Pyrophosphate Stimulates the Phosphate-Sodium Symporter of *Trypanosoma brucei* Acidocalcisomes and *Saccharomyces cerevisiae* Vacuoles

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**ABSTRACT** Inorganic pyrophosphate (PPi) is a by-product of biosynthetic reactions and has bioenergetic and regulatory roles in a variety of cells. Here we show that PPi and other pyrophosphate-containing compounds, including polyphosphate (polyP), can stimulate sodium-dependent depolarization of the membrane potential and Pi conductance in *Xenopus* oocytes expressing a *Saccharomyces cerevisiae* or *Trypanosoma brucei* Na+/Pi symporter. PPi is not taken up by *Xenopus* oocytes, and deletion of the TbPho91 SPX domain abolished its depolarizing effect. PPi generated outward currents in Na+/H1001/Pi-loaded giant vacuoles prepared from wild-type or pho91Δ yeast strains but not from the pho91Δ strains. Our results suggest that PPi, at physiological concentrations, can function as a signaling molecule releasing Pi from *S. cerevisiae* vacuoles and *T. brucei* acidocalcisomes.

**IMPORTANCE** Acidocalcisomes, first described in trypanosomes and known to be present in a variety of cells, have similarities with *S. cerevisiae* vacuoles in their structure and composition. Both organelles share a Na+/Pi symporter involved in Pi release to the cytosol, where it is needed for biosynthetic reactions. Here we show that PPi, at physiological cytosomal concentrations, stimulates the symporter expressed in either *Xenopus* oocytes or yeast vacuoles via its SPX domain, revealing a signaling role of this molecule.

**KEYWORDS** SPX domain, *Saccharomyces cerevisiae*, *Trypanosoma brucei*, *Xenopus laevis*, acidocalcisome, phosphate-sodium symporter, pyrophosphate
acidocalcisomes is removed, indicating that PPi is a component of this organelle’s structure. Besides the acidocalcisomal V-H\(^+\)/PPase, other enzymes of \textit{T. brucei}, such as a soluble pyrophosphatase (11–13), and the glycosomal pyruvate-phosphate dikinase (14) can use PPi.

Recent work has shown that a phosphate-sodium symporter from both \textit{T. brucei} acidocalcisomes (TbPho91) and \textit{Saccharomyces cerevisiae} vacuoles (Pho91p) is stimulated to release P\(_i\) and Na\(^+\) to the cytosol by the binding of inositol hexakisphosphate (IP\(_6\)) or diphosphoinositol pentakisphosphate (5-PP-IP\(_5\) or 5-IP\(_7\)) to their SPX domain (15). PPi, is formed by biosynthetic reactions, like the synthesis of deoxynucleotide triphosphates (dNTPs) that are needed for yeast DNA duplication (16) or for the biosynthesis of phospholipids and nucleotides needed for cell duplication (17). These reactions require an abundant source of P\(_i\). We therefore considered a potential signaling role of PPi in the export of vacuolar P\(_i\), heterologously expressed TbPho91, with or without its SPX domain, in \textit{Xenopus} oocytes was tested by the two-electrode voltage clamp method to measure transmembrane currents in the presence of PPi, and polyphosphates. We also prepared giant vacuoles of yeast expressing either wild-type or \textit{T. brucei} Na\(^+\)/P\(_i\) symporters and patch-clamped them. We report that PPi stimulates TbPho91 and Pho91p, leading to P\(_i\), and Na\(^+\) release to the cytosolic side of the vacuoles, and that the presence of an SPX domain in TbPho91 is important for this stimulation to occur.

