Activity of the yeast vacuolar TRP channel TRPY1 is inhibited by Ca\textsuperscript{2+}–calmodulin binding

Mahnaz Amini\textsuperscript{1,2,6}, Yiming Chang\textsuperscript{2,4}, Ulrich Wissenbach\textsuperscript{1}, Veit Flockerzi\textsuperscript{1}, Gabriel Schlenstedt\textsuperscript{1}, and Andreas Beck\textsuperscript{1,*}

From the \textsuperscript{1}Experimentelle und Klinische Pharmakologie und Toxikologie/PZMS, Universität des Saarlandes, Homburg, Deutschland; \textsuperscript{2}Department of Medical Biochemistry and Molecular Biology/PZMS, Medical School, Saarland University, Homburg, Germany

Edited by Roger J. Colbran

The transient receptor potential (TRP) cation channels, which are conserved across mammals, flies, fish, sea squirts, worms, and fungi, essentially contribute to cellular Ca\textsuperscript{2+} signaling. The activity of the unique TRP channel in yeast, TRP yeast channel 1 (TRPY1), relies on the vacuolar and cytoplasmic Ca\textsuperscript{2+} concentration. However, the mechanism(s) of Ca\textsuperscript{2+}-dependent regulation of TRPY1 and possible contribution(s) of Ca\textsuperscript{2+}-binding proteins are yet not well understood. Our results demonstrate a Ca\textsuperscript{2+}-dependent binding of yeast calmodulin (CaM) to TRPY1. TRPY1 activity was increased in the cmd1\textsuperscript{6} yeast strain, carrying a non–Ca\textsuperscript{2+}-binding CaM mutant, compared with the parent strain expressing wt CaM (Cmd1). Expression of Cmd1 in cmd1\textsuperscript{6} yeast rescued the wt phenotype. In addition, in human embryonic kidney 293 cells, hypertonic shock-induced TRPY1-dependent Ca\textsuperscript{2+} influx and Ca\textsuperscript{2+} release were increased by the CaM antagonist ophio-bolin A. We found that coexpression of mammalian CaM impeded the activity of TRPY1 by reinforcing effects of endogenous CaM. Finally, inhibition of TRPY1 by Ca\textsuperscript{2+}–CaM required the cytoplasmic amino acid stretch E\textsubscript{33}–Y\textsubscript{92}. In summary, our results show that TRPY1 is under inhibitory control of Ca\textsuperscript{2+}–CaM and that mammalian CaM can replace yeast CaM for this inhibition. These findings add TRPY1 to the innumerable cellular proteins, which include a variety of ion channels, that use CaM as a constitutive or dissociable Ca\textsuperscript{2+}-sensing subunit, and contribute to a better understanding of the modulatory mechanisms of Ca\textsuperscript{2+}–CaM.

The transient receptor potential yeast (TRPY) channel 1 is a member of the superfamily of transient receptor potential (TRP) cation channels. Based on their amino acid sequence similarities, the TRP proteins fall into seven subfamilies, six of which are found in mammals: canonical (TRP canonical), vanilloid (TRP vanilloid [TRPV]), melastatin (TRP melastatin [TRPM]), mucolipin (TRP mucolipin), ankyrin (TRP ankyrin), and polycystin (TRP polycystin). The TRPN (NO-Mechano-Potential) has so far only been detected in worm, fly, and zebrafish and is proposed to be a mechanosensing channel. So far, no TRP channels have been identified in archaea or bacteria, and only few examples of TRP genes have been identified in nonland plants and fungi (1).

TRPY1 is the only TRP homolog in the yeast 	extit{Saccharomyces cerevisiae}. It is encoded by the gene YVC1 (yeast vacuolar conductance 1) and forms ion channels in the yeast vacuolar membrane (2–5). Recently, the Moissenkova–Bell group described the full-length structure of TRPY1 by cryo-EM with typical tetrameric TRP channel architecture, in which subunits are arranged in a four-fold symmetry around a central ion permeation path, as previously suggested (1, 6, 7) but with distinct structural folds for the cytosolic N and C termini (8).

TRPY1 is activated by osmotic stress (9–11), indole and related aromatic compounds (12), and Ca\textsuperscript{2+} acting from the cytosolic site (2, 13, 14). Reducing agents substantially lower the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) required for TRPY1 activation from the cytosol (2, 14) by targeting cysteine residue 624 (4) to EC\textsubscript{50} values of 391 to 498 μM (13). The resting level of cytoplasmic Ca\textsuperscript{2+} in yeast is about 200 to 400 nM (15, 16), and it has been suggested (17) that hyper-osmotic shock represents the initial physiological stimulus in intact yeast, which elevates [Ca\textsuperscript{2+}]\textsubscript{cyt} by Ca\textsuperscript{2+} release via TRPY1, and thereby reinforces TRPY1 activity (13). A negative charge cluster D\textsuperscript{573}DDD\textsuperscript{576} within the cytoplasmic C terminus has been postulated as binding site on TRPY1 for Ca\textsuperscript{2+}, although a deletion TRPY1 mutant, lacking this negative charge cluster including adjacent amino acid residues (amino acids 570–580) retains a residual Ca\textsuperscript{2+} dependence (17). Apparently, additional Ca\textsuperscript{2+}-dependent mechanisms are required to restrain TRPY1 activation.

Bertl and Slayman (18) proposed a Ca\textsuperscript{2+}–calmodulin (CaM)-dependent mechanism contributing to TRPY1 regulation, CaM is a ubiquitous and highly conserved Ca\textsuperscript{2+}-binding protein conveying changes of cytoplasmic Ca\textsuperscript{2+} concentrations to numerous target proteins including ion channels. \textit{In vitro}, single and multiple binding sites for Ca\textsuperscript{2+}–CaM have been identified in protein fragments of the mammalian TRPs (19) and of the founding members of the TRP channel family, \textit{Drosophila} TRP and TRP like (TRPL) (20–24). Ca\textsuperscript{2+}–CaM binding has been linked to TRPV1 desensitization (25), TRP ankyrin channel 1 potentiation and inactivation (26), TRPM4

\textsuperscript{6} These authors contributed equally to this study.

\textsuperscript{*} For correspondence: Andreas Beck, andreas.beck@uks.eu.

Present address for Yiming Chang: Evolva Group, 4153 Kanton Reinach, Switzerland.
Ca²⁺–calmodulin inhibits TRPY1

sensitization (27), and TRPV5 and TRPV6 inhibition (28–30). Different from in vitro binding studies and published CaM structures bound to ion channel fragments in vitro, according to recent cryo-EM structures, TRPV5 and TRPV6 inhibition required one CaM molecule per TRPV6 (30) or TRPV5 tetramer (31, 32). Up to six surface regions of TRPV6 or TRPV5 interact with the single CaM, which contacts all four subunits of the tetramer.

Mammalian CaM comprises two globular domains, the N and C lobes. Each lobe contains two EF-hand-type Ca²⁺-binding motifs (33, 34). The N and C lobes are connected by a central flexible linker that allows CaM and Ca²⁺–CaM to differently interact with target proteins including TRP channels. Sequence identity of the well-conserved mammalian CaMs and yeast CaM is 60% (34). Yeast CaM (Cmd1) has four potential high-affinity Ca²⁺-binding loops, but only the first three loops have all the residues required to bind Ca²⁺ (35). The yeast strain relying only on the non–Ca²⁺–binding CaM mutant Cmd1–6 is viable, whereas disruption of the wt CaM gene (cmd1) is lethal (35).

Here, we characterize TRPY1 as a Ca²⁺–CaM-binding protein. Ca²⁺ imaging and vacuolar patch clamp recordings in wt and cmd1–6 yeast reveal TRPY1 channel inhibition dependent on Ca²⁺–CaM. Similarly, activity of TRPY1 expressed in human embryonic kidney 293 (HEK-293) cells is inhibited by overexpressed mammalian CaM but not by the mammalian non–Ca²⁺–binding CaM mutant CaM(DSF1,2,3,4). Channel inhibition was abolished by deleting a stretch of 60 amino acids within the N terminus of TRPY1, apparently representing major sites of CaM’s contact with TRPY1.

