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ARTICLE

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Heterologously expressed fungal transient receptor potential channels retain mechanosensitivity in vitro and osmotic response in vivo

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Abstract The budding yeast *Saccharomyces cerevisiae* has a mechanosensitive channel, TrpY1, a member of the Trp superfamily of channels associated with various sensations. Upon a hyperosmotic shift, a yeast cell releases Ca^{2+} from the vacuole to the cytoplasm through this channel. The *TRPY1* gene has orthologs in other fungal genomes, including *TRPY2* of *Kluyveromyces lactis* and *TRPY3* of *Candida albicans*. We subcloned *TRPY2* and *TRPY3* and expressed them in the vacuole of *S. cerevisiae* deleted of *TRPY1*. The osmotically induced Ca^{2+} transient was restored in vivo as reported by transgenic aequorin. Patch-clamp examination showed that the TrpY2 or the TrpY3 channel was similar to TrpY1 in unitary conductance, rectification properties, Ca^{2+} sensitivity, and mechanosensitivity. The retention of mechanosensitivity of transient receptor potential channels in a foreign setting, shown here both in vitro and in vivo, implies that these mechanosensitive channels, like voltage-gated or ligand-gated channels, do not discriminate their settings. We discuss various mechanisms, including the possibility that stress from the lipid bilayer by osmotic force transmits forces to the transmembrane domains of these channels.

Keywords Mechanosensitive channel · Lipid forces · Osmolarity · Yeast vacuole · *Candida albicans* · Luminometry · Ca^{2+} release

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Abbreviations MS: Mechanosensitive · PCR: Polymerase chain reaction · TRP: Transient receptor potential

Introduction

The protein-free lipid bilayer is permeable to the solvent (water) but not to most of the solutes. This semipermeable nature of the bilayer enables it to partition different solutions and sustain osmotic pressure. With water being essential, it seems reasonable for molecular mechanisms to have evolved to gauge the osmotic pressure as a measure of dehydration or overhydration. Indeed, all extant organisms measure and respond to the water contents of their environments, as evident from bacterial osmoregulation (Wood et al. 2001) to regulated volume increase or decrease of mammalian cells (Lang et al. 1998). This measurement has been shown to be made by membrane proteins such as mechanosensitive (MS) channel proteins that gauge the bilayer tension created by the osmotic pressure (Levina et al. 1999, Martinac et al. 1987).

In *Escherichia coli*, overhydration activates MS channels MscL and MscS (Matinac 2001, Levina et al. 1999, Sukharev and Corey 2004, Sukharev et al. 1997). These channel genes and proteins have been cloned, purified, reconstituted into artificial bilayers of known lipid compositions and shown to retain their mechanosensitivity (Sukharev et al. 1997). Their structures at atomic resolutions have been solved through X-ray crystallography (Bass et al. 2003, Chang et al. 1998). They have also been further examined by electron paramagnetic spectroscopy (Perozo and coworkers 2002a, 2002b), computational modeling (Betanzos et al. 2002), molecular dynamic simulation (Colombo et al. 2003) and forward (Ou et al. 1998, Maurer and Dougherty 2003) and reverse genetics (Barlett et al. 2004) as reviewed in this symposium and elsewhere

(Martinac 2004, Sukharev and Anishkin 2004). Some models of mechanosensitivity focus on the channel lipid interface, where the local tension and pressure internal to the bilayer impinge on the MS channel protein. As the bilayer thins and bends by the osmotic or other physical forces, the internal force profile acting on the channel protein is changed and the protein may migrate to an energetically favored different conformation (e.g., open) (Perozo et al. 2002b). Consistent with this force-from-lipid model are lines of evidence from research on mammalian MS channels, such as the two-pore-domain K^+ channel of Patel and coworkers (1998, 1999, 2001) and the Ca^{2+} channels in astrocytes (Suchyna et al. 2004). Alternative models depict the MS channels being held by elements such as cytoskeletons or extracellular matrices, through which the gating force is delivered (Goodman and Schwarz 2003, Sukharev and Corey 2004).

Transient receptor potential (TRP) channels, discovered through the analysis of a near-blind *Drosophila* mutant, are a rapidly growing superfamily whose members include the well-known receptors of heat, cold, pressure and pain (Clapham 2003). Several channels belonging to different TRP subfamilies have been found in the last 4 years to be associated with mechanosensation. They include Osm-9 (osmotaxis 9 of *Caenorhabditis elegans*) (Tobin et al. 2002), TRPV4 (VR-OAC) (Liedtke and coworkers 2000, 2003, Liedtke and Friedman 2003), Nan (*Nanchung* of *Drosophila* hearing) (Kim et al. 2003), PKD-1, PKD-2 (polycystic kidney disease) (Nauli et al. 2003), and NOMPC (no mechanoreceptor potential C of *Drosophila* touch receptor) (Walker et al. 2000, Sidi et al. 2003). In these instances, mutations or deletions of the *TRP* genes lead to the loss of osmosensing, fluid-motion detection, touch, hearing or their physiological correlates in vivo. Direct demonstration of the mechanosensitivity of these Trp channel proteins in vitro remains difficult. *TRPV4*-expressing cultured mammalian cells show hypotonically induced steady-state currents and Ca^{2+} entry. Under patch clamp, the unitary conductance corresponding to TRPV4 has been reported (Liedtke et al. 2000, Strotmann et al. 2000). In one case, cell-attached patches showed hypotonically induced unitary current, but such current could not be induced by direct pressure on excised patches (Strotmann et al. 2000).