RESULTS

Modulation of the Na\(^+\)/P\(_i\) conductance of TbPho91 by pyrophosphate and polyphosphates. TbPho91 localizes to acidocalcisomes (18), and these organelles are rich in PPi (6). Therefore, we examined whether this compound induced net inward currents when applied to \textit{Xenopus} oocytes expressing the symporter. Figure 1A shows the inward current generated at holding potential (\(V_h = -60\) mV) by the addition of equimolar concentrations of P\(_i\) or PPi. The current amplitude induced by PPi was a few hundred nanoamperes and was larger than that induced by P\(_i\) (Fig. 1A and B). One possible reason for the induction of these inward currents is the cotransport of Na\(^+\) and P\(_i\) through TbPho91. However, while there is Na\(^+\)-dependent uptake of \(^{32}\)P\(_i\), there is no significant Na\(^+\)-dependent \(^{32}\)PPi uptake into oocytes expressing TbPho91 (Fig. 1C). The results suggest that while PPi is not transported, Na\(^+\) transport, which generates an inward current, is stimulated by PPi. Interestingly, when PPi was added before P\(_i\), P\(_i\) induced larger current amplitudes than when added alone, indicating that PPi has a modulating effect on Na\(^+\) transport through TbPho91 (Fig. 1D).

PolyPs of different lengths induce inward currents in a pH- and calcium-dependent manner in oocytes expressing TbPho91 and PHO91. When polyPs of different lengths were used, similar inductions of inward currents were observed. PolyP\(_3\) (tripolyphosphate or TPP) induced currents of larger amplitude than polyP\(_{100}\) or polyP\(_{700}\) (Fig. 2A), and similar results were observed when \textit{S. cerevisiae} Na\(^+\)/P\(_i\) cotransporter (PHO91) was expressed in oocytes (Fig. 2B). However, when we used the same concentration of PPi, and polyP\(_3\) in phosphate units as with the longer polyPs, the amplitude changes were not significantly different (data not shown). Peak amplitudes of inward currents in oocytes expressing TbPho91 (in nanoamperes) were as follows: 250.1 ± 40.4 (\(n = 4\)) for PPi, 416.3 ± 47.4 (\(n = 4\)) for polyP\(_3\), 119 ± 69.6 (\(n = 5\)) for polyP\(_{100}\) and 333.5 ± 45.6 (\(n = 4\)) for polyP\(_{700}\) (Fig. 2A, right panel). In oocytes expressing yeast PHO91, the amplitudes of inward currents (in nanoamperes) were as follows: 447.8 ± 84.1 (\(n = 5\)) for PPi, 771.6 ± 168.4 (\(n = 5\)) for polyP\(_3\), 312.4 ± 50.8 (\(n = 5\)) for polyP\(_{100}\) and 142.8 ± 23.5 (\(n = 5\)) for polyP\(_{700}\) (Fig. 2B, right panel). The control amplitudes of Na\(^+\)/P\(_i\) currents in TbPho91- and PHO91-expressing oocytes were 157.1 ± 32.8 nA and 146.8 ± 41.6 nA (\(n = 4\)), respectively (Fig. 2A and B, right panels).

Similar to P\(_i\)-induced currents (15), polyP-induced currents also depended on extracellular pH (Fig. 2C). Acidification of the extracellular medium inhibited TbPho91 currents induced by the application of 10 mM polyP\(_3\). The amplitude of the Na\(^+\)/polyP transient was significantly lower at pH 6.8 (203.5 ± 12 nA, \(P < 0.0001\), \(n = 8\)) and pH 6.2
(56 ± 5.7 nA, P < 0.0001, n = 8) than at pH 7.0 (424.1 ± 65 nA). This effect was reversible in the course of tens of minutes in medium at neutral pH. However, a shift to more alkaline pH values (up to pH 7.8) did not produce significant changes in current (Fig. 2D). Figure 2E shows means ± standard errors of the means (SEM) of the results of three experiments.

Decreasing the extracellular calcium concentration ([Ca²⁺]₀) from 1.8 mM to 100 and 10 μM induced an increase in polyP₃ current from 426.4 ± 36.6 nA to 564.8 ± 41 nA (P < 0.05, n = 8) and 534.8 ± 69.6 nA (P < 0.05, n = 4), respectively (Fig. 2F and G). In addition, the kinetics of the polyP₃ current transient was also changed, showing a slow decay in restoration to the basal level, especially at 10 μM [Ca²⁺]₀ (Fig. 2F). Thus, the half-width of the current transient increased (in seconds) from 23.9 ± 2.3 at 1.8 [Ca²⁺]₀ to 46.8 ± 6.4 (P < 0.05, n = 4) and 73.7 ± 10.8 (P < 0.01, n = 4) at 100 μM and 10 μM [Ca²⁺]₀ respectively (Fig. 2H). An increase of [Ca²⁺]₀ above 3.0 mM led to oocyte death within minutes.