Results

TRPY1 interacts with Ca²⁺–CaM in vitro

The yeast vacuolar TRP channel (TRPY1, Yvc1; hydrophilicity plot) (Fig. 1A) is activated by cytoplasmic Ca²⁺ (2, 5, 13, 14). Ca²⁺ was suggested to interact with the negative charge cluster D⁵⁷³DDD⁵⁷⁶ within the C terminus of TRPY1 assumed to be exposed to the cytoplasm (17). A 25-amino acid peptide covering this negative charge cluster and adjacent amino acid residues, spotted to a cellulose membrane, significantly bound ⁴⁵Ca²⁺ (Fig. 1B, top). The Ca²⁺ binding was significantly reduced when D⁵⁷³DDD⁵⁷⁶ were replaced by alanine residues. As positive and negative controls, 25-mer peptides of the fourth Ca²⁺-binding loop of mammalian CaM and its D⁷⁹,11A, E¹⁷,¹⁸A mutant version were treated in the same way (Fig. 1B, bottom). Next, we fused the TRPY1 N and C terminus (224 and 137 amino acids) to glutathione-S-transferase (GST) and tested the fusion proteins TRPY1-N and TRPY1-C (Fig. 1C) for direct Ca²⁺ binding. GST or GST-fused yeast CaM (GST-Cmd1) and its non–Ca²⁺–binding mutant version Cmd1–6 (35) served as controls. In the mutant Cmd1–6, the aspartate and glutamate residues at the first and 12th position of the three Ca²⁺-binding loops were replaced by alanine residues. While all proteins were detected by Coomassie blue (Fig. 1C, left), only GST-Cmd1 significantly bound ⁴⁵Ca²⁺ (Fig. 1C, right). Apparently, the negative charge cluster D⁵⁷³DDD⁵⁷⁶ involved in Ca²⁺ binding of the 25-mer peptide is buried in protein domains present in the C terminus of the 137 amino acids (GST-TRPY1-C (538–675)) and thus could not be accessed by Ca²⁺.

We therefore analyzed for a potential interaction of Ca²⁺–CaM and TRPY1. Pull-down assays were performed using recombinant TRPY1-N and TRPY1-C fused to maltose-binding protein (MBP) and GST-fused Cmd1 and Cmd1–6 proteins. Purified MBP-TRPY1-N and MBP-TRPY1-C were immobilized to amylose resin and incubated with GST-Cmd1 or GST-Cmd1–6 in the absence and presence of 2 mM Ca²⁺. Cmd1 but not Cmd1–6 significantly bound to both protein fragments of TRPY1 but only in the presence of Ca²⁺ (Fig. 1D). To confirm the latter interactions, we produced TRPY1-N and TRPY1-C with additional tags. GST-tagged TRPY1-C but not GST alone binds 6His-Cmd1 in the presence of Ca²⁺ (Fig. 1E, left). Similarly, the SUMO-TRPY1-N fusion protein strongly bound GST-Cmd1, but not GST alone, again in a Ca²⁺-dependent manner (Fig. 1E, right). The data suggest at least one interaction site of CaM at both the N and C terminus of TRPY1. This binding requires Ca²⁺ and a CaM molecule with intact Ca²⁺-binding sites.

Ca²⁺–CaM inhibits the activity of TRPY1 in yeast

To study the effect of CaM on TRPY1 activity in intact yeast cells, we expressed the luminescent Ca²⁺ reporter aequorin in wt yeast, in yeast where endogenous cmd1 was replaced by cmd1–6 and in TRPY1 knockout yeast (Δyvc1). TRPY1 activity was challenged by increasing the osmolarity in the bath solution (3, 5, 13, 14). Application of 1.5 M NaCl (hyperosmotic shock) induced a cytoplasmic Ca²⁺ signal in wt yeast, which was not detectable in Δyvc1 cells but massively increased in cmd1–6 cells (Fig. 2, A and C). Overexpression of cmd1 slightly reduced the hyperosmotic shock–induced Ca²⁺ signal in wt cells but significantly diminished the Ca²⁺ signal in the cmd1–6 cells (Fig. 2, A–C). Thus, the ability of CaM to bind Ca²⁺ is required to inhibit TRPY1 activity.

TRPY1 is located in the yeast vacuolar membrane, and next, we recorded TRPY1 currents from wt and cmd1–6 yeast vacuoles by patch clamp experiments (Fig. 2D). For intracellular organelles, like the vacuole, membrane potentials refer to the cytosolic side and inward currents, for example, at –80 mV, represent movement of positive charges out of the vacuole into the cytosol (36). Application of 1 mM Ca²⁺ or 1 mM Ba²⁺ from the cytosolic side induced inward and outward currents, which were significantly increased in cmd1–6 compared with wt vacuoles (Fig. 2, E, F, and H and Fig. S1, A–C). Vacuoles, isolated from Δyvc1 cells, did not reveal any inward current upon application of 1 mM cytosolic Ca²⁺ (Fig. 2G, blue trace) or Ba²⁺ (Fig. S1D). The outward current (Fig. 2G and Fig. S1D) represents a vacuolar Cl⁻ conductance (13), which is also present when TRPY1 activity is abolished by 1 mM vacuum Ca²⁺ (Fig. 2G, red trace, and Fig. 2H). The TRPY1-independent Cl⁻ current is slightly increased by
cytosolic application of Ca\(^{2+}\) (Fig. 2G) and even more by Ba\(^{2+}\) (Fig. S1D).

**CaM modulates the activity of TRPY1 expressed in HEK-293 cells**

TRPY1 retains its channel properties when expressed in HEK-293 cells (13), and we established a Fura-2-based Ca\(^{2+}\) imaging protocol to monitor TRPY1 activity in the absence and presence of increasing osmolarity of the Ca\(^{2+}\)-containing bath solution by application of sorbitol. TRPY1 complementary DNA (cDNA) expression was induced in transfected HEK-293 cells by ponasterone A. The TRPY1 protein was detectable in induced but not in noninduced transfected HEK-293 cells (Fig. 3A). For Ca\(^{2+}\) imaging, HEK-293 cells were identified by their GFP expression, that is, green fluorescence.

Application of sorbitol dose-dependently caused a cytoplasmic increase of Ca\(^{2+}\) in TRPY1-expressing cells (Fig. 3B) but not in control cells only expressing GFP (Fig. 3C). After depleting intracellular Ca\(^{2+}\) stores by thapsigargin, this cytosolic Ca\(^{2+}\) increase in the presence of sorbitol remained (Fig. 3D), whereas sorbitol had no effect on HEK-293 control cells (Fig. 3E). Thus, TRPY1, present in the plasma membrane, is responsible for the hyposmotic shock--induced Ca\(^{2+}\) influx. Application of sorbitol in the absence of extracellular Ca\(^{2+}\) still elicits an increase of cytoplasmic Ca\(^{2+}\), which equals the sorbitol-induced Ca\(^{2+}\) increase in the presence of extracellular Ca\(^{2+}\) when the plasma membrane TRPY1 is blocked by La\(^{3+}\), a Ca\(^{2+}\) flux inhibitor (Fig. 3F). Such Ca\(^{2+}\) signals are not seen in HEK-293 control cells upon stimulation with 1 M sorbitol even in the presence of extracellular Ca\(^{2+}\) (Fig. 3C). Apparently, TRPY1, transiently expressed in HEK-293 cells, is also
present in the membrane of the endoplasmic reticulum (ER) and, in addition to Ca\textsuperscript{2+} influx (see aforementioned), mediates a hyperosmotic shock–induced Ca\textsuperscript{2+} release.