In the genome of the budding yeast, *Saccharomyces cerevisiae*, we found a single gene, *TRPY1* (formerly *YVC1*) that encodes a protein homologous to the animal TRP channels. Deleting this gene removes a conductance of approximately 400 pS in the vacuolar membrane (Palmer et al. 2001). This TrpY1 channel is unique among the TRP channels in being sensitive to mechanical forces directly under patch clamp (Zhou et al. 2003). Aequorin acting as a transgenic Ca^{2+} reporter indicates a rise of internal Ca^{2+} immediately after a hyperosomotic challenge, and this pulse of Ca^{2+} is entirely absent in *trpy1*Δ mutants (Denis and Cyert 2002). We postulated that osmotic perturbations mechanically activate TrpY1, thereby releasing Ca^{2+} into the cytoplasm to initiate downstream osmotic defense(s). Thus, the TrpY1 protein appears to function as the detector and the first responder to the change in osmotic pressure in vitro and in vivo.

The budding yeast *S. cerevisiae* has become the most powerful model for the cell biology of eukaryotes. To add to the prowess of this research system, the genomes of other fungi have been sequenced for the purpose of evolutionary comparison (Cliften et al. 2003, Dujon et al. 2004, Kellis et al. 2003). These genomes reveal *TRPY1* homologs in various fungi (Fig. 1). We have begun examining these homologs, in part, to see whether and how compatible they are with the vacuolar membrane of *S. cerevisiae*.

Materials and methods

Plasmids, yeast strains and media

Yeast strains of W303 and BY4742 backgrounds were used throughout. *TRPY1* knockout strains were either purchased from Invitrogen/Resgen (Carlsbad, CA, USA) or created using a standard deletion protocol (Ausubel et al. 2003). Cloning plasmids and yeast expression plasmids, such as p416 GALL (inducible but weakened GAL1 promoter, ATCC, MD, USA), p415 GDP (constitutive GDP promoter, ATCC, MD, USA), pCM190 (constitutive *CYC1* prometer, ATCC, MD, USA), pBluescript (Stratagene, La Jolla, CA, USA) and their derivatives, were used. The yeast and bacterial culture media were the same as those described elsewhere (Palmer et al. 2001, Ausubel et al. 2003, Zhou et al. 2003).

Cloning of *TrpY1* homologs

TRPY2 gene from *Kluyveromyces lactis* in a ClaI-EcoRI fragment of its genomic DNA was first cloned into pBluescript II KS. Its open reading frame alone was then amplified by polymerase chain reaction (PCR) using oligonucleotides with appropriate restriction sites for cloning in the yeast expression plasmids. *TRPY3* gene from *Candida albicans* (SC5314 strain from ATCC, MD, USA) was amplified by PCR using oligonucleotides CGGGATCCATGCCTGAACTTGATTTGGA and CCGCTCGAGCTAAGCTTTATTTTCAAGTTTC as primers. Upon sequencing, differences from the genome-project sequence were found at T543C, TG954CA and C992T. Because of the diploidy of the *C. albicans* genome, we also recovered a second PCR fragment of *TRPY3*, with which all except for C4T substitutions were accounted for in the genome-project sequence (Jones et al. 2004). Because of the unusual condon usage *Candida*, the former *TRPY3* gene was mutated with the standard PCR mutagenic protocol at CTG1780TCT to express Ser in *S. cerevisiae* and then cloned in yeast expression plasmids with additional appropriate

