WILD-TYPE AND BRACHYOLMIA-CAUSING MUTANT TRPV4 CHANNELS RESPOND DIRECTLY TO STRETCH FORCE.

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Summary

Whether animal ion channels functioning as mechanosensors are directly activated by stretch force or indirectly by ligands produced by the stretch is a crucial question. The transient receptor potential channel subtype V4 (TRPV4), a key molecular model, can be activated by hypotonicity (1,2), but the mechanism of activation is unclear. One model has this channel being activated by a downstream product of phospholipase A₂, relegating mechanosensitivity to the enzymes or their regulators (3). We expressed rat TRPV4 in Xenopus oocytes and repeatedly examined >200 excised patches bathed in a simple buffer. We found that TRPV4 can be activated by tens of mm Hg pipette suctions with open probability rising with suction even in the presence of relevant enzyme inhibitors. Mechanosensitivity of TRPV4 provides the simplest explanation to its various force-related physiological roles, one of which is in the sensing of weight load during bone development. Gain-of-function (GOF) mutants cause heritable skeletal dysplasias in human (4,5). We therefore examined the brachyolmia-causing R616Q GOF channel and found increased whole-cell current densities compared to wild-type channels. Single-channel analysis revealed that R616Q channels maintain mechanosensitivity but have greater constitutive activity and no change in unitary conductance or rectification.

Introduction

In stark contrast with vision, smell, and most tastes, which are based on G-protein coupled receptors, the mechanical senses (hearing, touch, balance, monitoring blood pressure or systemic osmolarity etc.) are poorly understood in molecular terms. Although prokaryotic mechanosensitive (MS) channels are clearly activated directly by membrane stretch force (6), how eukaryotic MS channels are activated is still under debate. Models range from direct activation by stretches from membrane and/or cytoskeleton to indirect activation through stretch-produced ligands (7,8). The mammalian 2-pore K⁺ channels TREK1 and TAASK are activated both by membrane stretch and polyunsaturated fatty acids (PUFAs) (9). There, cytoskeletal disruption actually increases mechanosensitivity suggesting that the cytoskeleton does not transmit stretch force to these channels (10).

Among the Ca²⁺-permeable transient-receptor-potential channels, yeast TRPY1 (11) and animal TRPV4 (12) have been studied extensively for their response to osmotic or mechanical stimuli. TRPY1 can be activated directly by suctions applied to excised membrane patches (11,13). Results from such a test from limited
preliminary studies found in the TRPV4 literature, however, are inconsistent and even contradictory (1,2,14) likely reflect mechanical complexities of patches excised from animal cells and/or molecular heterogeneity.

Rodent TRPV4s were first cloned repeatedly by following hypotonicity-induced Ca\textsuperscript{2+} signal (1,2). Rat \textit{trpv4} complements the mechano- and osmosensitivity defects of \textit{osm-9} mutant worm (15). How osmotic force activates TRPV4, however, is unclear. Besides hypotonicity, this polymodal channel is also activated by PUFAs (16) among other stimuli (12). One study used enzyme inhibitors to block the hypotonicity response and concluded that swelling activates TRPV4 through a specific PUFA, namely 5',6'-epoxyeicosatrienoic acid (5',6'-EET), produced by an enzyme pathway comprising phospholipase A2 (PLA\textsubscript{2}) and cytochrome-P450 epoxygenase (EPG) (3,16). This model therefore has the PUFA-producing enzyme(s) or their upstream regulator(s) as the force sensor and TRPV4 a ligand-gated and not a mechanically gated channel. Rat TRPV4 expressed in the budding yeast can also be activated by hypotonicity. This finding questions this model since yeast has no PLA\textsubscript{2} or PUFAs (17). Because stretching TRPV4-expressing patches should provide a direct test and because TRPV4 expresses strongly in \textit{Xenopus} oocyte (18), we have undertaken a systematic examination on the possible direct mechanosensitivity of TRPV4 so expressed. Here, we report that TRPV4 is in fact directly mechanosensitive.

Among its many functions, TRPV4 appears to gauge forces sustained by bones. Unloading-induced bone loss is suppressed in \textit{trpv4}\textsuperscript{-/-} mice (19). Recently, TRPV4 gain-of-function (GOF) mutations were found to cause autosomal dominant blockage of bone development in human. In heterozygotes, clinical manifestations range from post-natal brachyolmia (4), spondylometaphyseal dysplasias and metatropic dysplasia (5,20) to infantile and neonatal lethality (5). Expressed in HEK cells, some, though not all, of these GOF alleles present larger whole-cell currents than the wild type (4,20). No patch-clamp examination of these mutant channels has been reported to date. We conducted an extensive analysis of the brachyolmia-causing R616Q allele and found it to have an increased constitutive activity but also display direct force activation.

