effect, the effect of NaCl was examined for a subset of the mutants (the cax, MscS, nca-1, nca-2, and nca-3

FIG 2 Growth phenotypes of the Ca$^{2+}$ transport mutants. (A) Effects of Ca$^{2+}$ depletion or high Ca$^{2+}$ concentrations on colony growth. The mutant strains were inoculated at the centers of agar plates containing either YPD plus 1 mM BAPTA (ca. 100 nM Ca$^{2+}$), YPD plus 1 mM BAPTA and 1.1 mM CaCl$_2$ (ca. 0.23 mM Ca$^{2+}$), YPD alone (0.13 mM Ca$^{2+}$), or YPD plus 25, 200, or 400 mM CaCl$_2$. Colony diameters were measured every 1 to 2 h and are shown as increases in diameter (in centimeters per hour). Means (horizontal bars) and individual data (open triangles) are shown. The gray horizontal bar represents the mean growth rate of the wild type in YPD. The most notable phenotype was the strong inhibition of growth by elevated Ca$^{2+}$ concentrations in strains containing the nca-2 mutation. (B) Osmotic sensitivities of Ca$^{2+}$ transport mutants. The mutant strains were grown on YPD supplemented with various concentrations of NaCl. Large symbols represent means; small symbols represent results of individual experiments. Linear regressions for the data are shown. All of the Ca$^{2+}$ transport mutant strains examined (the cax, MscS, nca-1, nca-2, and nca-3 mutants) exhibited osmotic sensitivity similar to that of the wild type. (C) The nca-2 mutant strain exhibits Li$^+$ and Zn$^{2+}$ sensitivities similar to those of the wild type but increased growth at low levels of Mg$^{2+}$ (25 mM).
mutants) (Fig. 2B). All exhibited osmotic sensitivity similar to that of the wild type up to 1.2 M NaCl. Thus, the Ca$^{2+}$ sensitivity of the nca-2 mutant is not due to an osmotic effect. Furthermore, any disruptions of Ca$^{2+}$ transport and homeostasis in these mutants does not affect their response to osmotic stress.

Given the Ca$^{2+}$ sensitivity of the nca-2 mutant, the effects of other ions on its growth were examined (Fig. 2C). Its Li$^+$ and Zn$^{2+}$ sensitivities were similar to those of the wild type. There was a notable (and significant [P = 0.016]) increase in the growth of the nca-2 mutant at 25 mM Mg$^{2+}$ that was not observed in the wild type.

Inhibitors of various components of Ca$^{2+}$ signaling were examined (Fig. 3). Inhibition of the nca-2 mutant by an inhibitor of inositol 1,4,5-triphosphate (IP$_3$)-activated Ca$^{2+}$ channels (2-APB) and an inhibitor of Ca$^{2+}$ sequestration (cyclopiazonic acid) are known to affect Ca$^{2+}$ gradients at growing hyphal tips (37) but had similar inhibitory effects on the wild type and the nca-2 mutant. The nca-2 mutant exhibited less sensitivity to TMB-8 (P = 0.0011), but greater sensitivity to the Ca$^{2+}$-ATPase inhibitor thapsigargin, than the wild type (P = 0.0005).

**FIG 3** Effects of inhibitors on the wild type and the nca-2 mutant. The fungus was treated with inhibitors by perfusion with 2 ml of OM containing either 2-APB (50 μM), cyclopiazonic acid (100 μM), TMB-8 (200 μM), or thapsigargin (100 μM). Growth rates of individual hyphae (usually 4 to 5 per treatment) for 10 min before and after inhibitor treatment were measured using ImageJ. Data are shown as percentages of the control value (before inhibitor treatment). An inhibitor of IP$_3$-activated Ca$^{2+}$ channels (2-APB) and an inhibitor of Ca$^{2+}$ sequestration (cyclopiazonic acid) are known to affect Ca$^{2+}$ gradients at growing hyphal tips (37) but had similar inhibitory effects on the wild type and the nca-2 mutant. The nca-2 mutant exhibited less sensitivity to TMB-8 (P = 0.0011), but greater sensitivity to the Ca$^{2+}$-ATPase inhibitor thapsigargin, than the wild type (P = 0.0005).