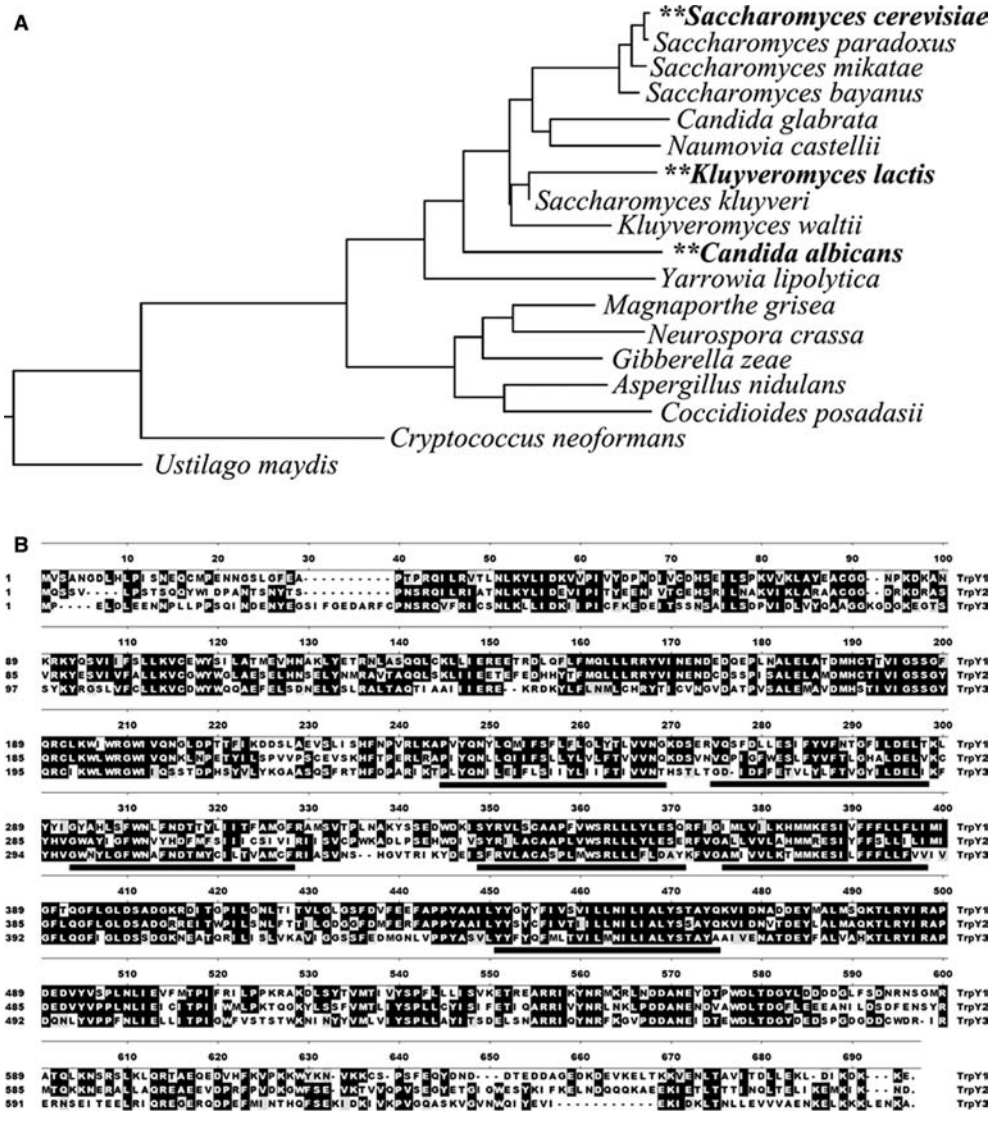

Fig. 1 Members of the fungal Trp-channel protein family. **a** A dendrogram showing the relatedness in amino acid sequence of the 18 TrpY members generated using the MegAligh program from the DNAStar suite (DNAStar, Madison, WI, USA). The remotely related homologs are from the basidiomycetes *Cryptococcus neoformans* and *Ustilago maydis*. The remainders are all from ascomycetes, including TrpY1 from *Saccharomyces cerevisiae*, TrpY2 from *Kluyveromyces lactis* and TrpY3 from *Candida albicans* (*bold* with *asterisks*). **b** Amino acid sequence comparison among TrpY1, TrpY2 and TrpY3. *Black boxes* indicate identity; *gray boxes* indicate similarity; *no boxes* indicate dissimilarity. The six putative transmembrane domains are *underlined* sequentially with *black bars*. Note that the transmembrane domains and their vicinities are highly conserved, although conservations are also seen elsewhere

restriction sites. *S. cerevisiae* with *TRPY1* deletion was transformed with the yeast expression plasmids as described earlier in this work (Palmer et al. 2001, Ausubel et al. 2003, Zhou et al. 2003).

Luminometry

The protocol for the luminometric measurement of the Ca^{2+} response through TrpY1p was as described previously (Denis and Cyert 2002). Briefly, a dense overnight culture of yeast cells carrying TrpY plasmids and also aequorin plasmids (PEVP11/AEQ) was subcultured by 1/10 dilution into an appropriate medium containing 1–2 μM coelenterazine as well as inducers for TrpY expression when necessary. After overnight expression of TrpY channels, 50–100 μl of cell culture was placed in a plastic tube, which was then placed in a Berthold luminometer (Berthold, Bad Wildbad, Germany). Luminescence was measured continuously for 3 min. At 30 s into the measurement, an equal volume of fresh culture medium containing 3 M sorbitol was manually added to the cells. For the control, an equal volume of fresh medium without sorbitol was used.

Patch clamp

The methods of yeast-cell subculturing, cell-wall digestion, spheroplast formation, vacuole liberation, seal formation, establishment of the whole-vacuole recoding mode, recording conditions, signal filtration, and digitized data analyses were as described previously (Palmer et al. 2001). The membrane potential was referred to that of the cytoplasmic side with reference to the vacuolar side. Unless otherwise stated, the patch-clamp pipets were filled with 180 mM KCl, 0.1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (Hepes), pH 7.2, and the bath contained 100 mM K_3 citrate, 20 mM $MgCl_2$, 5 mM Hepes, pH 7.2. All solutions contained 2 mM dithiothreitol.

Results

TRPY1 orthologs

We searched the partially or fully sequenced fungal genomes for *TRPY1* homologs and found one each in 18 fungal genomes reported in the public domain. They span the two major fungal divisions: the ascomycetes (yeasts, molds, etc.) and the basidiomycetes (mushrooms, etc.), including free-living and parasitic species. Figure 1a summarizes the relatedness of these *TRPY1* orthologs. We chose to study *TRPY2* of *K. lactis*, a free-living haploid aerobic yeast, and *TRPY3* of *C. albicans*, the infectious diploid yeast. The conceptually translated TrpY2 protein is 56% identical to TrpY1, and TrpY3 is 45% identical to TrpY1 (Fig. 1b) in terms of amino acid sequence.

TRPY2 and *TRPY3* can be expressed in the vacuolar membrane of *S. cerevisiae*

We have previously shown that the MS 400-pS cation conductance is completely removed from strains of *S. cerevisiae* in which the chromosomal copy of the *TRPY1* gene has been knocked out. Refurnishing *TRPY1* from a plasmid restores this conductance completely (Palmer et al. 2001). We employed the knockout strain, *trpy1*Δ, to test the heterologously expression of *TRPY2* and *TRPY3* as diagramed in Fig. 2. The results of expressing the two fungal homologous transgenes are similar. Results mostly from *TRPY3* of *C. albicans* will be used to illustrate the findings, since it is more distantly related to *TRPY1* of *S. cerevisiae* than *TRPY2* of *K. lactis* is.