**Experimental Procedures**

\textit{Oocyte Expression:} Wild-type or mutant alleles were PCR amplified using high-fidelity PfuUltra polymerase (Stragene) and integrated into pGH19, an oocyte-specific cRNA expression plasmid (21). cRNA was synthesized from Xho1-linearized templates using a mMessage mMACHINE T7 kit (Ambion). Stage V and VI \textit{X. laevis} oocytes were injected with 40 nl of diluted RNA solution. Since TRPV4 expression, particularly of GOF alleles is toxic to oocytes, 1\textmu M ruthenium red (Sigma) was added to the ND96 incubation buffer (22). Channel activities increased over several days. Currents were measured after 1 to 5 days depending on experimental requirement.

\textit{Electrophysiological Techniques:} Two-electrode voltage-clamp recording used HS2A head stages and a VG-2Ax100 virtual bath clamp connected to a Gene Clamp 500 amplifier interfaced through
a Digidata 1440A digitizer acquired using pClamp10 software (all Axon Instruments). The base bath solution contained (in mM) 66 KCl, 100 sorbitol, 1.8 BaCl₂, and 5 K⁺-HEPES, pH 7.2 (all Sigma). Three μM 4α-phorbol 12,13-didecanoate (4αPDD, Sigma) was added directly to the bath. Sorbitol was omitted from the base solution to form the hypotonic solution. Data was analyzed using both pClamp10 and Sigma Plot 2000 (SPSS) software. Patch clamp used borosilicate glass pipette with ~ 1-μm diameter opening at the tip, recorded in excised inside-out mode with symmetric 98 KCl, 1 MgCl₂, ad 10 K⁺-HEPES, pH 7.2 (all Sigma), unless otherwise stated. Data acquired at 10 kHz through an eight-pole Bessel filter at 1 kHz, played back at >5 kHz for analysis. Suctions were applied through a syringe and gauged with a PX140 pressure sensor (Omega).

Results

Wild-type and R616Q Macroscopic Currents

We examined whole-oocyte currents under two-electrode voltage clamp. *Xenopus* oocytes 3 days after injection of 4 ng of wild-type TRPV4 cRNA present small currents that can be greatly amplified by the addition of 3 μM of the synthetic phorbal-ester activator 4αPDD (23) (n > 30, Fig. 1a, top). As expected of TRPV4, these currents show prominent outward rectification (24). Un-injected oocytes (n > 10) or those injected with TRPV4 cRNA with a M680K mutation engineered at the presumed filter (15) showed no such currents (n > 10, Fig. 1a, bottom). Perfusion of a hypotonic solution also activates these currents over minutes, which subside upon the return to isotonicity (n > 10). This cycle can be repeated with the same oocyte except when blocked by TRP blocker ruthenium red (Fig. 1b). Although a native stretch activated channel is observed in excised patches from oocytes, hypotonicity fails to activate currents in the majority of whole oocytes examined by two-electrode voltage clamp (25).

Oocytes injected with 4 ng of cRNA of the brachyolmia-causing R616Q mutant allele, present very large currents even without 4αPDD or hypotonicity, the magnitudes of which are greater than oocytes injected with 10-fold more wild-type cRNA (Fig. 1c). Despite variations, expressed currents clearly rise with the amount of cRNA injected. Assuming the same production of channels per unit injected RNA, comparisons show that the R616Q channel has about thirty times the steady-state activities of the wild-type channel (Fig. 1d). R616Q-expressing oocytes exhibit macroscopic currents that show outward rectification, ruthenium-red blockage and stimulation by 4αPDD and hypotonicity like the wild type (data not shown). Oocytes rarely survive the injection of more than 40 ng wild-type TRPV4 cRNA or more than 4 ng of R616Q cRNA. Toxicity of TRPV4 stronger GOF mutants selected through yeast genetics has also been observed (23).

Unitary Conductance.