Elevated cytoplasmic Ca$^{2+}$ sensitivity of calcium transport mutants. To assess the ability of the Ca$^{2+}$ transport mutants to regulate cytoplasmic Ca$^{2+}$ levels, we examined the effect of the calcium ionophore A23187 on the growth of the knockout mutants (the MscS, cac, nca-1, nca-2, and nca-3 mutants). In *Aspergillus awamori*, A23187 is reported to increase cytoplasmic Ca$^{2+}$ levels, as monitored by aequorin fluorescence (30). The technique—growth into an A23187-containing zone—has been used to demonstrate the sensitivity of a mechanosensitive channel mutant (the mid-1 mutant) to the Ca$^{2+}$ ionophore in *N. crassa* (25).

The mutant strain phenotypes were the same as the wild-type phenotype except for the nca-2 mutant. For all mutants (in contrast to the mid-1 mutant), growth was not arrested when the colony edge grew into the A23187-containing zone. Except for the nca-2 mutant (which grew more sparsely than the other strains), aerial hyphae were absent in a zone extending back from the A23187-containing region (Fig. 4). Thus, the nca-2 mutant exhibits no response to elevated cytoplasmic Ca$^{2+}$ levels, in contrast to the other Ca$^{2+}$ transport mutants examined.

To explore whether the lesions in transport for the various mutants were reflected in ion transport, the current-voltage properties of the mutants were examined.

**Electrical properties of calcium transport mutants.** To determine the relative contributions of active transport (the electrogenic H$^+$-ATPase) and passive ion distributions (the Goldman-Hodgkin-Katz potential for permeant ions), the hyphae were treated with cyanide to deplete cytoplasmic ATP, thereby inhibiting H$^+$-ATPase activity. The use of cyanide as a respiratory inhibitor in *N. crassa* to deplete ATP and determine the activity of the electrogenic pump (H$^+$-ATPase) at the plasma membrane is well established (13, 41). Measurements of each mutant strain were interspersed with measurements of a wild-type control. The current-voltage relations were shown in Fig. 5. The resting potential and conductance measurements are shown in Fig. 6 and 7, respectively.

The current-voltage relations were fairly similar for all mutants examined (Fig. 5). Two mutants (the MscS and nca-2 mutants) had significantly depolarized resting potentials compared to that of the wild type (Fig. 6). The MscS mutant potential was $-107 \pm 18$ mV ($n = 16$), compared to the wild-type control potential of $-131 \pm 20$ mV ($n = 13$) (P < 0.002). The nca-2 mutant potential was $-116 \pm 13$ mV ($n = 15$), compared to the wild-type control potential of $-137 \pm 13$ mV (P < 0.0002). The resting potential was also significantly depolarized in the nca-2 nca-3 and nca-2 cac mutants.
double mutants but not in the nca-3 cax mutant, indicating that the nca-2 knockout was responsible. The cyanide-induced change in the potential was significantly smaller for the nca-2 mutant (84 ± 15 mV) than for the wild-type control (100 ± 11 mV) (P < 0.003), suggesting that the cause is lower electrogenic ATPase activity (either electrogenic Ca\(^{2+}\)-ATPase or H\(^{+}\)-ATPase [see Discussion]).

The conductances of the mutants versus that of the wild type were very similar before and after cyanide treatment. Only the nca-2 cax and nca-3 cax double mutants exhibited statistically significant differences from the wild type (P < 0.007 and < 0.002, respectively), but only in the cyanide-induced change in conductance (Fig. 7).

Because the growth of the nca-2 mutant was sensitive to elevated Ca\(^{2+}\) concentrations, the effect of elevated extracellular Ca\(^{2+}\) concentrations on its potential was explored in greater detail. Hyphae were impaled with a single-barrel micropipette so that only the potential was monitored. Extracellular [Ca\(^{2+}\)] was increased by adding 0.5 ml of 0.5 M CaCl\(_2\) (in BS) dropwise around the water immersion objective (final concentration, 71 mM). Under this elevated-Ca\(^{2+}\) concentration, the effect of elevated extracellular Ca\(^{2+}\) concentrations on tip growth and mitochondrial distributions at growing tips was examined. Tip-localized mitochondria play a role in Ca\(^{2+}\) sequestration (18); therefore, mitochondrial distributions at growing hyphal tips were examined before and after treatment with elevated Ca\(^{2+}\) concentrations (Fig. 8). Mitochondria were normally vermiform, with a higher density just behind the hyphal tip. There were no dramatic differences in the tip localization of mitochondria between the nca-2 mutant and the wild type, and elevated Ca\(^{2+}\) concentrations did not affect distributions in either strain.

