Small Molecule Channels Harness Membrane Potential to Concentrate Potassium in *trk1Δtrk2Δ* Yeast

Jennifer Hou¹, Page N. Daniels¹, and Martin D. Burke^{1,2,3,4,5*}

¹ Department of Biochemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Ave., Urbana, IL 61801

² Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Ave., Urbana, IL 61801

³ Carle Illinois College of Medicine, 807 South Wright Street, Champaign, IL 61820

⁴ Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, 1206 West Gregory Dr., Urbana, IL 61801

⁵ Arnold and Mabel Beckman Institute, University of Illinois at Urbana-Champaign, 405 North Mathews Ave., Urbana, IL 61801

ABSTRACT

Many protein ion channels harness membrane potential to move ions in opposition to their chemical gradient. Deficiencies of such proteins cause several human diseases, including cystic fibrosis, Bartter Syndrome Type II, and proximal renal tubular acidosis. Using yeast as a readily manipulated eukaryotic model system, we asked whether, in the context of a deficiency of such protein ion channel function *in vivo*, small molecule channels could similarly harness membrane potential to concentrate ions. In yeast, Trk potassium transporters use membrane potential to move potassium ions from a compartment of relatively low concentration outside cells (~15mM) to one of >10 times higher concentration inside (150-500mM). *trk1Δtrk2Δ* yeast are missing these potassium transporters and thus cannot concentrate potassium or grow in standard media. Here we show that potassium permeable, but not potassium selective, small molecule ion channels formed by the natural product amphotericin B can harness membrane potential to concentrate potassium in *trk1Δtrk2Δ* cells and thereby restore growth. This finding expands the list of potential human channelopathies that might be addressed by a molecular prosthetics approach.

INTRODUCTION

Many protein ion channels use membrane potential to move ions from compartments of relatively low concentration to those of higher concentration.¹ These include the CFTR anion channel (which concentrates chloride), the ROMK potassium channel (which concentrates potassium), and the NBCe1 sodium bicarbonate cotransporter (which concentrates bicarbonate). Deficiencies of these protein functions cause the challenging to treat human diseases cystic fibrosis, Bartter Syndrome Type II, and proximal renal tubular acidosis, respectively.^{2,3} Replacing protein ion channels with small molecule mimics⁴ can in some cases restore physiology in cell and animal models of human diseases.^{5,6,7} With the goal of better understanding the potential scope and mechanistic underpinnings of this molecular prosthetics approach, we questioned

whether small molecule channels, which tend to be less ion selective than their protein counterparts, could similarly harness membrane potential to concentrate certain ions in the context of a deficiency of protein ion channel function *in vivo*. We hypothesized that in such settings, increased membrane potential and/or compensatory actions of other protein transporters could enable imperfect small molecule ion channel mimics to be sufficient.

Yeast represent an excellent eukaryotic model system for asking such questions. In the plasma membrane of yeast, Trk1 and Trk2 potassium transporters^{8,9,10} harness membrane potential generated by the proton pump Pma1¹¹ to move K⁺ from a compartment of relatively low concentration outside cells (~15mM) to ≥ 10 times higher concentration inside cells (150-500mM), (Fig. 1a).^{12,13,14,15} Deletion of Trk1 and Trk2 K⁺ transporters (*trk1Δtrk2Δ*)¹² leads to decreased [K⁺]_{intracellular} (abbreviated [K⁺]_i) and lack of cell growth under standard experimental conditions (Fig. 1b).^{12,16} Interestingly, the plasma membranes of *trk1Δtrk2Δ* cells are hyperpolarized relative to wild type (Fig. S3),^{12,13,17} suggesting an amplified driving force for potassium import in the context of the potassium transporter deficiency. Moreover, there are a number of other protein transporters in yeast, including Tok1, Nha1, and Ena1¹⁸ that may be able to compensate for the relative lack of ion selectivity of a small molecule channel.

Figure 1. (a) Pma1 pumps H⁺ (purple spheres) out of cells and the resulting membrane potential is harnessed by Trk transporters to concentrate K⁺ (green spheres) inside cells. (b) *trk1Δtrk2Δ* yeast are hyperpolarized and yet unable to concentrate K⁺. (c) We hypothesized that small molecule mimics of the Trk potassium transporters can harness the membrane potential generated by Pma1 to concentrate K⁺ inside cells and thereby restore cell growth.