Taken together, the results suggest that PP₁ and polyPs might be modulating the opening of the Na⁺/P₁ cotransporter and facilitating Na⁺ transport and generation of the currents in a pH- and calcium-dependent manner.

**Modulation of the Na⁺/P₁ conductance of TbPho91 by pyrophosphate is dependent on the SPX domain.** It has been recognized that the SPX domains present in the N termini of vacuolar transporter chaperones, signaling proteins, and phosphate transporters can function as polyphosphate sensor domains (19). They bind to phosphate-containing ligands like PP₁, polyP₃, and IP₆ at micromolar levels and to 5-IP₇.
at nanomolar concentrations. Similarly, we found that IP6 and 5-IP7 stimulate the yeast and *T. brucei* 
Na$^+$/H$^+$/Pi symporter through its SPX domain (15). We therefore investigated 
whether this was also the case with PPi.

In addition to the ability of PPi to directly activate TbPho91, it can also modulate the 
Na$^+$/H$^+$/Pi current. When oocytes were preincubated for 5 to 6 min with PPi, in the 
micromolar range (Fig. 3A), there was an induction of slow inward currents, followed by 
amplification of the Na$^+$/H$^+$/Pi-transmembrane current evoked by 10mM Pi. The thresh-
olds for statistically significant amplification of the Na$^+$/H$^+$/Pi current were 100 
M for PPi (13.5% ± 0.87% higher than the reference value, $P < 0.05; n = 5$) (Fig. 3B) and 200 
M for polyP3 (15.7%, $P < 0.05; n = 4$) (Fig. 3C and D).

To examine the role of the SPX domain of TbPho91 in this stimulation by PPi, we 
expressed the protein with a deletion of this domain (TbPho91-ΔSPX) (15) and mea-
sured its response to PPi. When *TbPho91*-ΔSPX-expressing oocytes were tested, no 
amplification of the currents induced by 10mM Pi occurred by the addition of PPi 
(Fig. 3B), which confirms previous findings on the role of the SPX domain in regulating 
Pho91p conductance.

**FIG 2** Currents elicited by PPi and polyPs in oocytes expressing TbPho91 and Pho91p. (A) Representative 
currents recorded after the addition of 10mM Na$^+$/PPi, Na$^+$/polyP$_5$, Na$^+$/polyP$_{100}$ and Na$^+$/polyP$_{700}$ to 
oocytes expressing TbPho91. The right panel shows the quantification of currents elicited from four 
experiments. (B) Representative currents recorded after the addition of 10mM Na$^+$/PPi, Na$^+$/polyP$_5$, 
Na$^+$/polyP$_{100}$ and Na$^+$/polyP$_{700}$ to oocytes expressing Pho91p. The right panel shows the quantification 
of currents elicited from four experiments. (C to E) Currents recorded in response to the addition of 
10mM Na$^+$/polyP$_5$ at different pH levels (C and D) and quantification of the results of three experiments 
(E). (F to H) Currents recorded in response to the addition of 10mM Na$^+$/polyP$_5$ at different Ca$^{2+}$ 
concentrations (F) and quantification of the current intensity (G) or current duration (H) of several 
experiments. Values in panels E, G, and H are means ± SEM; $n = 4$. *, $P < 0.05; **, $P < 0.01; ***$, $P < 0.001; 
n.s., not significant (Student’s *t* test). Concentrations of Na$^+$/polyP$_{100}$ and Na$^+$/polyP$_{700}$ are expressed in phosphate units.
Stimulation of Na\(^+/\)P\(_i\) release by PP\(_i\) from yeast vacuoles. We applied the spheroplast incubation method to prepare giant cells of *S. cerevisiae* by using 2-deoxyglucose to inhibit cell wall synthesis (20). The giant cells were treated by moderate hypsomotic shock to disrupt the plasma membrane and release the enlarged vacuoles. A patch pipette was then attached to the vacuolar membrane, and after formation of a gigaseal, the patch membrane was ruptured by high-voltage pulses. The lumen of the vacuole was connected to the pipette (whole-vacuole configuration) and was loaded with a solution containing Na\(^+/\)/H\(_{11001}\)/P\(_i\), to record transmembrane currents. We used *pho91* cells to express TbPHO91.

