Hypotonic shocks activate rat TRPV4 in yeast in the absence of polyunsaturated fatty acids

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Abstract

Transient-receptor-potential channels (TRPs) underlie the sensing of chemicals, heat, and mechanical force. We expressed the rat TRPV1 and TRPV4 subtypes in yeast and monitored their activities in vivo as Ca²⁺ rise using transgenic aequorin. Heat and capsaicin activate TRPV1 but not TRPV4 in yeast. Hypotonic shocks activate TRPV4 but not TRPV1. Osmotic swelling is modeled to activate enzyme(s), producing polyunsaturated fatty acids (PUFAs) to open TRPV4 in mammalian cells. This model relegates mechanosensitivity to the enzyme and not the channel. Yeast has only a single Δ9 fatty-acid monodesaturase and cannot make PUFAs suggesting an alternative mechanism for TRPV4 activation. We discuss possible explanations of this difference.

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1. Introduction

The seven subtypes of transient-receptor-potential channels (TRPs) are sensors of temperature, mechanical force, noxious chemicals, and are key elements in some G-protein-coupled receptor (GPCR)-mediated signal transductions [1,2]. Among the TRPV (vanilloid) subtype, TRPV1 is best known to respond to noxious heat and its surrogate capsaicin, and TRPV4 to hypotonic shocks [3,4]. All TRPs are promiscuous and can be activated by multiple means. Rat TRPV4 expressed in cultured mammalian cells can be activated by polyunsaturated fatty acids (PUFAs) [5,6], phorbol esters [5], warm temperatures [7–9], and hypotonic shock [3,4]. Placing a mammalian TRP channel in cultured mammalian cells provides it with a similar environment, likely preserving much of its physiology. However, such an environment is complicated, presumably having the channel in a complex network of native regulators, potentially making it difficult to tease apart the precise molecular mechanism by which specific stimuli lead to channel opening. This is particularly difficult for stimuli such as temperature, which has profound global effects on cell metabolism. Likewise, osmotic swelling over minutes globally induces many processes, making it difficult to know whether a TRP activation is due directly to membrane swelling itself or by a downstream consequence of swelling. Indeed, there is evidence that swelling activates enzyme(s) that produce PUFAs to activate TRPV4 in HEK cells [5,6]. We expressed rat TRPV1 and TRPV4 in yeast where the rat channels are much less likely to be affected by multiple regulatory network as in animal cells. Thus, yeast expression may make it easier to fathom the channels’ own molecular properties. We found that the yeast expression faithfully reproduces some key features: rat TRPV1 being sensitive to heat and capsaicin; TRPV4 to hypotonic shock. However, TRPV4 does not respond to warmth in yeast, as it was sometime reported to do in HEK cells [7–9]. Interestingly, there also seems to be another mechanism for TRPV4 activation by hypotonicity since yeast does not make PUFAs.

2. Materials and methods

2.1. Yeast strains and media

All experiments were conducted on BYYT, a yvcl::tok1::kanMX4 derivative of BY4742 (MATa, ura3Δ0, his3Δ1, leu2Δ0, lys2Δ0, yvcl::HIS3, tok1::kanMX4). Cells were cultured using a 30 °C shaker in leu−, ura− “DCD” medium [10], which was supplemented with 1 M sorbitol in the case of the hypotonic shock experiments. Cells modify the medium as they grow, so the actual total osmolarity was directly measured before shock. All cells were transformed with
the leu-selectable aequorin-expressing plasmid pEVPl1/Aeq [11] as well as ura-selectable TRP-channel-expressing plasmids. p416GPDV4 was created by amplifying the rTRPV4 ORF by PCR from a cDNA clone generously supplied by W. Liedtke and subcloned into the vector p416GPD [12]. rTRPV1, a generous gift of D. Julius, was similarly subcloned into p416GPDGFP to make the plasmid p416GPDV1. The M680K filter mutation of TRPV4 was generated by standard PCR site-directed mutagenesis techniques. All plasmids were transformed into BYYT by electroporation [13].

2.2. Luminometry

Luminometry was as described [11]. For hypotonic shocks, the osmolarity of the culture and other solutions were measured with a Wescor 5500 Vapor Pressure Osmometer (Logan, UT, USA). 20 \( \mu L \) of cell culture was placed in a tube in the luminometer and shocked with 200 \( \mu L \) of a solution containing between 500 mM and 100 mM NaCl and 25 mM NaEGTA or NaMES (pH 7.2). For the capsaicin experiments, 20 \( \mu L \) cultures were exposed to 200 \( \mu L \) DCD media containing 10 \( \mu M \) capsaicin (Cayman Chemical Company, Ann Arbor, MI, USA). Heat responses were evoked by injecting 1 ml of pre-warmed DCD media, measured with a thermocouple to 0.1 \( ^\circ C \) accuracy, onto 50 \( \mu L \) cells. The temperature of the injector was measured directly with a thermocouple.