To show the localization of TRPY1, we 3′ fused GFP cDNA to the cDNA of TRPY1. HEK-293 cells were transfected with the new cDNA to yield the TRPY1-C-GFP fusion protein (TRPY1-GFP). The cDNA of BiP, a chaperone located in the lumen of the ER, fused to mRFP (BiP-mRFP), was coexpressed as a marker for the ER (Fig. 3D). Independently, another marker for the ER, ER-CFP (pECFP-ER; Clontech), was expressed and compared with the expression of TRPY1-GFP (Fig. 3D). TRPY1-GFP and BiP-mRFP are colocalized (Fig. 3I, yellow cell) and TRPY1-GFP and ER-CFP exhibit the same cellular fluorescence pattern (Fig. 3I). The fluorescence images show that TRPY1 is present in ER membranes and functionally contributes to hyperosmotic shock–mediated Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores (Fig. 3F). Infusion of 1 μM Ca\textsuperscript{2+} via the patch pipette revealed whole-cell currents in TRPY1-GFP–expressing cells but not in HEK-293 control cells (Fig. S2, A and B), showing that the TRPY1-GFP fusion protein forms a functional channel in the plasma membrane, as unfused TRPY1 does.

The cells shrink in the hyperosmotic bath solution, and we analyzed volume changes by exciting Fura-2 fluorescence at the isosbestic wavelength 360 nm and monitored the emitted light using the same filter system as for Ca\textsuperscript{2+} measurements (Fig. 3, G and H). Hypertonic shock mediated a fluorescence increase, that is, volume reduction, of HEK-293 control cells in a dose-dependent manner (Fig. 3G), which was more transient and significantly reduced after an initial peak in TRPY1-expressing cells (Fig. 3H). The initial volume reduction activates TRPY1, and cation influx into the cell occurs, counteracting the hyperosmotic shock–mediated volume reduction only in TRPY1-expressing cells.

These results show that in HEK-293 cells, TRPY1 present in the plasma membrane and in the membranes of intracellular Ca\textsuperscript{2+} stores mediate the hyperosmotic shock–induced increase of cytoplasmic Ca\textsuperscript{2+} and that TRPY1 at both places may contribute to a TRPY1 function in osmoregulation.

Next, HEK-293 cells were cotransfected with the TRPY1 cDNA and the cDNAs of wt mammalian CaM or the mammalian non–Ca\textsuperscript{2+}-binding CaM(D\textsubscript{EF1,2,3,4A}) mutant, carrying a D-to-A mutation in the first position of all four EF hands (37). The hyperosmotic shock–induced Ca\textsuperscript{2+} signal was significantly reduced in cells coexpressing TRPY1 and CaM compared with cells expressing TRPY1 alone or cells coexpressing TRPY1 and the CaM(D\textsubscript{EF1,2,3,4A}) mutant (Fig. 4, A and C). Neither CaM nor CaM(D\textsubscript{EF1,2,3,4A}) expression revealed any effect on the cytosolic Ca\textsuperscript{2+} upon application of 1 M sorbitol in HEK-293 control cells (Fig. 4B). Inhibition of
Ca\textsuperscript{2+}–calmodulin inhibits TRPY1

TRPY1 expressed in HEK-293 cells by endogenous CaM depends, like in yeast, on Ca\textsuperscript{2+} binding, and is prevented by ophiobolin A, a CaM inhibitor (Fig. 4, D and E). In addition, TRPY1 whole-cell currents in HEK-293 cells, activated by 1 μM Ca\textsuperscript{2+} in the patch pipette, were significantly reduced after coexpression of CaM but not the CaM(DF1,2,3,4A) mutant (Fig. 4, F–H). We also recorded TRPY1 currents induced by hyperosmotic shock (500 mM sorbitol) after overexpression of the CaM cDNA (Fig. S3, H–K) or activated by 1 μM intracellular Ca\textsuperscript{2+} and hyperosmotic shock (500 mM sorbitol) after cytosolic infusion of 10 μM recombinant mammalian CaM via the patch pipette (Fig. S3, A–G). In all protocols, cytosolic CaM decreased the TRPY1 current. In contrast, coexpression of the non–Ca\textsuperscript{2+}-binding CaM mutant (CaM(DF1,2,3,A)) had little effect on TRPY1 currents. The hyperosmotic shock-induced currents were not seen in nontransfected HEK-293 cells (Fig. S3, L and M) and were virtually abolished in TRPY1-expressing cells in the absence of intracellular Ca\textsuperscript{2+} (10 mM BAPTA, 0Ca\textsuperscript{2+}; Fig. S3, N and O).

The data also show that mammalian CaM can functionally replace yeast CaM in inhibiting the activity of TRPY1. Such a functional replacement of yeast CaM by its mammalian homolog has been shown for several physiological functions in yeast (15, 34, 38, 39).

Cmd1-binding sites of the TRPY1 N terminus required for current inhibition

In vitro, Ca\textsuperscript{2+}–Cmd1 is bound by both the N and C terminus of TRPY1 (Fig. 1), and according to online databases (40, 41), multiple and partially overlapping binding sites for mammalian CaM within the N and C terminus of the TRPY1 protein are predicted. In order to identify the binding sites for yeast CaM (Cmd1), we performed a peptide scan. Peptides
corresponding to the N and C terminus of TRPY1, with a length of 20 amino acids each, were synthesized on a cellulose membrane, and the amino acid sequences were shifted by five amino acids from one spot to the other. After incubation of the cellulose membrane with $^{14}$C-labeled Cmd1 and exposure, we did not find clearly defined single sites but rather several binding domains. According to the relative intensity visualized on the autoradiograph (Fig. S4), two domains within the N terminus, residues 68 to 92, and less intense residues 28 to 52, were most prominent, compared with domains within the C terminus (residues 527–546 and residues 607–631).

Because most confirmed CaM-binding sites have a net positive charge, lysine residues of the major two domains at positions 43 and 48 as well as 86, 89, and 91 within the N terminus were replaced by alanine residues, and, in addition, both domains were deleted, generating TRPY1-K43 48A, TRPY1-K86 88 91A, and TRPY1-Δ33 to 92 cDNAs. The wt and the mutant cDNAs expressed in TRPY1 knockout yeast (Δyvc1) yielded comparable amounts of protein (Fig. S5A). Next, we recorded TRPY1 currents from yeast vacuoles by patch clamp experiments. Application of 1 mM Ca$^{2+}$ from the cytosolic side induced inward and outward currents in vacuoles expressing wt TRPY1 (Fig. 5B). The current amplitudes of TRPY1-K43 48A (Fig. 5C) and TRPY1-K86 89 91A (Fig. 5D) were not distinguishable to the current amplitudes obtained from the wt channel (Fig. 5, B–D, F, and G), whereas deletion of residues 33 to 92 (TRPY1-Δ33–92) including both domains increased current amplitude almost twofold (Fig. 5, E–G). As shown in Figure 2, E, F, and H, replacement of wt Cmd1 by cmd1–6 had a similar effect on wt TRPY1 currents.

Discussion

The present study identifies TRPY1 as a Ca$^{2+}$–CaM-binding protein. In vitro, purified recombinant TRPY1 protein fragments bound yeast CaM but not the non–Ca$^{2+}$–binding yeast CaM mutant cmd1–6 in pull-down assays. In vivo, patch clamp recordings from yeast vacuoles and HEK-293 cells expressing TRPY1 and Ca$^{2+}$ imaging experiments in yeast and HEK-293 cells demonstrate that Ca$^{2+}$–CaM inhibits TRPY1 activity and that a 60 amino acid domain within the N terminus of TRPY1, exposed to the cytoplasm, is required for this inhibition.