The vacuolar membrane of the *trpy1*Δ cell of *S. cerevisiae* exhibited no MS conductance in numerous independent trials ($n > 50$) (Palmer et al. 2001, Zhou et al. 2003, Fig. 6, left). Both *TRPY2* and *TRPY3* transgenes restored a cation-permeable conductance in the vacuolar membrane of *trpy1*Δ of *S. cerevisiae*. Although the level of expression varies from trial to trial, as gauged by the largest number of conducting units that can be elicited in the whole vacuole mode, the characteristics of the unitary conductance do not change. These conductances are remarkably similar to those of TrpY1. The *TRPY3* transgene supports a conductance, approximately 380 pS (a typical case shown in Fig. 3a, b), not significantly different from that of TrpY1 (400 pS, in the recording conditions used here) (Palmer et al. 2001, Zhou et al. 2003).

Inward rectification

TrpY1 is an inward rectifier, opening more frequently when the cytoplasm is more electronegative than the interior of the vacuole (Bertl and Slayman 1990, Wada et al. 1987, Palmer et al. 2001). The same property was registered for TrpY3. The steady-state activity of TrpY3 is given in Fig. 3c. The activation from 0 mV by negative voltages (cytoplasmic negative) (Fig. 4a) and the deactivation from steady-state activation by positive voltages (Fig. 4b) of TrpY3 were observed. Both processes are slow, with time constants on the order of seconds, similar to those known for TrpY1 (Bertl and Slayman 1990, Palmer et al. 2001).

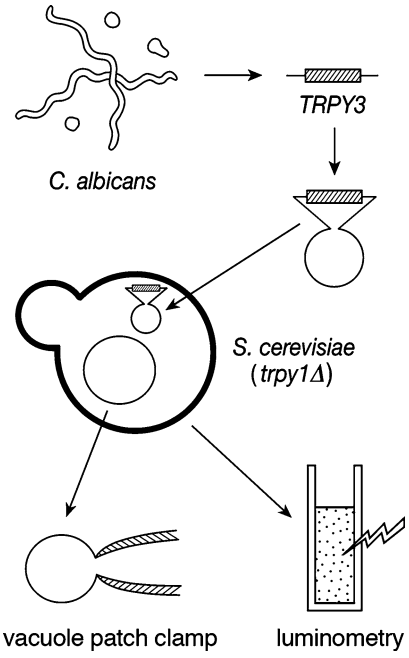

Fig. 2 Summary of the experimental strategy. A fungal *TRPY* homolog is retrieved and subcloned into an inducible plasmid. A budding yeast strain deleted of its own *TRPY1* gene is used as the host. Plasmid transformants are subcultured and examined in vivo through luminometry for hypertonically induced Ca^{2+} release from the vacuoles. Vacuoles of the transformants are examined individually under patch clamp for TrpY-channel activity. Depicted are procedures for *TRPY2* from *C. albicans*. Identical procedures were carried with *TRPY3* from *K. lactis*. See "Materials and methods" for detail

Activation by cytoplasmic Ca^{2+}

TrpY1 can be activated by cytoplasmic Ca^{2+} and seems to serve as a Ca^{2+}-induced Ca^{2+}-release (CICR) pathway that amplifies the flow of Ca^{2+} from the vacuole into the cytoplasm upon stimulation (Bertl and Slayman 1990, Wada et al. 1987, Palmer et al. 2001, Zhou et al. 2003). The heterologously expressed TrpY3 also has this property. Figure 5 shows that the addition of millimolar free Ca^{2+} greatly increases the open probability of TrpY3. Figure 5 also illustrates that large numbers of heterologous conducting units can be expressed in a foreign vacuole. In some cases, more than 100 unitary conductances could be discerned. That TrpY3, like the native TrpY1, can be activated by Ca^{2+} implies that either the Ca^{2+}-binding domain is in the TrpY protein itself or a partner Ca^{2+} binder from *S. cerevisiae* can