We recently reported that ion channels formed by the small molecule natural products amphotericin B (AmB) and nystatin A1 (Nyst) (Fig.S1) can restore growth in Trk potassium ion transporter-deficient yeast.⁵ We also noted that non-selective inhibition of Pma1 by ebselen blocks AmB- and Nyst-mediated growth rescue.⁵ However, in this prior study, we did not quantify intracellular potassium concentrations or relative membrane potential, nor did we have access to the recently reported more selective chemical inhibitor of Pma1 (Pma1i),¹⁹ thus limiting the mechanistic conclusions that could be made. To our knowledge, no prior studies determined whether small molecule channels can use membrane potential to concentrate ions. Here we test the mechanistic hypothesis that these imperfect small molecule mimics of Trk potassium ion transporters can harness the membrane potential in *trk1Δtrk2Δ* yeast to concentrate potassium inside these cells and thereby restore cell growth (Fig.1c).

RESULTS

We first tested whether ion channels formed by AmB and Nyst⁴ could promote the net movement of K⁺ into *trk1Δtrk2Δ* yeast in opposition to its chemical gradient.^{14,20} In the absence of small molecule treatment, *trk1Δtrk2Δ* cells show reduced [K⁺]_i relative to WT cells (Fig.2a,c,e).¹⁶ Natamycin (Nat) is similar in structure to AmB and Nyst but does not form ion channels and thus served as a negative control.²¹ Nat did not change [K⁺]_i (Fig.2a), nor did it restore cell growth in *trk1Δtrk2Δ* at concentrations up to 1μM (Fig. 2b). In contrast, AmB caused a dose-dependent increase in [K⁺]_i in *trk1Δtrk2Δ* yeast (Fig.2c), which was paralleled by an increase in cell growth (Fig.2d). Notably, there was no change in [K⁺]_i or cell growth when WT cells were treated with the same low concentrations of AmB (Fig.2c,d). Similar results were observed with Nyst (Fig.2e,f).

Figure 2. (a) Nat did not increase $[K^+]_i$, and (b) Nat did not rescue yeast growth. (c) AmB dose-dependently increased $[K^+]_i$ and (d) AmB similarly restored $trk1\Delta trk2\Delta$ growth. (e) Nyst increased $[K^+]_i$ and (f) Nyst restored cell growth. NS not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

We similarly quantified $[K^+]_i$ and cell growth as a function of time for a fixed concentration of each small molecule. We observed no changes in $[K^+]_i$ or cell growth over the course of 24h in $trk1\Delta trk2\Delta$ cells treated with 100nM Nat (Fig.3a,b) compared to DMSO control. Similar results were observed when Nat was tested at 1 μ M (Fig.S2a,b). In contrast, 100nM AmB treatment increased $[K^+]_i$ to wild type-like levels within 6h (Fig.3c), and this was paralleled by WT-like growth rates (Fig.3d). Similar results were observed with 1 μ M Nyst (Fig.3e,f).

Figure 3. Small Molecule Ion Channels Increase $[K^+]_i$ over Time. Total cellular K^+ content and growth were monitored in WT and small-molecule-treated $trk1\Delta trk2\Delta$ yeast. (a) Nat did not increase $[K^+]_i$ (N=3) nor (b) rescue $trk1\Delta trk2\Delta$ growth (N=3–4) when tested at 100nM, over the course of 24h. In contrast, both (c) AmB

(100nM) and (e) Nyst (1 μ M) quickly restored $[K^+]_i$, and (d and f) rescued growth of $trk1\Delta trk2\Delta$ cells (N=5 for (c),(d) and N=3 for (e),(f)). *NS not significant*, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