Patch-clamp recordings of the vacuoles were performed at a *V_h* of +60 mV. The bath solution had 10 mM HEPES, pH 7.1, containing 100 mM NaCl, 200 mM sorbitol, and 1 mM MgCl\(_2\), while the pipette solution contained a similar solution plus 10 mM Na\(_2\)HPO\(_4\)-Na\(_2\)HPO\(_4\) in order to detect outward currents generated by displacement of Na\(^+/\)/P\(_i\) to the bath solution (“cytosol”). After 10 mM PP\(_i\), was added to the bath solution (Fig. 4A), we registered outward currents of 60.3 ± 12.7 pA (n = 3) in vacuoles from wild-type cells. When vacuoles from *pho91Δ* cells were used, no significant currents were detected after PP\(_i\) application (Fig. 4B).

We then expressed TbPHO91 in giant vacuoles of *pho91Δ* cells. Application of 10 mM PP\(_i\) induced outward currents of 22.3 ± 3.7 pA (n = 3) (Fig. 4C and D). Our results demonstrate that PP\(_i\) triggers the release of Na\(^+/\)/P\(_i\) by the Pho91 symporters.

DISCUSSION

We report here that functional expression in *Xenopus laevis* oocytes of *T. brucei* or *S. cerevisiae* Na\(^+/\)/P\(_i\) symporter Pho91, followed by two-electrode voltage clamp recordings, showed that the application of PP\(_i\), or polyP resulted in the depolarization of the oocyte membrane potential and an increase in the P\(_i\) conductance. The stimulation
induced by PPi was abolished when the SPX domain of the symporter was deleted. Application of PPi to yeast giant vacuoles expressing TbPho91 or Pho91p but not to vacuoles of pho91/H9004 cells induced outward currents, suggesting a role of PPi in Na+/H+/Pi release. Pyrophosphate does not penetrate Xenopus oocytes, but it stimulates the Pho91 transporters that are expressed in them. If this happens through the SPX domain, the domain would have to be oriented toward the exterior of the oocyte. Plasma membrane orientation is essentially demonstrated by positive functionality. The best evidence that the topology of Pho91 and TbPho91 in Xenopus oocytes is inverted is that Na+ and Pi are transported into the oocytes, as demonstrated by electrophysiological recordings and 32Pi uptake experiments. This does not occur in the giant vacuoles, where we detected Pi release to the cytosolic side of the vacuole. The currents detected are due to the electrogenic nature of the transporter (Na+ is the charge carrier, and Pi without Na+ does not elicit currents [15]). The transfer of Na+ to the cytosol is favored by the higher Na+ concentration in the extracellular medium. In contrast, acidocalcisomes and yeast vacuoles have more Na+ than the cytosol and Na+ efflux is favored.

**FIG 4** PPi induces activation of Na+/Pi currents in Pho91- and TbPho91-expressing yeast vacuoles. (A) Activation of Na+/Pi outward currents in vacuoles from wild-type yeast after the addition of 10 mM PPi. (B) pho91Δ vacuoles do not produce currents after application of PPi. (C) Complementation of pho91Δ with TbPHO91 restores vacuole response to PPi. Data are representative of two to four independent experiments and are quantified in panel D. We used at least four successful current recordings for each experiment. About 80% of the vacuoles showed clear responses. WT, wild type; KO, knockout.
This inversion of the membrane topology in the plasma membrane of *Xenopus* oocytes indicates that the amino-terminal region containing the SPX domain is also inverted and oriented toward the outside, as demonstrated by the experiments with expression of truncated TbPho91. This is also in agreement with structural data available for other P<sub>i</sub> transporters (21) that showed that there is no reorientation of the carrier alternatively exposing the substrate binding sites to one or the other side of the membrane, as previously postulated (22, 23), but movement of ions within the transmembrane field. It is known that lipid composition can affect topology of a membrane protein, or orientation of its α-helices in a membrane, which underlies membrane protein function. Inversion of the membrane topology of vacuolar transporters expressed in the plasma membrane of *Xenopus* oocytes is not infrequent (24).