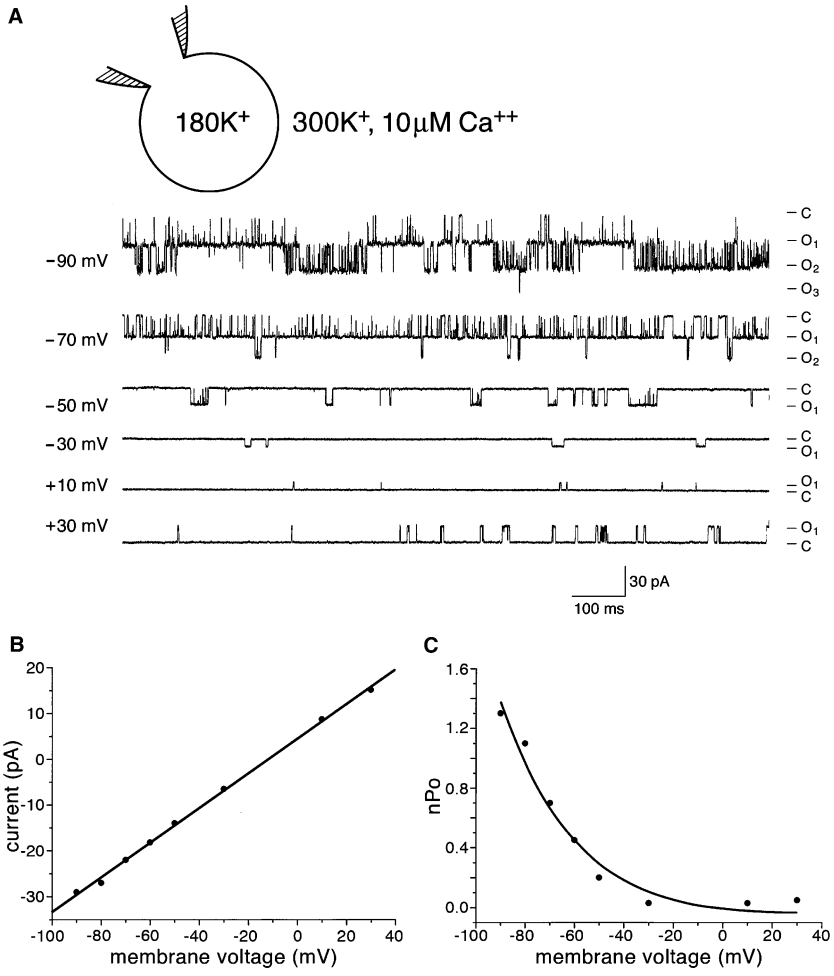

Fig. 3 *TRPY3*-encoded unitary conductances in the vacuolar membrane of *trpy1*Δ cells of *S. cerevisiae*. **a** Spontaneous TrpY3 conductances without pressure into the vacuole, recorded in the whole-vacuole mode, in the presence of free 10 μM Ca^{2+} added to the bath (the cytoplasmic side). Membrane voltage and current levels are marked. **b** Single-channel current/voltage plot yielding a unitary conductance of 380 pS. **c** The open probability (*nPo*) against the applied voltage, showing inward rectification characteristics previously shown for TrpY1. All are from one recording

transmit the Ca^{2+}-induced conformational change to the heterologously expressed TrpY3.

TrpY2 and TrpY3 are mechanosensitive

As reported previously vacuoles of yeast cells deleted of *TRPY1* do not display any signs of the TrpY1 conductance (Palmer et al. 2001, Zhou et al. 2003), regardless of the amount of pressure exerted on the vacuoles (Fig. 6, left) or the excised patches. In contrast, conductances were observed numerous times when negative pressure was applied to the surface of *S. cerevisiae* vacuole expressing *TRPY2* or *TRPY3* trangenes, in attempts to form the gigaseals and to reach the whole-vacuole mode. Pressure-induced conductances were better documented in whole-vacuole or excised cytoplasmic-side-out recording mode. Pressure pulses stronger than 10 mmHg, invariably activated the approximately 380-pS conductance of TrpY3 ($n > 20$) (Fig. 6, right).

Like the native channel, the transplanted TrpY3 channel in the heterologous vacuolar membrane shows no signs of fatigue or rundown upon repeated mechanical stimulation. The same vacuole responds to pressure pulses repeatedly numerous times over tens of minutes, where the degree of TrpY3 activation depends on that of the applied pressure (Fig. 7a). The size and the kinetics of TrpY3, like those of TrpY1, allow clear resolutions of single-channel events activated by force (Fig. 7b). With excised cytoplasmic-side-out patches where the membrane noise is smaller, the discrete, unitary nature of the TrpY3 conductances is especially clear (Fig. 7c). The approximate threshold and degree of activation by pressures from 10 to 50 mmHg did not differ significantly between TrpY3 and TrpY1. These vacuoles are between 2 and 5 μm in diameter. A 40-mmHg pressure on a vacuole of 4-μm diameter yields a force of approximately 5×10^{-3} N/m. Such a force activates a significant portion of TrpY1 in its native membrane setting (Zhou et al. 2003). A similar activation of TrpY3 is evident in Fig. 7. The yeast TrpY channels tested thus far appear to have mechanosensitivity similar to that of MscL, the bacterial MS channel, where half-maximal activation is reached at 12×10^{-3} N/m (Sukharev et al. 1999).

Release of Ca^{2+} through TrpY2 or TrpY3 in vivo

Denise and Cyert (2002) showed that increase of external osmolarity triggers a Ca^{2+} release from the vacuole

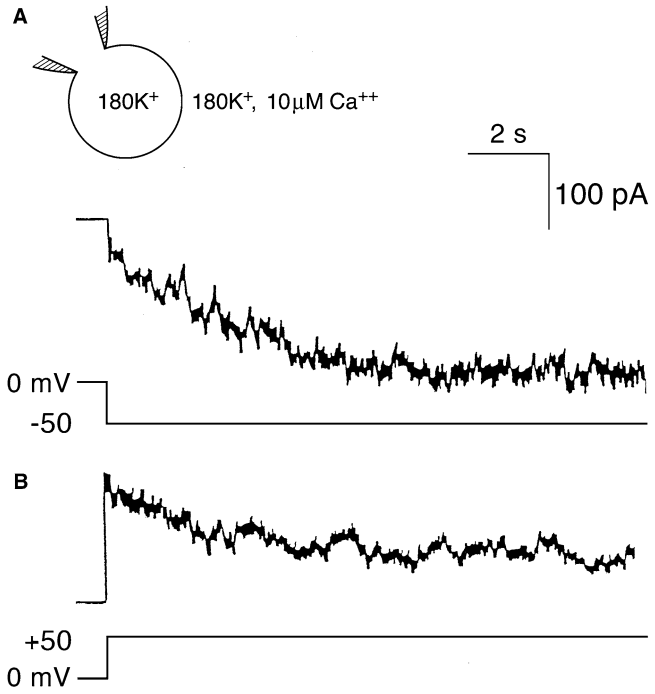

Fig. 4 Activation and deactivation of *TRPY3*-induced conductance. The pressure was kept constant after reaching the whole-vacuole recording mode. **a** Activation kinetics. The vacuolar membrane was first clamped at 0 mV and step-hyperpolarized to –50 mV. The inward current activates with a time constant on the order of seconds. **b** Deactivation kinetics for the same vacuolar membrane step depolarized from 0 to +50 mV