**Turgor in the nca-2 mutant.** Turgor regulation in *N. crassa* can rely on ion transport (24) and can be lowered in mutants with depolarized potentials (27). Therefore, we examined turgor in the nca-2 mutant. Turgor in the nca-2 mutant (525 ± 95 kPa) (n = 20) was similar to that in the wild type (544 ± 64 kPa) (n = 20) (P = 0.457).

**Ca\(^{2+}\) fluxes in the nca-2 mutant.** One possible phenotype of the nca-2 mutant is a lesion in Ca\(^{2+}\) export from the hyphae, since this Ca\(^{2+}\)-ATPase is located on the plasma membrane as well as the vacuole (5). This was tested directly by measuring net ion fluxes with Ca\(^{2+}\)-selective electrodes (scanning ion-selective electrode technique [SIET]) (31) (Fig. 9). Previous flux measurements did not identify localized regions of net Ca\(^{2+}\) flux along growing hyphae (20). By use of OM rather than BS for flux measurements,
background \([\text{Ca}^{2+}]\) was low, increasing the signal-to-noise ratio for flux measurements. In glucose-replete OM, the potentials of the WT and the \(\text{nca-2}\) mutant were more positive than those measured in BS (\(-83 \pm 23\) and \(-103 \pm 21\) mV, respectively) \((n = 24)\). The net \(\text{Ca}^{2+}\) fluxes were corrected for \(\text{Ca}^{2+}\) reservoirs in the cellophane used to “sandwich” the mycelium by subtracting fluxes over the cellophane layers from fluxes over the mycelium. There was no significant difference in net \(\text{Ca}^{2+}\) flux out of the hyphae between the WT (1.11 \pm 0.85 pmol cm\(^{-2}\) s\(^{-1}\)) \((n = 3)\) and the \(\text{nca-2}\) mutant (1.76 \pm 1.38 pmol cm\(^{-2}\) s\(^{-1}\)) \((n = 3)\) \((P = 0.534, 2\text{-tailed } t\text{ test})\).

**DISCUSSION**

The complete genomic sequence of *Neurospora crassa* \((11)\) and the creation of knockout mutants \((9)\) have revolutionized our ability to explore the roles of practically all genes in the life of the fungus. With these tools in hand, we undertook an electrophysiological characterization of \(\text{Ca}^{2+}\) transporter mutants in *Neurospora crassa*—the \(\text{cax}, \text{nca-1}, \text{nca-2},\) and \(\text{nca-3}\) mutants—for which localization and functional phenotypes are available \((5, 6)\), as well as the mechanosensitive channel \(\text{MscS}\) mutant.

Of the four \(\text{Ca}^{2+}\) transporter mutants, the \(\text{nca-2}\) mutant is sensitive to extracellular \(\text{Ca}^{2+}\) levels and contains elevated intracellular \(\text{Ca}^{2+}\) concentrations \((6)\). The sensitivity to extracellular \(\text{Ca}^{2+}\) was confirmed for the \(\text{nca-2}\) mutant and for double mutants containing the \(\text{nca-2}\) mutation \((2)\). Elevated \(\text{Mg}^{2+}\) levels increased the growth of the \(\text{nca-2}\) mutant, suggesting that divalent cation replacement alleviates the impact of elevated cytoplasmic calcium concentrations. None of the mutants exhibited a delete-
Our research on the electrical properties of the Ca\(^{2+}\) transport mutant provides an analysis of mutant phenotypes that complements the work of Bowman et al. (5, 6). For all of the mutant strains, the effects on the electrical properties of the hyphae could be direct or indirect. If the transporter contributed to electrogenic transport at the plasma membrane, its absence could affect the resting potential and/or conductance. Indirect effects would be due to modifications in calcium signaling that, in turn, modulate electrogenic transport at the plasma membrane. It has been reported that elevated cytoplasmic Ca\(^{2+}\) concentrations activate the plasma membrane H\(^+\)-pump (19). Ca\(^{2+}\) signaling pathways (mediated, for example, by IP\(_3\), calmodulin, and calcineurin) that regulate electrogenic transport could be affected in the calcium transport mutants due to dysfunctional Ca\(^{2+}\) homeostasis. The overall electrical potential (\(E_m\)) of the hypha depends on contributions from both the H\(^+\)-ATPase current and passive ion distributions across the membrane. This can be summarized by the equation:

\[
E_m = I_{\text{pump}}/g_m + E_C,
\]

where \(I_{\text{pump}}/g_m\) is the H\(^+\)-ATPase current divided by the conductance and \(E_C\) is the Goldman-Hodgkin-Katz potential for permeant ions (43). The conductance (\(g_m\)) is a measure of the overall electrogenic flux across the plasma membrane, which was unaffected in all of the mutants. We tested directly for an effect on the Goldman-Hodgkin-Katz potential caused by ion distributions across the membrane versus an effect on the H\(^+\)-ATPase current by using the metabolic inhibitor cyanide to deplete ATP (8). Cyanide causes the potential to depolarize to the level of the Goldman-Hodgkin-Katz potential.