Inside-out patches excised from TRPV4-expressing oocytes exhibit a large unitary conductance, not observed in uninjected oocytes or those injected with filter-mutant M680K RNA (data not shown). Spontaneous activities of wild-type or R616Q channels tend to run down immediately after patch excision.
Channel activity was allowed to relax to a more stable level before quantification (Fig. 2a). i-V plots of both the wild-type and the R616Q channels show unitary-conductance rectification, being 98 pS outward and 45 pS inward (Fig. 2b), easily distinguishable from the ~30 pS conductance native to the oocyte (26) (supplementary Fig. S1), which sometimes appeared in the same patches. TRPV4 activity could also be distinguished from that of this native channel by its 4αPDD activation and time-dependent blockage by ruthenium red back-filled in the patch pipette (data not shown). Ensemble open probabilities (nPo) of wild-type and mutant channels are small at negative voltages and rise steeply at positive voltages (Figs. 2a, c). Matching the amount of cRNA injected and incubation period, the R616Q patches invariably have higher basal activities than the wild-type patches. Activation of multiple R616Q channels at positive voltage gives a noisy impression (Fig. 2a), however, unitary conductance of R616Q can nonetheless be measured from basal activities from oocytes injected with less RNA (Fig. 5) or at negative potential (Fig. 2a).

**Suction Activates TRPV4**

Tens of mm Hg suction pulses applied to membrane patches excised from expressing oocytes directly activate TRPV4 channels (from >200 patches of >50 oocytes). Such activations are shown in sample traces in Fig. 3a for the wild-type and Fig. 5 a,b,f,g, for the R616Q-mutant channels. From the same excised patches, after the run down of spontaneous activities, nPo rises with the pipette suction (Fig. 3b,c). This increased nPo maintained over the course of 10-20s of pressure exposure (e.g. Figs 5 a, b, f, g) and in the cases where pressure sensitivities were assessed before patch excision, no significant changes was observed after excisions (data not shown). In no instances could TRPV4 nPo reach saturation before the applied suction ruptured the patch or the seal at about 60 to 200 mmHg. Starting with sizable spontaneous activities, the nPo of R616Q TRPV4 increases 3 to 4 folds, with a 10 to 15% s.e.m. between 0 and 60 mm Hg suction (Fig. 3c, open symbols, Fig. 5b,j). A similar rise in the wild-type nPo is seen between 30 to 60 mm Hg suction (Fig. 3c, closed symbols). The R616Q basal activities appear to cause a left shift of the nPo vs. force response curve but variability and other technical limitations preclude slope comparison (Fig. 3c). The basal activities of the wild type among different patches are low and variable. Estimate of mechanosensitivity as the fold increase in nPo by suction is therefore carried out with R616Q instead of the wild type.

A **PLA₂** Inhibitor Blocks General Activation of TRPV4 in intact Oocytes.

It has been reported that hypotonic but not 4αPDD activation of TRPV4 expressed in cultured cells can be blocked by pre-incubating them for 20 minutes in the presence of 100 μM of the PLA₂ inhibitor bromophenacyl bromide (BPB) (27). Similar treatment had no effect on the ability of hypotonicity or 4αPDD to activate TRPV4 in whole oocytes. Because of the possibility that BPB’s access was restricted by the oocyte’s large size and complex geometry, we extended the incubation time for several hours. Indeed we found that the hypotonic response was greatly inhibited by extended incubation (Fig. 4a, c).
However, we also found that the 4αPDD response was also reduced (Fig. 4b, d) in fact to a greater extent (Fig. 4e). It thus appears that BPB can greatly inhibit the ability of either 4αPDD or hypotonicity (but not depolarization) to activate TRPV4 in intact oocytes. In the previous work the apparent specificity of BPB inhibition of the hypotonic response could have reflected a partial inhibition of PLA2 due to the shorter incubation resulting in obvious inhibition of only the weaker hypotonic response there. It is notable that depolarization activation was not substantially affected by BPB in the absence of additional stimulation, underscoring the independence of voltage- from chemical- and mechanical-dependent gating (23). Anthranilic acid (ACA), another PLA2 inhibitor at 20 μM for 15 min (27) also had no effect on TRPV4’s hypotonic response. 1 hour treatment with 50 μM ACA is toxic to oocytes.

Blockers of 5’,6’-EET Synthesis have no Effect on the Direct Mechanosensitivity of TRPV4 in Excised Patches.