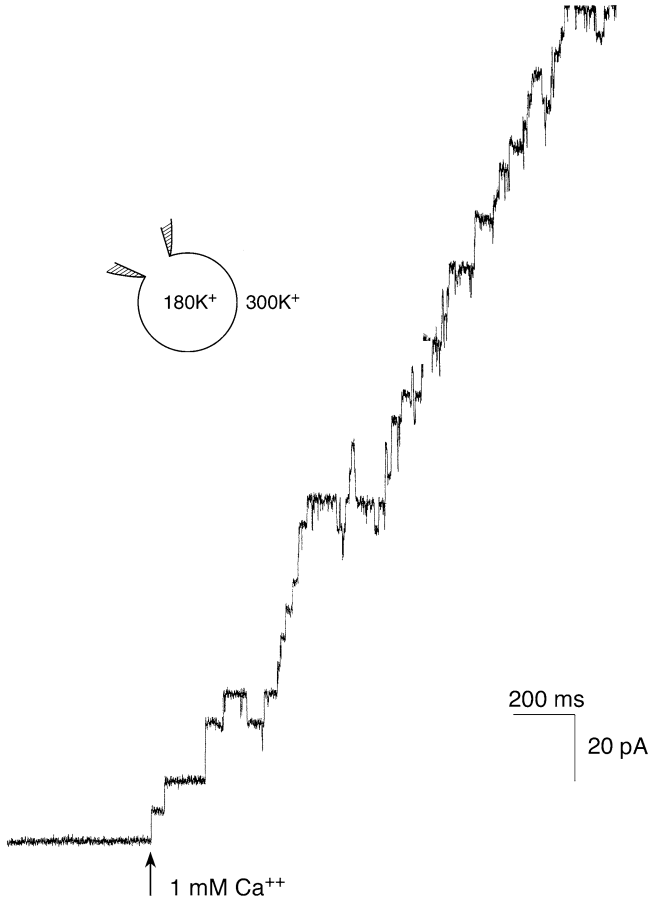

Fig. 5 Ca^{2+} activation the heterologously expressed TrpY3 in the whole-vacuole recording mode. The experiment began with the standard solutions where the bath contained no additional Ca^{2+} (≤ 1 μM free). The addition of $CaCl_2$ (*arrow*) to a final concentration of 1 mM free Ca^{2+} in the bath, which is equivalent to the cytoplasmic side, activated more than 30 conducting units, saturating the recording system. The membrane voltage was set at –30 mV throughout; the inward current is shown upward here

in live wild-type *S. cerevisiae* cells and that this release is abolished in the *trpy1*Δ strain but is restored by a *TRPY1* transgene. These observations indicate that TrpY1 function in vivo in a physiologically meaningful manner. This observation is robust as we have replicated their results with independently constructed strains (Loukin, Lin, Zhou, Kung and Saimi, unpublished results). To test whether the transgenic TrpY2 or TrpY3 also function as osmotically operated Ca^{2+} release channels, we examined the *trpy1*Δ strains of *S. cereviceae* containing plasmids bearing *TRPY2* or *TRPY3*. Using transgene-produced aequorin as a reporter, the concentration of Ca^{2+} was monitored upon osmotic upshock using a luminometer. As shown in Fig. 8, both the *TRPY2* from *K. lactis* and the *TRPY3* from *C. albicans* restore the upshock-induced Ca^{2+} signal to *trpy1*Δ. The light signal varies in different experiments, possibly owing to variation in the expression of aequorin and the uptake of the cofactor ceretarizine. Within the experimental error, we observed no clear difference among the signals from TrpY1, TrpY2 and TrpY3.

Discussion

To further examine the characteristics of the fungal TRP channels, we heterologously expressed the *TRPY2* of *K. lactis* and *TRPY3* of *C. albicans* in *trpy1*Δ strains of *S. cereveciae*. We found the channel activities expressed from the transgenes to be very similar to those associated with the native channel gene *TRPY1*. Most importantly, like TrpY1, the mechanosensitivity is found in TrpY2 or TrpY3 expressed heterologously.

Previous work supports a hypothesis that TrpY1 releases Ca^{2+} upon osmotic upshocks to initiate a long-term defense against dehydration (Denis and Cyert 2002). We have further hypothesized that hypertonic stimulation to the cell causes mechanical perturbations in the vacuolar membrane that lead to the activation of TrpY1 channels and thereby elicit Ca^{2+} release from the vacuole to the cytoplasm (Zhou et al. 2003). So far, we have found that each of the 18 fungal genomes contains a *TRPY1* ortholog, with the exception of *Schizosaccharomyces pombe*. Because fungal genomes are relatively streamlined, compared with those of animals, this widespread presence of fungal *TRPY1* homologs (Fig. 1a) indicates that TRP channels confer an evolutionary selective advantage and therefore a physiological importance.

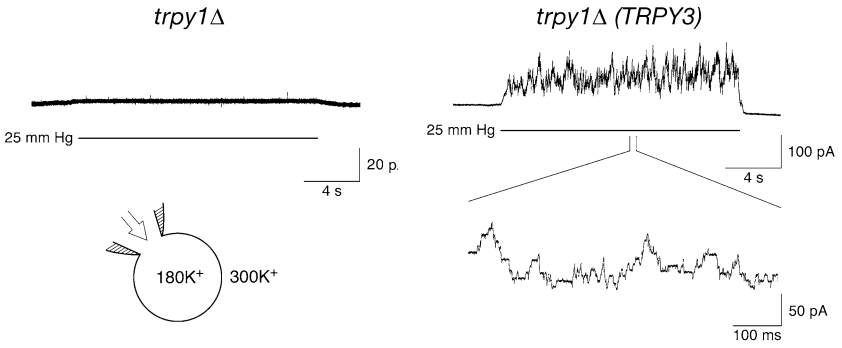

Fig. 6 *TRPY3* restores mechanosensitivity to the vacuole of *S. cerevisiae* deleted of *TRPY1*. Recordings were in whole-vacuole mode. *Left* No currents can be evoked by 25-mmHg pressure exerted into a vacuole from a *trpy1*Δ cell. *Right* The same pressure pulse activates multiple unitary conductances in a vacuole from a *trpy1*Δ cell transformed with a plasmid bearing the *TRPY3* gene from *C. albicans*