We next tested whether the AmB-mediated concentration of K^+ ions in $trk1\Delta trk2\Delta$ cells is driven by the membrane potential generated by Pma1. We employed the recently reported small molecule inhibitor of Pma1 (Pma1_i)¹⁹ and flow cytometry to quantify the percentage of yeast cells that are relatively depolarized upon Pma1 inhibition. Cells with FITC-A signal above 10^3 were characterized as depolarized (to the right of black line, Fig. 4a-c). In the absence of Pma1 inhibition a minimal percentage of $trk1\Delta trk2\Delta$ cells were depolarized (<1%) (Fig. 4a,d). Modest inhibition of Pma1 by 15 μ M Pma1_i did not cause a statistically significant increase in the number of depolarized $trk1\Delta trk2\Delta$ yeast cells (Fig. 4b,d). In contrast, most of the cells treated with 30 μ M Pma1_i were depolarized (78%, $p \leq 0.001$, Fig. 4c,d).

Accordingly, $trk1\Delta trk2\Delta$ yeast cells treated with 100nM AmB and either 0 or 15 μ M Pma1_i showed increases in $[K^+]_i$ (Fig. 4e) and cell growth (Fig. 4f) relative to non-AmB-treated $trk1\Delta trk2\Delta$ yeast controls. In contrast, $trk1\Delta trk2\Delta$ cells treated with 100nM AmB and 30 μ M Pma1_i showed no increase in $[K^+]_i$ nor cell growth (Fig. 4e and f, respectively). AmB-treated $trk1\Delta trk2\Delta$ yeast cells were also significantly more sensitive to this [Pma1_i] than their similarly AmB-treated WT counterparts. Such disproportionate effects on $[K^+]_i$ and cell growth in $trk1\Delta trk2\Delta$ and WT yeast were not observed when AmB-treated $trk1\Delta trk2\Delta$ and WT yeast were alternatively treated with doxorubicin, which inhibits yeast growth via a non-ion channel-related mechanism (DNA intercalation) (Fig. S4). These results are consistent with the conclusion that Pma1-generated membrane potential drives the AmB-mediated concentration of K^+ and restored cell growth in $trk1\Delta trk2\Delta$ yeast.

Figure 4. (a-c) Chemical inhibition with 30 μM Pma1_i caused a substantial increase in the percentage of depolarized cells (N=3), representative flow cytometry data. (d) The corresponding % depolarized cells was determined as the percentage of cells for which cells with FITC-A signal above 10^3 were characterized as depolarized (right of black line), over the total number of live singlet cells (N=3). (e) Pma1 inhibition caused a reduction in total cellular K⁺ content. (f) Pma1 inhibition caused a reduction in cell growth in $\text{trk1}\Delta\text{trk2}\Delta$ +100nM AmB relative to WT+100nM AmB cells. NS not significant, * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

DISCUSSION

Thus, small molecule ion channels can harness membrane potential to concentrate ions inside cells and thereby restore physiology *in vivo*. These data suggest that the cellular mechanisms for concentrating K⁺ inside cells are sufficiently robust to enable such functional complementation with an imperfect small molecule mimic of the Trk proteins. It is also notable that AmB channels are not selective for K⁺; they can also transport Na⁺, H⁺, Cl⁻, and HCO₃⁻.⁶ We speculate that other protein ion pumps and channels in the plasma membrane, such as Tok1, Nha1, and Ena1,¹⁸ may play a role in correcting for this imperfect ion selectivity, and this possibility will be interrogated in a future study. Collectively, these results further encourage the pursuit of small molecule surrogates for deficient protein ion channels that underlie a wide range of challenging to treat human diseases, including those that harness membrane potential to concentrate specific ions.

METHODS

Yeast Growth Conditions. Isogenic *Saccharomyces cerevisiae* strains BY4741(WT) and BYT12(*trk1Δtrk2Δ*) were maintained on 100mM KCl YPD agar plates at 4°C. Yeast were streaked every 1–3 weeks from frozen glycerol stocks. Overnight media was either 50 or 100mM KCl Yeast Nitrogen Base (YNB) pH 5.8 prepared by combining 4g (NH₄)₂SO₄, 1.63g Translucent K⁺ Free YNB (ForMedium, CYN7505), 1.285g BSM (ForMedium, DBSM225), and the corresponding amount of KCl/L. pH of media was adjusted to 5.7 with citric acid and ammonium hydroxide before autoclaving. Following, 50mL of sterile 40% dextrose was added per liter. The same formulation was followed for K⁺ limiting media (15mM K⁺ final), with 1.11g of KCl added per liter.