We quantified the effects of PLA2 or EPG inhibitors on the mechanosensitivities of R616Q and wild-type alleles of oocyte expressed TRPV4 in excised patches as well. Ten R616Q membrane patches were sampled and their responses to 60-mm Hg pipette suctions were registered as controls. This procedure was repeated on oocytes pre-incubated in BPB (Fig. 5, top) or 17-octadecynoic acid (17-ODYA), an inhibitor of EPG (3,16) (Fig. 5, bottom) for between 1 and 3 hours. The inhibitors were also present in the recording chamber where they had direct access to the excised membrane patches. No significant changes in channel responses to suctions were observed by the incubation of these inhibitors. Qualitatively similar results were also obtained with the wild-type channels, with no difference in the mechanosensitivity observed after inhibitor exposure (data not shown). Thus, the direct mechanosensitivity of TRPV4 in excised patches seems to be independent of the production of AA or its metabolite 5’,6’-EET.

Discussion

We found rat TRPV4 to respond directly to pipette suctions that stretch the excised membrane patches. This mechanosensitivity is robust and consistent, observed in more than 200 wild-type or R616Q mutant patches (Figs. 3, 5). TRPV4’s response to stretch force in excised patches bathed in a simple buffer rules out any covalent chemistry dependent on diffusible cytoplasmic factors being involved in transduction.

Inhibition of either PLA2 by BPB or EPG by 17-ODYA had no effect on the direct mechanosensitivity of TRPV4 in excised patches (Fig. 5). However, prolonged exposure to BPB strongly inhibits both the hypotonic response and the 4αPDD response of intact oocytes (Fig. 4). This could reflect a Ca2+-dependent feedback mechanism which could facilitate an all-or-none response of TRPV4 to stimulation. PLA2 requires Ca2+ binding to its C2 domain for activity (28). Threshold initial Ca2+ entry through the force-gated TRPV4 could subsequently activate the Ca2+-PLA2-EPG-5’,6’-EET pathway to provide a positive feedback to enhance TRPV4's Ca2+ influx. BPB would block such a feedback. Such a Ca2+-induced Ca2+-release (CICR) feedback is fairly
common and has been documented for the mechanosensitive TRPY1 (11,13).

Beyond the control of systemic osmolarity (1,29), TRPV4 has been implicated in other force-related transduction \textit{in vivo}, including flow-mediated dilation of arteries (30), strain-induced endothelial cell reorientation (31), viscosity-coupled epithelial ciliary activity (32,33) and osteoclast response to weight load on bones (19). In these instances, a direct mechanosensitivity of TRPV4 channel itself provides the simplest explanation.

We encountered patch-to-patch heterogeneity, which may reflect differences in patch elasticity or other geometric and mechanical complexity of the membrane (34) with its subtending cytoskeleton (35,36), and/or heterogeneity of the channel populations (e.g. degree of phosphorylation?). These intricacies are beyond present technical resolution to sort out and may underlie the statistical variances reported within studies (3,16) and contradictory claims between studies (1,2,14) found in the TRPV4 literature.

Recent discoveries on heritable human diseases due to TRPV4 mutations illustrate the medical importance of TRPV4 (4,37). We found the brachyolmia-causing R616Q allele to retain wild-type unitary conductance and rectification but have constitutive activities in the absence of applied stimuli (Figs 1,2,3,4). R616 is predicted to be at the base of the 5th transmembrane helix (S5) in the TRPV4 subunit. Recent forward-genetic screen for TRPV4 mutations that are toxic to yeast yielded GOF alleles nearby (L619P, L624P). These yeast-selected mutations had similar but much stronger GOF phenotypes than R616Q (23). These findings support a general \textit{Shaker}-like topology for TRPV4, with S5 being a part of the main gate. Because the mutant channel has direct mechanosensitivity and constitutive activities, it likely behaves in osteoclasts or other relevant cells as a wild-type channel under mechanical stress, leading to the GOF phenotypes \textit{in vivo} (4,5). High-resolution analyses of other disease-causing alleles under patch clamps should further our understanding of the disease process.