We found the activities of TrpY2 and TrpY3 to be quite similar to that of TrpY1. This is rather remarkable, especially for TrpY3, since it is only 45% identical to TrpY1 at the level of the amino acid sequence. We are most interested in the conservation of the mechanosensitivity of TrpY2 and TrpY3 when they are placed in a foreign arena: the vacuolar membrane of *S. cerevisiae*. Remarkably, the mechanosensitivity is observed

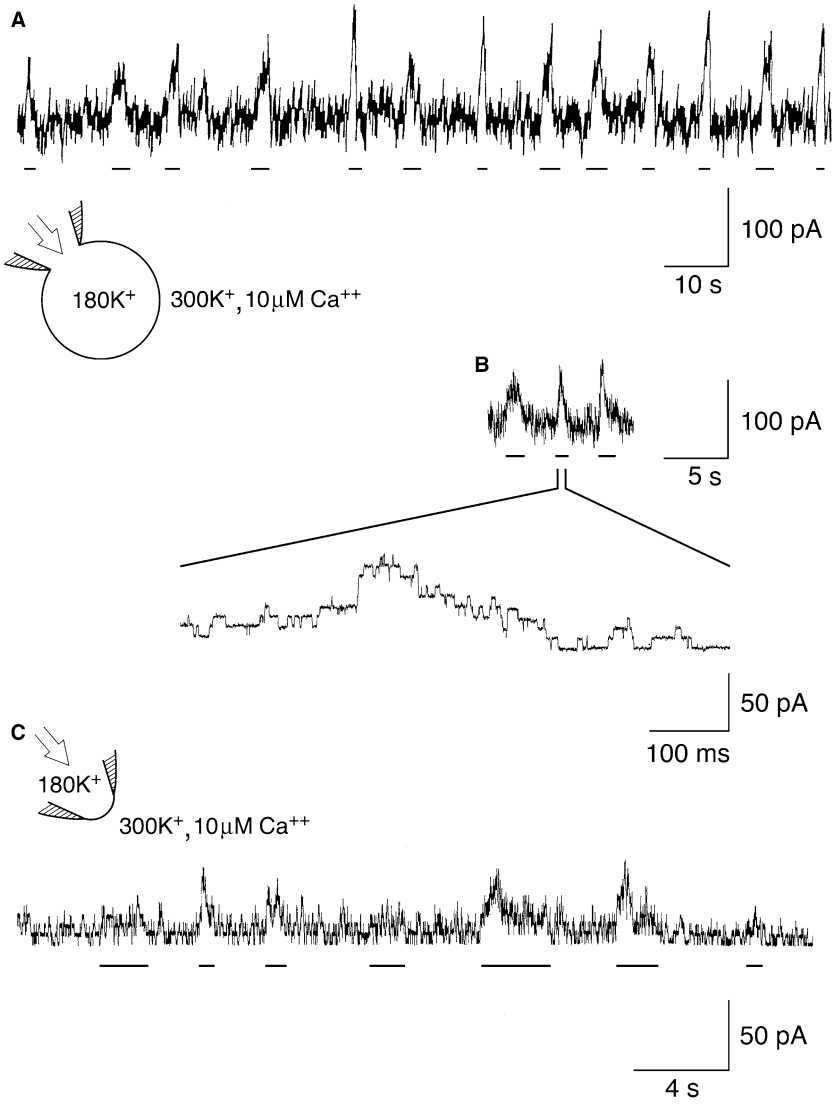

Fig. 7 The robust mechanosensitivity of the conductance generated by the *TRPY3* transgene. **a** Repeated pressure pulses at tens of millimeters of mercury delivered into the vacuole evoke currents over a 2-min period. No rundown is evident. The discrete and unitary nature of the mechanically evoked additional currents can be observed when recording from the whole-vacuole mode (**b**), or the excised cytoplasmic-side-out mode (**c**)

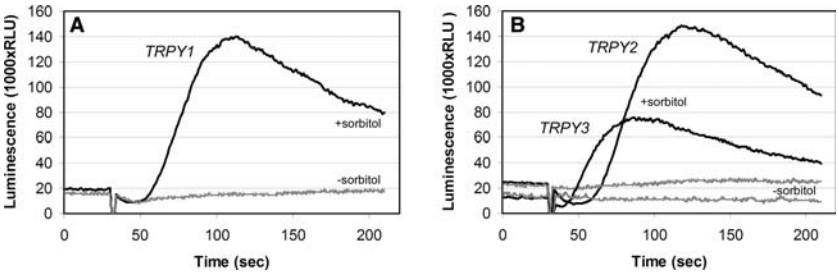

Fig. 8 Ca^{2+} response from yeast cells expressing *TRPY1* of *S. cerevisiae*, *TRPY2* of *K. lactis* and *TRPY3* of *C. albicans* upon hypertonic stimulation. Yeast cells of *trpY1*Δ background harboring plasmids bearing *TRPY1* (**a**), *TRPY2* or *TRPY3* (**b**) were cultured in the modified complete minimal medium with glucose (lacking uracil and leucine for *TRPY1* and *TRPY2* expression) or in an identical medium except for galactose instead of glucose (for *TRPY3* expression) in the presence of 1–2 μM coelenterazine overnight at room temperature. (*TRPY1* and *TRPY2* were in PCM190-derived pZUC plasmid bearing a *URA3* marker, a *CYC1* promoter and a *CEN6*/*ARS4* origin; *TRPY3* was in p416 GALL plasmid.) These cells were also expressing constitutively aequorin from PEVP11/AEQ (with a *LEU2* marker) (see "Materials and methods", and also Denis and Cyert 2002 for more detail). A 100-μl aliquot of cell suspension was taken out of the culture in a tube, which was then placed in a Berthold luminometer for the Ca^{2+} response assay. During the measurement (at the inflections in the graphs), 100 μl of the fresh culture medium with 3 M sorbitol (*thick lines*) or without (*thin lines*) was manually injected into the tube. Ca^{2+} responses, though variable among cell cultures but consistent within the culture, were measured as aequorin–coelenterazine luminescence in relative units (*RLU*)