3. Results

3.1. Hypotonic stress mobilizes Ca\(^{2+}\) through TRPV4 in yeast

We used transgenic aequorin to follow the activity of rat TRPV4 expressed in yeast cells deleted of their native TRP-channel gene (yvc1) [11] (Fig. 1A). A 750-mOsM hypotonic shock from 1400 to 650 mOsM causes a rise of cytoplasmic Ca\(^{2+}\) peaking within 1 min of TRPV4 transformants (Fig. 1B). Such a robust response has been repeatedly observed (n > 50). The peak response is proportional to the degree of hypotonic shock above 500 mOsM (Fig. 1C). Empty-plasmid transformants gave little response (Fig. 1B and C). Transformants with an M680K mutation in the channel filter that blocks conduction [14] also gave little response, strongly indicating that the hypototically induced Ca\(^{2+}\) flux is due to TRPV4 channel activity (Fig. 1B and C). rTRPV4 does not affect aequorin production: no difference in luminescence measured after digitonin permeation was seen between cultures expressing the empty or the experimental plasmids (data not shown). Like that of the native Yvc1 [10], the rTRPV4 response required yeast cells to be in a post-logarithmic state of growth.

3.2. TRPV4 releases Ca\(^{2+}\) exclusively from internal store(s)

EGTA (25 mM) was included in the solutions in the above experiment (Fig. 1B and C), indicating that Ca\(^{2+}\) there was released from an internal store(s). Presence of 25 mM MES or 25 mM EGTA made little difference to the response of 750 mOsM shock, indicating that the Ca\(^{2+}\) is entirely from internal release (Fig. 2A). Stronger (950 mOsM) hypotonic shocks, however, induce in the TRPV4 transformant as well as the empty-plasmid control a chelatable component (Fig. 2B) [11,15], which may reflect the 35-pS mechanically activated conductance in the yeast plasma membrane [16]. Direct addition of 10 \( \mu M \) PUFA 5\( \,\)6-epoxyeicosatrienoic acid or 10 \( \mu M \) 4z-phorbol 12,13-didecanoate elicited no response [5], presumably because they cannot access the internal membranes over the course of the experiments (data not shown). GFP-tagged TRPV1, when visible, labels the cell periphery uniformly as a faint
halo, suggestive of its residence in the plasma membrane. Neither GFP-tagged wild-type nor the M680K TRPV4 shows any discernable peripheral signal. Labels, when detected, are all internal. In some cases, they are in small particles reminiscent of the ER-associated compartment (ERAC) often induced by the over-expression of foreign proteins (data not shown).

3.3. Stimulus-appropriate responses from TRPV1 and TRPV4

For comparison, we have also expressed in yeast the rat TRPV1, which responds to capsaicin addition (Fig. 3A, black). TRPV1 is apparently located largely in the plasma membrane of yeast [18], since external EGTA addition removes the signal (Fig. 3A, red). Hypotonic shock did not induce significant Ca\(^{2+}\) release through TRPV1 in logarithmic (Fig. 3B, black) or post-logarithmic cells (data not shown) in the presence or absence (not shown) of EGTA. Conversely, the TRPV4 transformants responded to hypotonic shock (Fig. 3B, blue). They did not respond to capsaicin either in logarithmic (Fig. 3A, blue) or post-logarithmic phase (data not shown). Presence of 10 \(\mu\)M ruthenium red, the TRP-channel blocker [19], had no effect on the hypoosmotic response of TRPV4-expressing cells (Fig. 3B, green), further indicating TRPV4’s internal localization, but blocked the capsaicin response in TRPV1-expressing cells (Fig. 3A, green), showing that the cell wall is not a barrier for compounds such as ruthenium red.

3.4. Heat does not activate TRPV4 in yeast

As shown in Fig. 4, TRPV4-expressing yeast cells do not respond to heat above background (empty-plasmid control). On the other hand, TRPV1-expressing cells respond to noxious heat above 40 °C. As was the case with the capsaicin responses, the TRPV1 response is only evident with logarithmic but not post-logarithmic cultures, where neither TRPV1 nor TRPV4 responded to heat (data not shown).