Determination of Relative Plasma Membrane Potential. This protocol was performed as described,¹² with the following modifications. WT and *trk1Δtrk2Δ* cells were grown overnight in 50mM KCl supplemented YNB media, pH 5.8. After 18h, cells were harvested, washed, and re-suspended in 15mM KCl supplemented YNB, pH 5.8. UV-VIS spectrophotometry was used to determine the absorbance at 600nm (OD₆₀₀). Cells were then diluted to an OD₆₀₀=0.2 and incubated for indicated times. After incubation, cells were diluted to an OD₆₀₀=0.2, if necessary. Each sample was pipetted into a 96-well black microplate. 3,3-dipropylthiacarbocyanine iodide (DiSC₃(3)) dye was added to the wells, with a final concentration=0.2μM. Samples were incubated for 30min., 200XRPM, 30°C and then a fluorescence spectra, excitation 531nm, emission 549-589nm, was taken on a BioTek Synergy H1 microplate reader. The ratio of 580/560 was recorded for each sample.

Flow Cytometry Analysis of Inhibition of Plasma Membrane H⁺-ATPase Pma1. Two to three colonies each of BY4741(WT) and BYT12(*trk1Δtrk2Δ*) were cultured overnight in 50mM KCl YNB pH 5.8, 200XRPM, 30°C. The following solutions were prepared before each experiment: 1mg/mL propidium iodide (PI) in H₂O (final concentration=0.0033mg/mL) and 1.7mg/mL bis-(1,3 – dibutylbarbituric acid) trimethine oxonol (DiBAC) in DMSO (final concentration=0.001mg/mL)¹⁹ and sonicated. The following media were prepared: media (15mM KCl YNB pH 5.8, H₂O, DMSO), PI media (15mM KCl YNB pH 5.8, PI stock, DMSO), DiBAC media (15mM KCl YNB pH 5.8, DiBAC stock, H₂O), and PI and DiBAC media (15mM KCl YNB pH 5.8, PI stock, and DiBAC stock). 35mL of cells were pelleted at 1000XG, 23°C, for 5min. The supernatant was removed, and cells were resuspended in the corresponding volume of sterile milli-Q H₂O. Then the cells were recentrifuged and washed. Afterwards, cells were resuspended in 15mM KCl YNB pH 5.8 to an OD₆₀₀=0.090-0.110.

Next, 2mL of cell suspension were combined with the corresponding media condition. 6μL of DMSO or Pma1 inhibitor (Pma1i)¹⁹ stock solution was added to give the final concentrations of 15 and 30μM. Samples were incubated for 40-60min., 200XRPM, 30°C, and then analyzed on a BD LSRII Flow Cytometry Analyzer. Quality control samples were run before each experiment. Just before analyzing the sample, 30μL of AccuCheck counting beads were added to 270μL of sample and vortexed briefly. 2000 bead events were collected per sample. Afterwards, data was processed with FCS Express 7 software. First, cells were gated to exclude counting beads. Then based on PI

staining intensity, dead cells were gated out (FCS-A vs. PI-A). The singlet cell population was analyzed, and cells with FITC-A signal above 10^3 were characterized as depolarized (to the right of black line, Fig. 4a-c). The same parameters were used to analyze data from three separate experiments.