For the two mutants with aberrant electrical properties, the MscS and nca-2 mutants, the normal membrane potential is depolarized relative to that of the wild type, but conductance is unaffected. The net Ca\(^{2+}\) efflux measured in the nca-2 mutant (nca-2 encodes a Ca\(^{2+}\)-ATPase) was similar to that for the wild type, so a direct effect on Ca\(^{2+}\) transport at the plasma membrane is unlikely. The depolarized potential after cyanide treatment was the same for the nca-2 mutant as for the wild type and was significantly more positive for the MscS mutant. Thus, there is less ATP-dependent electrogenic pumping in the nca-2 mutant. Since Ca\(^{2+}\) efflux is unaffected, the phenotypic effect of the nca-2 deletion on electrical properties is an effect on H\(^+\)-ATPase activity rather than a direct effect of the absence of the Ca\(^{2+}\)-ATPase. For the MscS mutant, the phenotypic effect is likely an effect on passive ion distributions across the plasma membrane that would affect the Goldman-Hodgkin-Katz potential.

Tip growth in Neurospora relies on a tip-high Ca\(^{2+}\) gradient (39). The elevated Ca\(^{2+}\) level at the tip is generated from internal stores and is sequestered behind the tip by tip-localized mitochondria (18), among other mechanisms. In the nca-2 mutant, hyphal tip morphology and tip-localized mitochondrial distributions are unaffected, even by elevated external Ca\(^{2+}\) concentrations. Bowman et al. (6) reported that the internal Ca\(^{2+}\) concentration in the nca-2 mutant is elevated, but it appears that the tip-high Ca\(^{2+}\) gradient can still be maintained, allowing growth, albeit at a rate slightly lower than that of the wild type.

Overall, in combination with the results of Bowman et al. (5, 6), our results reveal a more complicated view of Ca\(^{2+}\) regulation than would be expected, one in which Ca\(^{2+}\) transport mutants affect other transport processes at the plasma membrane, but this is true only for the nca-2 mutant, which exhibits lower H\(^+\)-ATPase activity, and the MscS mutant, which exhibits a depolarized Goldman-Hodgkin-Katz potential. To what extent these phenotypes are due to modulation of the expression of other genes in the knockout mutants, or to other, unknown mechanisms regulating Ca\(^{2+}\) homeostasis, remains to be explored.

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FIG 9 Ca\(^{2+}\) fluxes in the wild type and the nca-2 mutant. The fluxes were measured with a Ca\(^{2+}\)-selective electrode, as described in Materials and Methods. The cells were grown between two layers of cellophane to create a 2-dimensional mycelial network in YPD (containing about 0.13 mM Ca\(^{2+}\)) to allow direct measurements of net efflux from Ca\(^{2+}\)-replete mycelia. (A) Diagram of the measuring technique. (B) Top-down view of the probe and mycelial network. The electrode was positioned as near the mycelium as possible and 50 \(\mu\)m away. The differences in concentration were used to calculate the flux as follows:

\[
j = -D \left( [Ca^{2+}]_{\text{near}} - [Ca^{2+}]_{\text{far}} \right)/d,
\]

where \(D\) is the diffusion coefficient, while \([Ca^{2+}]_{\text{near}}\) and \([Ca^{2+}]_{\text{far}}\) are the calcium concentrations sampled near the hyphae and 50 \(\mu\)m (d) away from the hyphae. The cellophane contained a large reservoir of Ca\(^{2+}\). This was corrected for by subtracting the “flux” measured above the cellophane in an area without mycelium from the fluxes measured above the mycelial network (the correction ranged from 8 to 29% of mycelial flux). The fluxes were normalized to the relative area filled with hyphae in the regions being sampled. (C) The net fluxes of Ca\(^{2+}\) from the hyphae were outward (22) and were similar for the wild type and the nca-2 mutant.
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