Our results concerning the role of the SPX domain in the yeast Pho91p is at variance with its role in the plasma membrane low-affinity P<sub>i</sub> transporters Pho87p and Pho90p (25). When the SPX domain was removed to generate a truncated form of Pho90p, there was increased accumulation of phosphate, which was proposed as evidence that SPX is a regulatory domain that inhibits phosphate transport under normal conditions (25). However, the SPX removal experiments did not provide mechanistic evidence on how SPX regulates the transporters. Electrophysiological characterization of Pho90p and Pho87p could reveal whether polyphosphate-containing molecules (or the Slp2 protein), acting on the SPX domain, regulate phosphate uptake by these low-affinity transporters.

It was shown before that 1 mM PPi “primes” (26) or stimulates the catalytic domain of the polyP polymerase vacuolar transporter chaperone 4 (VTC4) of *S. cerevisiae* but inhibits the catalytic domain of *T. brucei* VTC4 (27). PP<sub>i</sub> and polyP<sub>3</sub> also bind to the SPX domain of *S. cerevisiae* VTC2, as determined by isothermal titration calorimetry, with dissociation constants (K<sub>d</sub>) of 154 ± 62 and 11.1 ± 1.7 μM, respectively, but PP<sub>i</sub>, does not significantly stimulate VTC-catalyzed polyP synthesis by isolated yeast vacuoles at millimolar concentrations (26). We found that the threshold for PP<sub>i</sub> for statistically significant amplification of the Na<sup>+</sup>/P<sub>i</sub> current in *Xenopus* oocytes expressing TbPho91 was 100 μM, which is within the physiological levels of cytosolic PP<sub>i</sub> in several cell types. For example, the PP<sub>i</sub> concentration in the cytosol of plant cells is about 0.2 to 0.3 mM (28), while in exponentially growing *Escherichia coli* K-12 cells, the intracellular PP<sub>i</sub> concentration is about 0.5 mM, even after varying the amount of pyrophosphatase from 15 to 2,600% of the control amount (29). In addition, the PP<sub>i</sub> content in *E. coli* can be increased up to 2.5 mM when the growth of cells is limited by inhibition of the synthesis of nucleotides (30). The concentration of PP<sub>i</sub> in different species has been reviewed extensively, and, for example, it has been estimated to be at about 100 to 200 μM in rat liver (3).

Although polyP<sub>3</sub> and other polyPs are able to induce inward currents in *Xenopus* oocytes expressing TbPho91, their physiological relevance is relative, as most of these compounds are compartmentalized in the acidocalcisomes (6, 7), nucleolus, and glycosomes (31). In this regard, it has been demonstrated the polyP is toxic when in the yeast cytosol (32). We do not think that polyPs could have a physiological role, and we attribute their stimulatory effect to their chemical similarities to PP<sub>i</sub>.

In conclusion, our work revealed that PP<sub>i</sub> stimulates the Na<sup>+</sup>/P<sub>i</sub> symporter of *T. brucei* acidocalcisomes, and that of its yeast ortholog localized in the vacuole, through its SPX domain. This stimulation results in the release of P<sub>i</sub> and Na<sup>+</sup> to the cytosolic side of the vacuoles. Our hypothesis is that as result of enhanced PP<sub>i</sub> production by anabolic reactions, the increase in PP<sub>i</sub> would stimulate the P<sub>i</sub> release needed for these anabolic reactions (Fig. 5). The results reveal an unrecognized role of PP<sub>i</sub> in cell signaling.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Integrated DNA Technologies (Coralville, IA) provided the primers used. All other reagents of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell cultures.** *T. brucei* (Lister 427 strain procyclic forms [PCF]) were grown at 28°C in SDM-79 medium (33) with 10% heat-inactivated fetal bovine serum and hemin (7.5 μg/ml).
FIG 5 Schematic representation of PP, signaling function. Large amounts of P, are needed for biosynthetic pathways, which generate PP, as a by-product. PP, stimulates the vacuolar Pho91 Na\(^+\)/P, symporters through their SPX domains, increasing the release of P, needed for ATP biosynthesis.