CaM is a universal Ca$^{2+}$ sensor, which translates changes of the [Ca$^{2+}$]$_{cyt}$ to target proteins including ion channels and thereby regulates target protein function. Based on the results of pull-down experiments with recombinant and purified fragments of TRPY1 and yeast CaM (Fig. 1, D and E), we performed a peptide scan in order to identify Ca$^{2+}$–CaM binding sites within the N and C terminus of TRPY1. We did
Ca\(^{2+}\)-calmodulin inhibits TRPY1

Figure 5. Cmd1 binding sites of the TRPY1 N terminus required for current inhibition. A, Western blot of protein lysates from wt yeast strain (wt), TRPY1 knockout (Δyvc1) yeast strain (control), and Δyvc1 transformed with single copy plasmids of wt YVC1, YVC1-K86 89 91A, YVC1-K43 48A, and YVC1-Δ33 to 92 incubated in the presence of the monoclonal anti-TRPY1 antibody. The filter was stripped and incubated with antibody for Yrb1 (lower panel) as loading control. B–F, inward and outward currents at −80 and 80 mV, extracted from 200 ms voltage ramps (0.5 Hz) spanning from 150 to −150 mV, Vh = 0 mV, plotted versus time (B–E) and corresponding IVs of the maximal currents (I\(_{\text{max}}\)), recorded from vacuoles isolated from Δyvc1 yeast strain transformed with single copy plasmids of wt TRPY1 (B), YVC1-K43 48A (C), YVC1-K86 89 91A (D), and YVC1-Δ33 to 92 (E). The bar indicates cytosolic application of 1 mM Ca\(^{2+}\). G, amplitudes of the net plateau currents upon activation by 1 mM Ca\(^{2+}\) at −80 and 80 mV from experiments in B–E, with current amplitudes right before application of 1 mM Ca\(^{2+}\) subtracted. The numbers in brackets indicate the number of measured vacuoles. Data represent means (F) and means ± SD (G) with p values in G calculated by one-way ANOVA (ANOVA values: +80 mV, F = 4.638, p = 0.0077; −80 mV, F = 9.80, p < 0.0001) with Bonferroni's multiple comparison test. Cmd1, yeast calmodulin; TRPY1, transient receptor potential yeast channel 1.

not find clearly defined single sites but rather several CaM-binding domains (Fig. S4). This is not an exceptional finding because CaM dynamically binds to its targets, which may not be represented by a single linear binding motif as illustrated by recent structures of the small-conductance Ca\(^{2+}\)-activated K\(^{+}\) (SK) channel (42) or TRPV6 and TRPV5 channels in complexes with Ca\(^{2+}\)-CaM (30–32). TRPV5 and TRPV6 channels undergo Ca\(^{2+}\)-induced inactivation, proposed to contain a fast Ca\(^{2+}\)-dependent and a slow Ca\(^{2+}\)-CaM-dependent component. More than five Ca\(^{2+}\)-CaM binding sites have been identified in TRPV5 and TRPV6 proteins in vitro (28–30, 43–45). Synthesized as peptides or recombinant channel protein fragments, most of these different sites bound at least one CaM molecule in vitro, whereas in the TRPV5–CaM and TRPV6–CaM channel structures (30–32), one channel tetramer binds one single CaM molecule. It contacts a tryptophan residue adjacent to the channels' lower gate and various hydrophobic patches on the N terminus and on the C terminus of single subunits. In the human SK4 (KCNN4) channel–CaM complex, four CaM molecules bind to one channel tetramer with each single CaM molecule communicating with three channel subunits (42). Based on these structure data, single subunits of homotetrameric channels may assume different functions in terms of CaM binding.

Neither the tryptophan nor the sequence motifs of the hydrophobic patches are conserved in TRPY1, which shares less than 16% sequence identity with TRPV6 or TRPV5. We therefore replaced the lysine residues 43 and 48 (TRPY1-K43 48A) and the lysine residues 86, 89, and 91 (TRPY1-K86 89 91A) by alanine residues within those domains of TRPY1, which were most prominent in the peptide scan (domains E28–30, 43, 4–45). Synthesized as peptides or recombinant protein fragments, most of these different sites bound at least one CaM molecule in vitro, whereas in the TRPV5–CaM and TRPV6–CaM channel structures (30–32), one channel tetramer binds one single CaM molecule. It contacts a tryptophan residue adjacent to the channels' lower gate and various hydrophobic patches on the N terminus and on the C terminus of single subunits. In the human SK4 (KCNN4) channel–CaM complex, four CaM molecules bind to one channel tetramer with each single CaM molecule communicating with three channel subunits (42). Based on these structure data, single subunits of homotetrameric channels may assume different functions in terms of CaM binding.

For vacuolar current recordings, we activated TRPY1 by applying Ca\(^{2+}\) to the bath solution. We have previously shown that Ca\(^{2+}\) applied from the cytosolic site activates vacuolar TRPY1 outward and inward currents at EC\(_{50}\) values of 391 and 498 μM, but the mechanism of Ca\(^{2+}\)-mediated activation is yet not fully understood (2, 5, 10, 13, 14, 46, 47). As shown in Figure 1B, in vitro, Ca\(^{2+}\) binds to a linear 25-mer peptide containing the negative charge cluster D\(^{573}\)DDD\(^{576}\) and adjacent amino acid residues from the cytosolic C terminus of
**Ca<sup>2+</sup>–calmodulin inhibits TRPY1**

TRPY1, suggested to be crucial for its cytosolic Ca<sup>2+</sup>-mediated activation (17). Four additional aspartate residues are located in close vicinity of the cluster, which might cause the residual Ca<sup>2+</sup> binding to the peptide containing the A<sup>573</sup>AAA<sup>576</sup> mutation (Fig. 1B). We did not observe any Ca<sup>2+</sup> binding to the complete recombinant TRPY1 C terminus K<sub>538</sub> to E<sub>675</sub> where the accessibility of the cluster for Ca<sup>2+</sup> might be prevented by structural restraints (Fig. 1C). Maintained TRPY1 channel activity was shown after replacing D<sub>573</sub>DDD<sub>576</sub> by asparagine residues (4) and a residual Ca<sup>2+</sup> dependence of the deletion mutant TRPY1<sup>Δ</sup>570G-S<sup>580</sup> (17), whereas TRPY1<sup>Δ</sup>570G-I<sup>690</sup> failed to respond to Ca<sup>2+</sup> (12).

In addition to TRPY1, TRPV5, and TRPV6, a variety of additional TRP channel proteins contain CaM-binding sites. The founding members of the TRP channel superfamily, the fly photoreceptor TRPs, TRP and TRPL, were the first TRPs characterized as Ca<sup>2+</sup>–CaM-binding proteins. The photoreceptor TRP protein has at least two Ca<sup>2+</sup>–CaM binding sites in the C terminus. In vitro, peptides or recombinant fragments carrying these binding sites bind CaM only in the presence of Ca<sup>2+</sup> (20, 22, 48). The second fly photoreceptor TRP channel, TRPL, was initially identified in a screen for CaM-binding proteins (49). Two CaM-binding sites were identified in TRPL fragments of its C terminus (23, 24). Both fly photoreceptor TRP channels are positively and negatively regulated by Ca<sup>2+</sup> (50, 51), but whether CaM is involved in this regulation is not known and might depend on the heterologous expression systems, insect Sf9 cells, or mammalian CHO cells (21, 23, 52, 53).

We show here that TRPY1 in the yeast *S. cerevisiae* is under inhibitory control of Ca<sup>2+</sup>–CaM. This inhibition is maintained when TRPY1 is expressed in mammalian HEK-293 cells and when yeast CaM is replaced by mammalian CaM. In intact yeast, Ca<sup>2+</sup>–CaM might restrain excessive TRPY1 responses to Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release caused by hyperosmotic stimuli.

**Experimental procedures**

**Protein purification, pull-down assays, and Western blots**

The production of proteins in *Escherichia coli* and the preparation of cell lysates were performed as described (54, 55). *E. coli* cells were transformed with plasmids as listed in the resources table (Supporting information). Recombinant fusion proteins were purified by affinity chromatography using glutathione sepharose (GE Healthcare), amylase resin (NEB), or Ni–NTA agarose (Qiagen).

Pull-down assays were performed as described (55). Typically, 30 μl of glutathione sepharose affinity beads were pre-equilibrated and incubated with 12 μg of the purified protein of interest at 4 °C for 60 min in Tris-buffered saline buffer (150 mM NaCl, 50 mM Tris–HCl, and pH 7.5). The beads were washed three times with buffer, and potential binding proteins were incubated with the resin at 4 °C for 1 h. After washing, bound proteins were eluted with SDS sample buffer and analyzed by SDS-PAGE and Coomassie blue staining or visualized by immunoblotting using anti-GST antibodies (1:1000; rabbit, polyclonal, made inhouse).