(Figs. 6, 7). It is as yet technically not feasible to consistently reach current saturation by pressure alone with the yeast vacuolar preparation, whether it is expressing *TRPY1*, *TRPY2* or *TRPY3*. This technical limitation obviates detailed comparisons of pressure activation among the three homologous channels. Nonetheless, as evident from previous (Zhou et al. 2003) and current work, no significant difference in mechanosensitivity between TrpY1 and TrpY3 can be detected within the resolution of our current technology. The mechanosensitivity of foreign TrpY in the *S. cerevisiae* vacuolar membrane is not likely an artifact of spheroplast formation, vacuolar isolation, or patch-clamp manipulations in vitro (Fig. 2) because the ability to release vacuolar Ca^{2+} upon hypertonic stimulation, missing the yeast cells of *trpy1*Δ background, is re-conferred by the *TRPY2* or *TRPY3* transgene in vivo (Fig. 8).

That heterologously expressed TrpY channels retain their mechanosensitivity is reminiscent of the channel-autonomous properties of voltage-gating or ligand-gating in other channels expressed in foreign systems. These channels, in numerous experiments, retain their ability to be activated by ligand or voltage when heterologously expressed in oocytes, in cultured cells, in yeast, in bacteria, or upon reconstitution into artificial lipid bilayers. We could interpret these observations to mean that ligand-binding sites or the voltage sensors are on the bilayer-embedded channel proteins themselves and not on other proteins. The mechanosensitivty of the bacterial MS channels are also clearly channel-autonomous: Purified MscL or MscS proteins reconstituted into pure lipid bilayers retain their mechanosensitivity (Sukharev et al. 1997), as reviewed in the "Introduction". In these cases, the MS channels do not require other proteins (partners, cytoskeleton, etc.) to be MS.

As yet, no purified TRP channels have been reported to function upon reconstitution into pure lipids. Without such a definitive proof, it remains possible that the gating force for TRP MS channels is delivered partly or solely through partner proteins such as the cytoskeleton. Nonetheless, there are less direct observations pertinent to the origin of the gating force. The *TRPV4* genes of several vertebrates have been expressed in CHO and other cultured cells and found to retain their ability to respond to hypotonic stimulations, as evidenced by electric currents and Ca^{2+} imaging (Liedtke et al. 2000, Strotmann et al. 2000). Thus, the heterologous new surrounding continues to deliver the mechanical force to these TrpV4 channels. If this force is delivered through protein–protein connection, say, from the attached cytoskeleton, the results imply that the cytoskeleton and the channel protein from different sources remain compatible. TrpV4, like other members of the TrpY or TrpC family, include large N-terminal and C-terminal peptides modeled to be in the cytoplasm, flanking the membrane-embedded channel proper (Clapham 2003). These are likely the sites of interaction with other proteins. For example the N-terminal domain contains several ankyrin repeats known to bind other proteins, probably cytoskeleton. Interestingly, TrpV4 deleted of its ankyrin repeats continues to support fully the hypotonically induced steady-state current in transfected cells under voltage-clamp, although the imaged Ca^{2+} response seems less robust (Liedtke et al. 2000). A parallel experiment has been performed in the nematode *C. elegans*, where a rat homolog (*TRPV4*) complements the osmotactic behavior defect of the host worm missing its own TrpV channel (Osm-9). Not only are the two identical to only 22%, but the *TRPV4* transgene deleted of its entire corresponding N-terminal domain, including all the ankryin repeats, as well as the entire C-terminal peptide remains potent in that complementation (Liedtke et al. 2003)! The simplest interpretation of these results seems to be that the physical force delivered to the Trp channels is through the lipid bilayer instead of the cytoskeleton. Unlike TrpYs, however, TrpV4 channels have not been shown to be activated by forces directly applied through the patch pipets (Liedtke et al. 2000, Strotmann et al. 2000).

The direct patch-clamp results and the in vivo Ca^{2+}-response lumenometry results presented in the present paper add weight to the notion that the gating force to the Trp channel comes directly from the lipid bilayer, whose physical properties are not expected to vary much among fungal species. However, our results do not constitute a definite proof. Structural conservations outside the transmembrane domains are apparent among TrpY1, TrpY2 and TrpY3 (Fig. 1b). Even though TrpYs have no ankyrin repeats, attachment to heterologous partner proteins with approximately 50% identity for force transmission, though unlikely, remains a possibility. In this context, one should be reminded that partner proteins may serve functions other than force transmission. Attachment to other proteins or cytoskeletons may be important in channel adaptation, Ca^{2+} sensitivity, protein deployment, etc.

Like the cases of the bacterial MS channels MscL and MscS, channel proteins purified and reconstituted into the artificial lipid bilayer can be examined more definitively for mechanosensitivity, leaving no room for force transmission through other elements. Such experiments with TRP channels will be desirable.

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