Yeast strains. We used S. cerevisiae strain BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0). Generation of pho91Δ was as described previously (15).

Preparation and isolation of giant yeast vacuoles. Preparation of giant yeast vacuoles from the wild type and pho91Δ mutants was done as described before (20), with minor modifications (15). The vacuoles were attached to a poly-l-lysine-coated chamber for patch-clamp recording. The micropipette solution contained 10 mM HEPES, pH 7.1, 100 mM NaCl, 200 mM sorbitol, 1 mM MgCl\(_2\), 5 mM NaH\(_2\)PO\(_4\) and 5 mM Na\(_2\)HPO\(_4\). The bath solution was similar, but without NaH\(_2\)PO\(_4\) and Na\(_2\)HPO\(_4\).

Preparation and maintenance of oocytes. Xenopus laevis oocytes were obtained from Xenoocyte (Dexter, MI). Oocytes collected at stage IV or V were manually defolliculated and devitellinized with collagenase (1 mg/ml) for 1 h at room temperature and then maintained in filtered modified Barth’s solution containing 88 mM NaCl, 1 mM KCl, 0.82 mM MgSO\(_4\), 0.41 mM CaCl\(_2\), 2.4 mM NaHCO\(_3\), 0.33 mM Ca(NO\(_3\))\(_2\), and 10 mM HEPES, plus 50 μg/ml gentamicin, pH 7.4, at a density of less than 100 per 60-mm plastic petri dish. Barth’s solution was replaced daily.

cRNA production, oocyte injection, and electrophysiology. PrimeSTAR HS DNA polymerase (Clontech) was used to amplify by PCR full-length TbPHO91 (Tb427tmp.01.2950), truncated TbPHO91 (TbPHO91-SPX) by removal of the 606-nucleotide sequence encoding the N-terminal putative SPX domain, and PHO91 (GenBank accession number NM_001183190) open reading frames (15) from T. brucei or S. cerevisiae genomic DNA, using the corresponding gene-specific primers indicated in Table 1. The PCR products were purified as described previously (15), and the nucleotide sequences were confirmed by sequencing. cRNAs were obtained by in vitro transcription using the purified PCR products as the templates with an mMESSAGE mMACHINE kit (Ambion Life Technologies, Thermo Fisher Scientific, Inc., Waltham, MA), in accordance with the manufacturer’s protocol, and verified as described previously (15). cRNA injection was done exactly as described before (15). Equal amounts of cRNA from control and mutant transporters were injected into the Xenopus oocytes. For electrophysiology, the standard two-electrode voltage-clamp technique was used, as described previously (15). At least four oocytes from two different frogs were used in each experiment. All recordings were obtained at room temperature. Oocytes were bathed in ND96 buffer solution containing 96 mM NaCl, 2 mM KCl, 5 mM MgSO\(_4\), 1mM Ca\(^{2+}\), and 2.5 mM HEPES, pH 7.5, with a continuous perfusion speed of ~2 ml/min. Low-calcium solutions were prepared by adding Ca\(^{2+}\) and EGTA at proportions calculated with MaxChelator software (Stanford University, CA). The required pH of ND96 was adjusted either with NaOH or HCl. The effect of PP, and polyphosphates was studied by their addition to ND96 with subsequent pH readjustments. To