The anti-TRPY1-4C9-A3 monoclonal rat serum antibody was generated inhouse (3, 13). Proteins of yeast cell lysates, prepared as described previously (56), were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Thermo Fisher Scientific). Proteins were detected with horseradish peroxidase–coupled secondary antibodies (1:1000 dilution; Sigma–Aldrich) and the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer) and the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The antibody against Yrb1 was described (57).

**CaM binding**

Purified MBP-fused proteins (12 μg) were immobilized to amylose resin and incubated with 12 μg of GST fusion proteins containing wt yeast CaM (Cmd1) or the non–Ca<sup>2+</sup>-binding yeast CaM mutant (Cmd1Δ6) in the presence (2 mM Ca<sup>2+</sup>) or the absence of Ca<sup>2+</sup> (5 mM EGTA) for 60 min at 4 °C. After washing, bound proteins were eluted with SDS sample buffer, analyzed by SDS-PAGE blotted, and detected by anti-GST antibodies.

GST or GST-Cmd1 (12 μg) was immobilized to glutathione sepharose, and 6His-Sumo–TRPY1-N fusion proteins were added. The Sumo moiety was inserted to increase solubility. Alternatively, immobilized GST or GST-TRPY1-C was supplemented with 12 μg of 6His-Cmd1. After incubation with or without Ca<sup>2+</sup> (2 mM CaCl<sub>2</sub> or 5 mM EGTA) for 60 min at 4 °C, the resins were washed, and bound proteins were eluted with SDS and analyzed by SDS-PAGE and Coomassie blue staining.

**45Ca<sup>2+</sup> binding assay**

The 45Ca<sup>2+</sup> binding assay of blotted proteins was performed as described (38). The recombinant proteins were run on SDS-PAGE and blotted onto a nitrocellulose membrane, which was soaked for 120 min in buffer (60 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM imidazole–HCl, and pH 6.8), changed every 30 min, followed by incubation in the same buffer containing 1 μCi/l 45Ca<sup>2+</sup> for 10 min. Thereafter, the membrane was rinsed with deionized water, dried at 21 °C, and exposed to a phosphor-imager screen (BAS-IP MS 2040; Fujifilm) for 12 to 24 h. The screen was scanned by a Typhoon FLA 9500 (GE Healthcare Life Sciences). The 45Ca<sup>2+</sup> binding to spotted peptides was essentially performed as described (13).

**Peptide scan assay and 14C-Cmd1 binding**

About 20-mer peptides of the TRPY1 N and C terminus were synthesized on an Intavis ResPepSL peptide spot synthesizer and spotted onto hardened cellulose membranes at 16 nmol per spot. The amino acid sequence was shifted by five amino acids from one spot to the next. The membranes were equilibrated with binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100) at room temperature for 60 min followed by incubation in the same buffer including 2 mM CaCl<sub>2</sub> and 60 cpm of 14C-GST–Cmd1 per peptide spot at 4 °C with gentle shaking for 16 h. Unbound proteins were washed...
away using binding buffer, and membranes were air dried. Membranes were exposed to phosphorimagery screens for 48 h, and screens were scanned by using the Typhoon FLA 9500 (GE Healthcare Life Sciences).

**Yeast strains and plasmids**

The TRPY1 knockout yeast strain (Δyvc1) GSY1180 (MATa YVC1::TRPY1) and the CaM mutant strain cmd1–6 (MATa cmd1–6) (35) are isogenic to the wt strain W303 (GSY170, MATa ura3 leu2 his3 trp1 ade2 can1). The cmd1–6 strain is a kind gift from Dr Trisha Davis, Seattle, WA. For Ca²⁺ imaging or patch clamp experiments, yeast cells were transformed with plasmids as listed in the resources table (Supporting information). Cells were cultured overnight in liquid yeast extract peptone dextrose or synthetic defined media (both Sigma–Aldrich) at 30 °C with rotary shaking and harvested at an absorbance at 600 nm between 1.2 and 1.5.

**Cytosolic Ca²⁺ measurements in yeast**

wt, CaM-mutant cmd1–6, and yvc1-deficient (Δyvc1) yeast cells were transformed with plasmid pEVP11-1AEQ89 (9), encoding the photoprotein aequorin, to measure cytosolic-free Ca²⁺ concentrations. For some experiments, the yeast cells were cotransformed with the plasmid pRS426-CMD1, encoding the yeast CaM. Ca²⁺ imaging was carried out as described (13). In brief, cells were resuspended in fresh medium to a density of an absorbance of 10 at 600 nm. Coelenterazine (Synchem) was added at a final concentration of 60 μM. After incubation for 20 min at 30 °C, cells were pelleted and resuspended in fresh medium and incubated again for 45 to 90 min at 30 °C on a roller. Luminescence was detected at 30 °C at 470 nm using a microplate reader (Infinite M200; Tecan) and plotted as relative luminescence units over time using the i-control 1.7 microplate reader software (Tecan).

**Patch clamp experiments on yeast vacuoles**

The preparation of large vacuoles was performed as described (2, 13). Briefly, overnight yeast cell cultures of wt, CaM-mutant cmd1–6, and yvc1-deficient (Δyvc1, for some experiments cotransformed with plasmids as listed in the resources table [Supporting information]) were harvested at an absorbance of 1 to 1.5 at 600 nm and kept in incubation buffer (50 mM KH₂PO₄, 0.2% β-mercaptoethanol, and pH 7.2) for 15 min at 30 °C. To remove cell walls, protoplasting buffer (50 mM KH₂PO₄, 0.2% β-mercaptoethanol, 2.4 M sorbitol, and pH 7.2) was added with final concentrations of 1 mg/ml zymolyase 20T (ICN Biochemicals) and 150 mg/ml (324 mM) bovine serum albumin (fraction V protease free; Carl Roth). After 45 min of incubation, spheroplasts were pelleted and resuspended in stabilizing buffer (220 mM KCl, 10 mM CaCl₂, 5 mM MgCl₂, 5 mM MES, 1% [w/v] glucose, and pH 7.2). After 2 to 3 days, the spheroplasts revealed giant vacuoles.

To release vacuoles, spheroplasts were incubated in releasing buffer (100 mM potassium citrate, 5 mM MgCl₂, 10 mM glucose, 10 mM MES, and pH 6.8) for 2 to 5 min. Then releasing buffer was replaced by bath (cytosolic side) solution (150 mM KCl, 5 mM MgCl₂, 2 mM DDT, 10 mM Hepes, and pH 7.2). A Zeiss Axiovert 135 microscope equipped with a 40× LD Acroplan objective (Zeiss) was used to visualize the vacuoles. Patch pipettes were pulled from glass capillaries GB150T-8P (Science Products) at a PC-10 micropipette puller (Narishige) and filled with the same solution as in the bath (see aforementioned), leading to resistances between 2 and 4 MΩ. After reaching the giga-seal, short voltage pulses (850–1100 mV, 1–5 ms) were applied to break-in and reach the whole-vacuole configuration. About 1 mM CaCl₂ or BaCl₂ was applied directly onto the measured vacuole via an application pipette (cytosolic side), or 1 mM CaCl₂ was added into the patch pipette (vacuolar side). Currents were recorded from voltage ramps of 200 ms spanning from 150 to –150 mV from a holding potential of 0 mV applied every 2 s using an EPC-9 patch clamp amplifier (HEKA). Currents were normalized to the size (capacitance) of the vacuole and plotted as pA/pF (current densities). Inward and outward currents were extracted at –80 and 80 mV, respectively, and plotted versus time. Representative current–voltage relationships were extracted at indicated time points. Note that the membrane potentials refer to the cytosolic side, that is, inward currents at –80 mV represent movement of positive charges from the vacuole toward the cytosol (36).