Small Molecule Rescue Assay of Potassium Transporter Deficient Yeast. All experiments were conducted under sterile conditions. Overnight yeast cultures comprised of three to five single colonies were grown to saturation over 12-24h, 200XRPM, 30°C in 100mM KCl YNB pH 5.8. Afterwards 90mL of cell suspension was pelleted at 1000XG, 23°C, for 5min. Supernatant was removed, and cells were resuspended in 90mL of sterile milli-Q H₂O and vortexed. Cells were centrifuged and washed again. After removing the supernatant, cells were resuspended in 15mM KCl YNB, pH 5.8 and vortexed. Cells were diluted with 15mM KCl YNB media to OD₆₀₀=0.090-0.110. Natamycin, Amphotericin B, and Nystatin A1 fresh stock solutions were prepared in DMSO and concentrations were determined by UV-VIS. UV-VIS specifications in methanol include Natamycin: $\lambda_{\text{max}}=317\text{nm}$, $\epsilon=76,000\text{M}^{-1}\text{cm}^{-1}$; Amphotericin B: $\lambda_{\text{max}}=406\text{nm}$, $\epsilon=164,000\text{M}^{-1}\text{cm}^{-1}$; Nystatin A1: $\lambda_{\text{max}}=304\text{nm}$, $\epsilon=51,207\text{M}^{-1}\text{cm}^{-1}$. A 40-fold concentrated small molecule solution was prepared in DMSO for each treatment dose; and the small molecule solution was delivered as a 1:40 volume-to-volume ratio for small molecule to cell suspension volume. *Dose-response:* Per tested concentration, 100mL of cell suspension were treated with either DMSO control or small molecule. Cells were grown protected from light for 24h at 200XRPM, 30°C. *Time-course:* 500mL of cell suspension was treated with either DMSO or small molecule. Then 100mL of treated cell suspension was transferred into 250mL flasks designated for 6, 12, and 24h time points. The remaining 200mL per treatment group was designated for the 0h time point. Cells were cultured for each time point at 200XRPM, 30°C, protected from light.^{5, 12}

Chemical Blocking of Small Molecule Mediated Rescue. The *Small Molecule Rescue Assay* protocol was modified to investigate the effect of chemical inhibition on protein ion pump Pma1. A 5 μM AmB solution was prepared; the final ratio of AmB to cell suspension=1:50 and final ratio of chemical inhibitor to cell suspension=1:200. Doxorubicin and Pma1i stock solutions were prepared in DMSO to achieve desired concentrations of 10mM and 6mM respectively. Subsequent doses were made by serial dilution.

Cells were harvested, washed, and diluted in 15mM KCl YNB, pH 5.8 media to OD₆₀₀=0.090-0.110, as previously described. For DMSO controls, 2.5mL of DMSO were added to the respective 100mL each of WT+DMSO or trk1 Δ trk2 Δ +DMSO flasks. Separately, 500mL of WT and 500mL of trk1 Δ trk2 Δ cell suspensions were treated with 10mL each of 5 μM AmB to give a final concentration of 100nM AmB. Then 100mL of each cell suspension was measured and transferred into labeled 250mL flasks. 0.5mL of DMSO or chemical inhibitor was added to each flask. The order of sample preparation was varied to avoid batch effects. Samples were cultured at 200XRPM, 30°C, 12h.

Quantification of Total Cellular K⁺ Content via Inductively Coupled Plasma-Mass Spectrometry. At each time point, 1mL aliquots of each sample were utilized to determine OD₆₀₀ values by UV-VIS. Subsequently, samples were quickly filtered through

0.8µm membrane filters (EMD Millipore) and washed with ice-cold 20mM MgCl₂ (prepared with ultra-pure, molecule grade biology water). Filters were transferred into 6-well plates and 5mL of ice-cold 20mM MgCl₂ was added to each well. Cells were resuspended; the resulting cell suspension was transferred into conical tubes and flash frozen in liquid nitrogen. Samples were lyophilized for ≥72h. Next, under argon, samples were transferred into aluminum capsules and sealed. Samples were analyzed by inductively coupled plasma-mass spectrometry.¹⁶

Statistical Analysis. All statistical analyses were performed with GraphPad Prism software. Unpaired t-test with Gaussian distribution and one-way ANOVA with Dunnett's multiple comparisons test were used for quantifying statistical differences between sample groups. *Statistical designations: NS not significant, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P ≤ 0.0001.*

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge online.

Supporting data for transmembrane voltage and control studies.

AUTHOR INFORMATION

Corresponding Author

Martin D. Burke, email: mdburke@illinois.edu, 600 South Mathews Ave., Urbana, IL 61801

Notes

The University of Illinois has filed patent applications on small molecules that perform protein ion channel-like functions, and these have been licensed or optioned to Ambys Medicines and cystetic Medicines, companies for which M.D.B. is a founder and consultant.

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