**References**


Footnotes
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Figure Legends

Fig. 1 Macroscopic currents of rat TRPV4 expressed in oocytes. (a) Currents from oocytes expressing wild-type 3 days after 4 ng cDNA injection (top row) or 40 ng of the non-conducting M680K TRPV4 (bottom) upon voltage steps (from -60 mV hold, to -100 to +60 test, in 20 mV increments), before (left) or 20 min after (right) exposure to 3 μM 4αPDD. (b) Peak currents from an oocyte expressing very high levels of wild-type TRPV4 (5 days after 40 ng injection), upon 100 ms voltage steps (from -20 to +20 mV every 10 s) in response to the removal of 100 mM sorbitol from the 250 mOsM bath solution (open bars) and the addition of 3 mM ruthenium red (filled bar). Inset shows the raw traces from which peak currents were assessed. (Only every fifth trace from time a to a’ is displayed for clarity). (c) Typical currents from oocytes injected with 4 ng of R616Q cRNA (top) or with 4 ng or 40 ng wild-type cRNA (bottom). Injections of R616Q RNA above 4 ng kill the oocytes. Tested 3 days after injection with voltage steps as in (a) but with shorter durations. (d) Expressed currents (assessed at +60 mV) of 15 wild-type-expressing oocytes (filled circles) and 15 R616Q-expressing oocytes (open circles) injected with different amount of cRNA examined 66 to 72 hr after injection.

Fig. 2 Spontaneous microscopic TRPV4 currents. (a) Spontaneous single-channel activities from a patch excised from an oocyte highly expressing wild-type (left) and a typical one expressing R616Q (right), examined at +100 mV (top) or -100 mV (bottom).
Segments of the records are displayed at a faster time base showing discrete opening and closing events. C marks the closed levels. (b) i-V plots showing the unitary conductances of wild type (filled) and R616Q (open circles). (c) nPo-V plot from a typical wild-type TRPV4-expressing patch showing activation by positive voltages

**Fig. 3** Direct activation of wild-type TRPV4 by membrane stretch. (a) Sample of raw traces of averaged quality from three patches excised from three different oocytes, showing activation by 60 mm Hg suctions applied to excised patches held at +50 mV under a patch clamp. Top traces are displays at a faster time base to show unitary transitions between closed (C) and open (O1, O2) levels. (b) Sample traces from a high-quality wild-type patch subjected to a range of pipette suction. Recorded with a pipette solution of 98 mM KCl, 1 mM MgCl2, 20 mM Na+ citrate, pH 4.5. (c) Plots of nPo vs. suction of wild-type (filled) and R616Q (open symbols). nPo from three patches each are normalized to that at 60 mm Hg. Different symbols are different typical patches chosen to show the trend as well as variability; curves are segments of symmetric sigmoidal fits drawn by eye.

**Fig. 4** BPB inhibits both the hypotonic and 4αPDD response in intact oocytes. Oocytes either not exposed to (a and b) or incubated for between 5 and 9 hours in 100μM BPB (c and d) were subjected to 1s voltage steps of between -100 and 60 mV either before (circles) or after (squares) exposure to hypotonicity (as described in Fig. 1) for 10 min (a and c) or to 3μM 4αPDD for 20 min (b and d). BPB was present during experimentation as well in c and d. Plotted are peak currents vs. test potentials. (e) shows the relative increase in the peak response at 60mV in the absence (open bars) or presence of BPB (as in c and d, grey bars) to hypotonicity (left) or 4αPDD (right, mean ± s.e.m., n=4). Note that the apparent smaller response to hypotonicity in (e) compared to that shown in Fig. 1b reflects that it was assessed at 60 mV here as opposed to 20 mV in Fig. 1b, and hypotonicity appears to cause a slight leftward shift in the G/V relationship, as can be seen in (a).

**Fig. 5** Enzyme inhibitors do not alter TRPV4 mechanosensitivity in excised patches. Five patches were excised from each of two R616Q-expressing oocytes held at +50 mV before and another 5 x 2 patches excised after 30 min incubation in the presence of 200 μM BPB (top). (a and b) show sample traces of response to suctions. (c) shows nPo’s increase by 60-mm Hg suctions in five different patches from one oocyte (black lines) and five from a second oocyte (gray lines) before the BPB treatment. (d) shows nPo’s increase by the same suctions of five new patches from the first oocyte (black lines) and five others from the second oocyte (gray lines) sampled after the BPB treatment. (e) summarizes the fold-increase in nPo from 0 to 60 mm-Hg suction. (Those with nPo ~ 0 without suction are excluded in the calculation.) (f. through i) show parallel experimental results with 100 μM 17ODYA.
Figure 1

Figure 2

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Figure 3

Figure 4
Figure 5
Wild-type and brachyolmia-causing mutant TRPV4 channels respond directly to stretch force

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