**Cytosolic Ca²⁺ measurements in HEK-293 cells**

For cell transfection, we used either the ponasterone A–inducible expression system (Invitrogen) or the bicistronic expression vector pcAGGS-ires-GFP (13, 59). HEK-293 cells (American Type Culture Collection; CRL 1573) were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific) with 10% fetal bovine serum and 1% penicillin/streptomycin. HEK-293 cells were plated on glass coverslips and after reaching about 80% confluency cotransfected with pIND-TRPY1-ires-GFP (HEK TRPY1) or pIND-ires-GFP (HEK control) plus pVgRXR (Invitrogen) plasmids. About 24 h after transfection, 10 μM ponasterone A (Invitrogen) was applied to induce YVC1 expression. Fugene HD (Promega) was used as transfection reagent. Cells were used 24 to 72 h after transfection.

For Ca²⁺ imaging, cells were loaded in media with 5 μM Fura-2-acetoxy methanester (TEFLabs) for 30 min at 37 °C. Subsequently, cells were washed and placed into a bath chamber containing 140 mM NaCl, 2.8 mM KCl, 2 mM MgCl₂, 10 mM Hepes, 10 mM glucose, and pH 7.2 in the presence or the absence of 1 mM CaCl₂. Compounds were applied directly into the bath. The Ca²⁺-dependent Fura-2 fluorescence of single cells was monitored at a rate of 0.5 Hz with a dual excitation fluorometric imaging system (polychrome V; TILL Photonics) controlled by TILLvisION software (TILL Photonics), using a Zeiss Axiovert 200 M microscope equipped with a 20× Zeiss EC Plan Neofluar objective. Fura-2-loaded cells were excited at 340 and 380 nm for 30 ms every 2 s each, and the fluorescence emission above 450 nm was detected by an Andor iXon CCD camera. After background subtraction, positively transfected cells, identified...
**Ca^{2+}--calmodulin inhibits TRPY1**

by their GFP expression (green fluorescence), were marked as regions of interest, and their mean fluorescence at 340 nm (F_{340}) and 380 nm (F_{380}) excitation were computed into relative ratio units (F_{340}/F_{380}). To analyze hypertonic shock-mediated volume changes, cells were excited by the Fura-2 Ca^{2+}-independent wavelength 360 nm for 30 ms every 2 s and the fluorescence emission above 450 nm was detected and normalized to the fluorescence intensity just before the hypertonic shock (F/F_{120}o).

**Cellular localization of TRPY1**

HEK-293 cells were transfected with cDNA for TRPY1 3’ extended by the cDNA of GFP (pcDNA3-TRPY1-GFP) to yield TRPY1-C-GFP fusion proteins (TRPY1-GFP), the ER marker ER-CFP (pECFP-ER; catalog no. 6907-1; Clontech) and/or the ER chaperon BiP, fused to mRFP (pN1-BiP-mRFP-KDEL, subcloned from pN1-BiP-mGFP-KDEL, addgene plasmid #62231 (60); BiP-mRFP). About 48 h after transfection, images were captured with a Plan-Neofluar 63×/1.25 Oil or Plan-Neofluar 100×/1.3 Oil objective (both ZEISS) at a fluorescence microscope (Observer Z1; ZEISS), equipped with GFP, ECFP, and Cy3 filter sets (all from AHF Analysentechnik) and a CCD AxioCam MRm camera (ZEISS), using AxioVision software (ZEISS). Images were cropped and merged using Fiji (ImageJ; National Institutes of Health).

**Patch clamp experiments on HEK-293 cells**

HEK-293 cells were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific) with 10% fetal bovine serum and 1% penicillin/streptomycin, plated on glass coverslips (2.5 cm in diameter) and, after reaching about 80% confluency, transfected with pcAGGS-TRPY1-IRES-GFP, and for some experiments, with pcAGGS-CaM-IRES-GFP or pcAGGS-CaM (DFI1,2,3,4A)-IRES-GFP. About 24 h after transfection, cells were trypsinized and scattered on small glass coverslips (1 cm in diameter). Patch clamp experiments were performed 48 to 72 h thereafter. A Zeiss Axiovert 135 microscope equipped with a 40× LD Achromplan objective (Zeiss), a green LED (Rapp OptoElectronic) and a GFP filter set (AHF Analysentechnik) were used to visualize and identify green cells. The bath solution contained 140 mM NaCl, 2.8 mM KCl, 3 mM MgCl2, 10 mM Hepes, and 10 mM glucose (pH 7.2, adjusted with NaOH). Patch pipettes were pulled from glass capillaries GB150T-8P (Science Products) at a PC-10 micropipette puller (Narishige) and filled with pipette solution comprising 120 mM cesium glutamate, 8 mM NaCl, 1 mM MgCl2, 10 mM Hepes, 10 mM Cs-BAPTA (pH 7.2, adjusted with CsOH), leading to resistances between 3 and 4 MΩ. For activation of TRPY1, the pipette solution either contained 1 μM free Ca^{2+} (10 mM Cs-BAPTA + 8.2 mM CaCl2) or bath solution, supplemented with 500 mM sorbitol (hypertonic shock), was applied via an application pipette directly onto the measured cell with 300 nM free Ca^{2+} (10 mM Cs-BAPTA + 5.7 mM CaCl2) in the patch pipette. Intracellular-free Ca^{2+} concentrations were calculated using webmaxc standard (https://somapp.ucdmc.ucdavis.edu/pharmacology/bers/maxchelator/webmaxc/webmaxc5.htm). For some experiments, 10 μM recombinant bovine (mammalian) CaM (Sigma–Aldrich) was added to the patch pipette, or Ca^{2+} was omitted (0Ca). For tip coating, patch pipettes were dipped into signacote (Sigma–Aldrich). Whole-cell currents were recorded from voltage ramps of 400 ms spanning from −100 to 100 mV from a holding potential of 0 mV applied every 2 s using an EPC-9 patch clamp amplifier (HEKA). Currents were normalized to the size (capacitance) of the cell and plotted as pA/pF (current densities). Inward and outward currents were extracted at −80 and 80 mV, respectively, and plotted versus time. Representative current–voltage relationships were extracted at indicated time points.

**Analysis and statistics**

The hydrophilicity plot was performed using Protein (DNASTAR). Luminometric Ca^{2+} imaging data were saved as Excel files. HEK-293 cells Ca^{2+} imaging data were initially analyzed in TILLvisiON. Fluorescence images were captured by the AxioVision software (ZEISS) and processed in ImageJ. Patch clamp data were analyzed in Patchmaster or Fitmaster (HEKA). All data were finally transferred to IgorPro (WaveMetrics) for further analysis and graphical presentation. GraphPad Prism (GraphPad Software, Inc) was used to prepare bar graphs, scatter plots, and box plots as well as to test for statistical differences. All data were first tested for parametric or nonparametric distribution using Anderson–Darling, D’Agostino and Pearson, Shapiro–Wilk, and Kolmogorov–Smirnov tests. Means of parametric data were compared by Student’s t test for two groups and one-way ANOVA with Bonferroni’s multiple comparison post hoc test for more groups. Means from nonparametric data were compared by Mann–Whitney test for two groups and Kruskal–Wallis test with Dunn’s multiple comparison post hoc test for more groups. The respective tests are indicated in the legends to the figures. Parametric data are presented as bar graphs, and nonparametric data as Tukey’s box and whiskers with the boxes extend from the 25th to the 75th percentile (interquartile range), and the line inside the box shows the median. Whiskers are extended to the most extreme data point that is no more than 1.5× interquartile range from the edge of the box, and outliers beyond the whiskers are depicted as dots. Error bars represent the SEM or SD as indicated in the legends to the figures. Final figures were prepared in CorelDRAW (Corel Corporation).

**Data availability**

All relevant data are contained within the article or the supporting information.

**Supporting information**—This article contains supporting information (61, 62).

**Acknowledgments**—The cmd1–6 strain and the CaM(DFI1,2,3,4A) cDNA were generous gifts of Dr Trisha Davis, University of
Washington, Seattle, WA and Dr John P. Adelman, Vollum Institute, Portland, OR; plasmid pCas28 was donated by Dr Günter Kramer, ZMBH Heidelberg. We thank Dr Stefanie Caesar for helpful discussions, Dr Martin Jung for peptide synthesis, Dr Adolfo Cavalié and Dr Tillman Pick for sharing ER-CFP and BiP-mRFP constructs and Silke Guthörl, Heidi Löhr, Stefanie Buchholz, and Christine Wesely for expert technical assistance.