| Primer sequencea | Useb |
|-----------------|------|
| AGGAAAAAATGCCGCTCAAAATCT | Knockout of yeast PHO91 |
| CAATACAAATGGGCAATGGCA | Knockout of yeast PHO91 |
| TTGGTACCGGCCCTTCCTCGAGGTGGCTTATCTCCGCTTATAT | Amplification of PHO91 for cloning in pRS413 |
| GATATCCCCGCGCTGAGAATTTGAAGTTCTGACATATCGAGAATGCA | Amplification of PHO91 for cloning in pRS413 |
| GACACCTAATACCTGACATGACATGAAGTTCTGACATATCGAGAATGCA | Amplification of TbPHO91 for fusing with PHO91 UTRs and cloning in pRS413 |
| TTTTTCATCTCTCATTGATAATCTCTACCTGAGTTGCTTATAAAAATCATCTATGTTTGCTTTTCAAACAC | Amplification of TbPHO91 for fusing with PHO91 UTRs and cloning in pRS413 |
| TTGGTACCG GCCCTTCCTCGAGGTGGCTTATCTCCGCTT | Amplification of PHO91 5’ UTR for fusing with TbPHO91 |
| GTCATGCTCAAGGTTACCGTGCACTCCACACATGTTCTTCTTTTGGT | Amplification of PHO91 5’ UTR for fusing with TbPHO91 |
| GATTATCCCATAGAGAAGAAAGTTCTAATAATAGATGTTGAGCT | Amplification of PHO91 3’ UTR for fusing with TbPHO91 |
| GGATTCCTGGCGCCTGAGAATTTGAAGTTCTGACATATCGAGAATGCA | Amplification of PHO91 3’ UTR for fusing with TbPHO91 |
| CCCGCCGAAATTAAAAATACCTACTATAGAGAAGACCCCAACTGAAATTGCGGATTGAGAACGCA | Amplification of PHO91 17TF (for Xenopus expression) |
| TGGACCAAGACACACATCTAGAGAAGACCCCAACTGAAATTGCGGATTGAGAACGCA | TbPHO91T17F (for Xenopus expression) |
| TGGACCAAGACACACATCTAGAGAAGACCCCAACTGAAATTGCGGATTGAGAACGCA | PHO9117TF (for Xenopus expression) |
| TGGACCAAGACACACATCTAGAGAAGACCCCAACTGAAATTGCGGATTGAGAACGCA | PHO9117T30R (for Xenopus expression) |

aFor the last five primers, T7 promoter or poly(T15) sequences are underlined. Kozak consensus sequences for increasing efficiency of translation initiation are in bold. Gene-specific sequences are italicized. Additional nucleotides upstream of the T7 promoter or the Kozak consensus sequence are incorporated into the primers for desirable in vitro transcription/translation in Xenopus laevis oocytes.

bUTR, untranslated region.

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prepare the phosphate solution, 300 mM stock solutions of mono- and dibasic sodium phosphates were mixed until pH 7.4 was obtained.

Yeast giant vacuole experiments were done exactly as described previously (15). All recordings were performed at a V_h of +60 mV. An Axopatch 200b amplifier was used for current registration, and data were filtered at 1,000 Hz, digitized with Digidata 1550A (Axon Instruments, USA), and analyzed offline using PClamp 10 software.

**2^P and 32^P uptake assays.** *Xenopus laevis* oocytes were injected with cRNA as described above and used after 3 days. Oocytes were incubated in standard ND96 solution or a modified ND96 solution with sodium replaced by an equimolar concentration of potassium or NMDG (ND96–Na). The healthiest looking oocytes were transferred to Eppendorf tubes (6 per tube) and incubated with 200 μL of ND96 or ND96–Na solutions containing 300,000 cpm of inorganic 2^P (60 Ci/mmol) or 32^P-labeled phosphate (60 Ci/mmol) (Perkin Elmer). Oocytes were then incubated for 30 min at room temperature and washed five times with 1 ml of ND96–Na. Prolonged incubation of oocytes under these conditions decreased the oocyte quality, probably due to strong and long-lasting depolarization of the cellular membrane. Oocytes were then lysed with 10% sodium dodecyl sulfate (SDS), and the total lysate was added to the scintillation cocktail (MP Biomedicals). 2^P radiation was measured using an LS 6500 multipurpose scintillation counter (Beckman Coulter). Each of three experiments was done using triplicate measurements.

**Statistical analysis.** All values are expressed as means ± SEM, unless indicated otherwise. Significant differences between treatments were compared using unpaired Student’s t tests. Differences were considered statistically significant at a P of <0.05, and n refers to the number of independent biological experiments performed. All statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

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