4. Discussion

The internal localization of TRPV4 made ineffective the external application of PUFAs, 4\(\alpha\)PDD, and ruthenium red. In want of pharmacological evidence, our claim that the Ca\(^{2+}\) luminometry response is indeed that of TRPV4 rests on two other types of
evidence. Physiologically, the robust response is to hypoosmolality, resembling TRPV4's responses in live worm, in mammals, and in cultured mammalian cells. Genetically, the response is absent with empty plasmid or when a point mutation is engineered at the filter of TRPV4.

PUFAs are needed for worm olfactory and nociceptive signaling with Osm-9, a TRPV homolog[20]. The specific PUFA, 5,6'-epoxyeicosatrienoic acid (5,6'-EET), not its isomers, is needed in hypotonic but not chemical or heat activation of TRPV4 in HEK cells[5,6]. It was proposed that hypotonic swelling activates phospholipase A2, releasing 5,6'-EET to activate TRPV4[6,21]. The Saccharomyces cerevisiae genome was sequenced in 1996, first among eukaryotes[22]. It has only a single fatty-acid desaturase, Ole1p, which produces A9 monounsaturated fatty acids[23,24]. Yeast can take in polyunsaturated phospholipids from the medium, but the cells were cultured without lipid supplement here. Yeast also could not have made PUFAs from such lipids, even if supplied, since it also lacks phospholipase A2 for the necessary hydrolysis. Our findings therefore show that hypotonic shocks can activate TRPV4 without PUFAs, including 5,6'-EET in some context.

It is formally possible that hypotonic swelling produces an element in yeast that activates TRPV4 in place of PUFA. However, it seems unlikely that yeast has an element that can fulfill the stringent chemical specificity of 5,6'-EET[5]. It is also possible that PUFA relieves TRPV4 from an innate inhibition in the mammalian membrane, which is absent in the yeast membrane. Yet another possibility is that TRPV4 itself senses the forces from the membrane. PUFAs have been shown to change the internal force profile of lipid bilayer in molecular dynamics simulation[25]. It has also been proposed that PUFAs enter the two monolayers differentially straining the bilayer to activate TREK1, the mechanosensitive two-pore-domain K+ channel[26], as in the activation of MscL by amphipaths[27]. The yeast membrane has a different composition and may have an internal force profile that can activate TRPV4 without the need of PUFA addition. Further experiments, including direct patch clamping of TRPV4 in yeast membrane, will be needed to test these possibilities.

The expression of TRPV4 in internal membrane(s) hampers direct patch-clamp examination in yeast. Traffic convolution is apparently a fairly common problem in heterologous expression. E.g. much of TRPV4 transiently expressed in HEK cells is found in intracellular membranes, while that in stably transformed cells in the plasma membrane[7]. TRPML1 and 3, normally expressed in endosomal and lysosomal membranes[28], are found in the plasma membrane when expressed in HEK cells[29]. Some re-routing of TRPV4 may be necessary in future investigations of TRPV4 in yeast. As to the in vivo assay here, water entry should swell the cytoplasm as well as the organelles therein. That a stronger shock is required to activate TRPV4 in yeast than in HEK cells may simply reflect the difference in cytology and TRPV4 location.

Myers et al.[18] has shown that TRPV1 in a multicopy 2μ plasmid stops yeast growth, when capsaicin is applied. Here, we show the time course of TRPV1 activation upon capsaicin addition in yeast cells transformed with TRPV1 in a centromeric plasmid (Fig. 3A). That TRPV1 is a heat sensor is well established. Whether heat activates TRPV4, however, is controversial[3–5,7,8,30]. Our

Fig. 4. Noxious heat above 45 °C causes a clear increase in cytoplasmic Ca2+ in TRPV1 transformants (upper three traces in each panel), whereas TRPV4 transformants (grey traces) do not have a response greater than yeast transformed with empty-plasmid (bottom black traces). All cultures were in logarithmic phase of growth. Heat is given as an estimate of the initial temperature at the point of injection (see Section 2), but little difference in the actual temperature between samples existed as evidenced by the consistency in responses, which are shown in triplicate. 1.2 × 10⁶ cells per test.
findings indicate that TRPV4, isolated from the context of mammalian cells, is not a heat sensor (Fig. 4). Such an isolation from the complex network of neighboring mammalian elements offers a particular advantage to study mechanical force and heat, which, unlike specific ligands, are blunt stimuli, capable of influencing multiple regulatory pathways that indirectly impact promiscuous TRP channels.

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