Author contributions—M. A., Y. C., V. F., G. S., and A. B. conceptualization; M. A., Y. C., U. W., and A. B. investigation; V. F. and G. S. resources; M. A. and A. B. writing—original draft; M. A., V. F., G. S., and A. B. writing—review and editing; V. F., G. S., and A. B. supervision; V. F. and G. S. project administration; V. F., G. S., and A. B. funding acquisition.

Funding and additional information—This work was supported by the Deutsche Forschungsgemeinschaft RTG 1326 (Y. C., M. A., G. S., V. F., and A. B.), Collaborative Research Center (SFB) 894 (A. B. and V. F.), the Homburg Forschungsförderungsprogramm HOMFOR (A. B.), and the Forschungsausschuss der Universität des Saarlandes (G. S., F. V., and A. B.).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: [Ca2+]cyt, cytosolic Ca2+ concentration; CaM, calmodulin; cDNA, complementary DNA; Cmd1, yeast calmodulin; ER, endoplasmic reticulum; GST, glutathione-S-transferase; HEK-293, human embryonic kidney 293; MBP, maltose-binding protein; TRP, transient receptor potential; TRPL, TRP like; TRPM, transient receptor potential melastatin; TRPV, transient receptor potential vanilloid; TRPY1, transient receptor potential yeast channel 1; YVCl, yeast vacuolar conductance 1.

References
1. Hilton, J. K., Kim, M., and Van Horn, W. D. (2019) Structural and evolutionary insights point to allosteric regulation of TRP ion channels. Acc. Chem. Res. 52, 1643–1652
2. Bertl, A., and Slayman, C. L. (1990) Cation-selective channels in the vacuolar membrane of Saccharomyces: Dependence on calcium, redox state, and voltage. Proc. Natl. Acad. Sci. U. S. A. 87, 7824–7828
3. Chang, Y., Schlenstedt, G., Flockerzi, V., and Beck, A. (2010) Properties of the intracellular transient receptor potential (TRP) channel in yeast, Yvcl. FEBS Lett. 584, 2028–2032
4. Hamamoto, S., Mori, Y., Yabe, I., and Uozumi, N. (2018) In vitro and in vivo characterization of modulation of the vacuolar cation channel TRPY1 from Saccharomyces cerevisiae. FEBS J. 285, 1146–1161
5. Palmer, C. P., Zhou, X. L., Lin, J., Loukin, S. H., Kung, C., and Saimi, Y. (2001) A TRP homolog in Saccharomyces cerevisiae forms an intracellular Ca(2+)-permeable channel in the yeast vacuolar membrane. Proc. Natl. Acad. Sci. U. S. A. 98, 7801–7805
6. Madej, M. G., and Ziegler, C. M. (2018) Dawning of a new era in TRP channel structural biology by cryo-electron microscopy. Pflugers Arch. 470, 213–225
7. Vangeel, L., and Voets, T. (2019) Transient receptor potential channels and calcium signaling. Cold Spring Harb. Perspect. Biol. 11, a035048
8. [preprint] Ahmed, T., Nisler, C. R., Fluck, E. C., Sotomayor, M., and Moiseenkova-Bell, V. Y. (2020) Structure of the ancestral TRPY1 channel from Saccharomyces cerevisiae reveals mechanisms of modulation by lipids and calcium. bioRxiv. https://doi.org/10.1101/2020.10.12.336495
9. Batiza, A. F., Schultz, T., and Masson, P. H. (1996) Yeast respond to hypotonic shock with a calcium pulse. J. Biol. Chem. 271, 23357–23362
10. Denis, V., and Cyert, M. S. (2002) Internal Ca(2+) release in yeast is triggered by hypertonc shock and mediated by a TRP channel homologue. J. Cell Biol. 156, 29–34
11. Zhou, X. L., Batiza, A. F., Loukin, S. H., Palmer, C. P., Kung, C., and Saimi, Y. (2003) The transient receptor potential channel on the yeast vacuole is mechanosensitive. Proc. Natl. Acad. Sci. U. S. A. 100, 7105–7110
12. John Haynes, W., Zhou, X. L., Su, Z. W., Loukin, S. H., Saimi, Y., and Kung, C. (2008) Indole and other aromatic compounds activate the yeast TRPY1 channel. FEBS Lett. 582, 1514–1518
13. Amini, M., Wang, H., Belkacemi, A., Jung, M., Bertl, A., Schlenstedt, G., Flockerzi, V., and Beck, A. (2019) Identification of inhibitory Ca(2+) binding sites in the upper vestibule of the yeast vacuolar TRP channel. iScience 11, 1–12
14. Wada, Y., Ohsumi, Y., Tanifuji, M., Kasai, M., and Anraku, Y. (1987) Vacuolar channel ion of the yeast, Saccharomyces cerevisiae. J. Biol. Chem. 262, 17260–17263
15. Davis, T. N., and Thorner, J. (1989) Vertebrate and yeast calmodulin, despite significant sequence divergence, are functionally interchangeable. Proc. Natl. Acad. Sci. U. S. A. 86, 7909–7913
16. Halachmi, D., and Elam, Y. (1989) Cytosolic and vacuolar Ca2+ concentrations in yeast cells measured with the Ca2+-sensitive fluorescence dye indo-1. FEBS Lett. 256, 55–61
17. Su, Z., Zhou, X., Loukin, S. H., Saimi, Y., and Kung, C. (2009) Mechanical force and cytoplasmic Ca2+ activate yeast TRPY1 in parallel. J. Membr. Biol. 227, 141–150
18. Bertl, A., and Slayman, C. L. (1992) Complex modulation of cation channels in the tonoplast and plasma membrane of Saccharomyces cerevisiae: Single-channel studies. J. Exp. Biol. 172, 271–287
19. Zuh, M. X. (2005) Multiple roles of calmodulin and other Ca(2+)-binding proteins in the functional regulation of TRP channels. Pflugers Arch. 451, 105–115
20. Chevesich, J., Kreuz, A. J., and Montell, C. (1997) Requirement for the PDZ2 domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex. Neuron 18, 95–105
21. Lan, L., Breton, H., and Barratt, G. J. (1998) The role of calmodulin-binding sites in the regulation of the Drosophila TRPL cation channel expressed in Xenopus laevis oocytes by Ca2+, inositol 1,4,5-trisphosphate and GTP-binding proteins. Biochem. J. 330, 1149–1158
22. Sun, Z., Zheng, Y., and Liu, W. (2018) Identification and characterization of a novel calmodulin binding site in Drosophila TRP C-terminus. Biochem. Biophys. Res. Commun. 501, 434–439
23. Trost, C., Marquart, A., Zimmer, S., Philipp, S., Cavalié, A., and Flockerzi, V. (1999) Ca2+-dependent interaction of the Trpvl cation channel and calmodulin. FEBS Lett. 451, 257–263
24. Warr, C. G., and Kelly, L. E. (1996) Identification and characterization of two distinct calmodulin-binding sites in the Trpvl ion-channel protein of Drosophila melanogaster. Biochem. J. 314, 497–503
25. Rosenbaum, T., Gordon-Shaag, A., Munari, M., and Gordon, S. E. (2004) Ca2+/calmodulin modulates TRPV1 activation by capsaicin. J. Gen. Physiol. 123, 53–62
26. Hasan, R., Leeson-Payne, A. T., Jaggar, J. H., and Zhang, X. (2017) Calmodulin is responsible for Ca(2+)-dependent regulation of TRP1 channel. Sci. Rep. 7, 45098
27. Nilius, B., Prener, I., Tang, J., Wang, C., Owsianik, G., Janssens, A., Voets, T., and Zhu, M. X. (2005) Regulation of the Ca2+ sensitivity of the nonselective cation channel TRPM4. J. Biol. Chem. 280, 6423–6433
28. de Groot, T., Kovalevskaya, N. V., Verkaart, S., Schilderink, N., Pelici, M., van der Hagen, E. A., Bindels, R. J., Vuister, G. W., and Hoenderop, J. G. (2011) Molecular mechanisms of calmodulin action on TRPV5 and modulation by parathyroid hormone. Mol. Cell. Biol. 31, 2845–2853
29. Niemeyer, B. A., Bergs, C., Wissenbach, U., Flockerzi, V., and Trost, C. (2001) Competitive regulation of CaT-like-mediated Ca2+ entry by protein kinase C and calmodulin. Proc. Natl. Acad. Sci. U. S. A. 98, 3600–3605
30. Singh, A. K., McGoldrick, L. D., Twomey, E. C., and Sobailevsky, A. I. (2018) Mechanism of calmodulin inactivation of the calcium-selective TRP channel TRPV6. Sci. Adv. 4, eaau6088
Ca2+-calmodulin inhibits TRPY1

31. Dang, S., van Goor, M. K., Asarnow, D., Wang, Y., Julius, D., Cheng, Y., and van der Wijst, J. (2019) Structural insight into TRPV5 channel function and modulation. Proc. Natl. Acad. Sci. U. S. A. 116, 8869–8878

32. Hughes, T. E. T., Pumroy, R. A., Yazici, A. T., Kasimova, M. A., Fluck, E. C., Huynh, K. W., Samanta, A., Molugu, S. K., Zhou, Z. H., Carnevale, V., Rohacs, T., and Moisescu-Koval, V. Y. (2018) Structural insights on TRPV5 gating by endogenous modulators. Nat. Commun. 9, 4198

33. Geiser, J. R., Van Tuinen, D., Brockerhoff, S. E., Neff, M. M., and Davis, T. N. (1991) Can calmodulin function without binding calcium? Cell 65, 949–959

34. Bertl, A., Blumwald, E., Coronado, R., Eisenberg, R., Findlay, G., Gradmann, D., Hille, B., Kohler, K., Kolb, H. A., MacRobbie, E., Meissner, G., Miller, C., Neher, E., Padle, P., Pantoja, O., et al. (1992) Electrical measurements on endomembranes. Science 258, 873–874

35. Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Mechanism of calcium gating in small-conductance calcium-activated potassium channels. Nature 395, 503–507

36. Cyert, M. S. (2001) Genetic analysis of calmodulin and its targets in Saccharomyces cerevisiae. Annu. Rev. Genet. 35, 647–672

37. Ohya, Y., and Anraku, Y. (1992) Yeast calmodulin: Structural and functional elements essential for the cell cycle. Cell Calcium 13, 445–455

38. Mruk, K., Farley, B. M., Ritacco, A. W., and Kobertz, W. R. (2014) Calmodulation meta-analysis: Predicting calmodulin binding via canonical motif clustering. J. Gen. Physiol. 144, 105–114

39. Yap, K. L., Kim, J., Truong, K., Sherman, M., Yuan, T., and Ikura, M. (2000) Calmodulin target database. J. Struct. Funct. Genomics 1, 8–14

40. Lee, C. H., and Mackinnon, R. (2018) Activation mechanism of a human SK-calmodulin channel complex elucidated by cryo-EM structures. Science 360, 508–513

41. Bate, N., Caves, R. E., Skinner, S. P., Goult, B. T., Basran, J., Mitcheson, J. S., and Vuister, G. W. (2018) A novel mechanism for calmodulin-dependent inactivation of transient receptor potential vanilloid 6. Biochemistry 57, 2611–2622

42. Derler, I., Hofbauer, M., Kahr, H., Fritsch, R., Muik, M., Keppinger, K., Hack, M. E., Moritz, S., Schindl, R., Groschner, K., and Romanin, C. (2006) Dynamic but not constitutive association of calmodulin with rat TRPV6 channels enables fine tuning of Ca2+-dependent inactivation. J. Physiol. 577, 31–44

43. Kovalevskaya, N. V., Bokhovchuk, F. M., and Vuister, G. W. (2012) The TRPV5/6 calcium channels contain multiple calmodulin binding sites with differential binding properties. J. Struct. Funct. Genomics 13, 91–100

44. Bonilla, M., and Cunningham, K. W. (2002) Calcium release and influx in yeast: TRPC and VGCC rule another kingdom. Sci. STKE 2002, pe17

45. Su, Z., Zhou, X., Haynes, W. J., Loukin, S. H., Anishkin, A., Saimi, Y., and Kung, C. (2007) Yeast gain-of-function mutations reveal structure-function relationships conserved among different subfamilies of transient receptor potential channels. Proc. Natl. Acad. Sci. U. S. A. 104, 19607–19612

46. Tang, J., Lin, Y., Zhang, Z., Tikuova, S., Birnbaum, L., and Zhu, M. X. (2001) Identification of common binding sites for calmodulin and inositol 1,4,5-trisphosphate receptors on the carboxyl termini of trp channels. J. Biol. Chem. 276, 21303–21310

47. Phillips, A. M., Bull, A., and Kelly, L. E. (1992) Identification of a Drosophila gene encoding a calmodulin-binding protein with homology to the trp phototransduction gene. Neuron 8, 631–642

48. Chi, B., Liu, C. H., Sengupta, S., Gupta, A., Raghu, P., and Hardie, R. C. (2013) Common mechanisms regulating dark noise and quantum bump amplification in Drosophila photoreceptors. J. Neurophysiol. 109, 2044–2055

49. Oubukhov, A. G., Schultz, G., and Luckhoff, A. (1998) Regulation of heterologously expressed transient receptor potential-like channels by calcium ions. Neuroscience 85, 487–495

50. Scott, K., Sun, Y., Beckingham, K., and Zaker, C. S. (1997) Calmodulin regulation of Drosophila light-activated channels and receptor function mediates termination of the light response in vivo. Cell 91, 375–383

51. Maurer, P., Redd, M., Solsbacher, J., Bischoff, F. R., Greiner, M., Podtelejnikov, A. V., Mann, M., Stade, K., Weis, K., and Schlenstedt, G. (2001) The nuclear export receptor Xpo1p forms distinct complexes with NES transport substrates and the yeast Ran binding protein 1 (Yrb1p). Mol. Biol. Cell 12, 539–549

52. Schlenstedt, G., Smirnova, E., Deane, R., Solsbacher, J., Kutay, U., Gorblich, D., Ponstingl, H., and Bischoff, F. R. (1997) Yrb4p, a yeast Ran-GTP binding protein involved in import of ribosomal protein L25 into the nucleus. EMBO J. 16, 6237–6249

53. Wright, A. P., Bruns, M., and Hartley, B. S. (1989) Extraction and rapid inactivation of proteins from Saccharomyces cerevisiae by trichloroacetic acid precipitation. Yeast 5, 51–53

54. Schlenstedt, G., Wong, D. H., Koepf, D. M., and Silver, P. A. (1995) Mutants in a yeast Ran binding protein are defective in nuclear transport. EMBO J. 14, 5367–5378

55. Maruyama, K., Mikawa, T., and Ebashi, S. (1984) Detection of calcium binding proteins by 45Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. J. Biochem. 95, 511–519

56. Phillips, S., Trost, C., Warrat, J., Rautmann, J., Himmerkus, N., Schroth, G., Kretz, O., Nastainczyk, W., Cavale, A., Hoth, M., and Flockerzi, V. (2000) TRP4 (CCE1) protein is part of native calcium release-activated Ca2+-like channels in adrenal cells. J. Biol. Chem. 275, 23965–23972

57. Lai, C. W., Aronson, D. E., and Snapp, E. L. (2010) BiP availability distinguishes states of homeostasis and stress in the endoplasmic reticulum of living cells. Mol. Biol. Cell 21, 1909–1921

58. Chang, H. F., Mannebach, S., Beck, A., Ravichandran, K., Krause, E., Frohweiler, K., Fecher-Trost, C., Schirra, C., Patru, V., Flockerzi, V., and Rettig, J. (2018) Cytotoxic granule endocytosis depends on the Flower protein. J. Cell Biol. 217, 667–683

59. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122, 19–27