Human TRPA1 is an inherently mechanosensitive bilayer-gated ion channel

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A B S T R A C T
The role of mammalian Transient Receptor Potential Ankyrin 1 (TRPA1) as a mechanosensor is controversial. Here, we report that purified human TRPA1 (hTRPA1) with and without its N-terminal ankyrin repeat domain responded with pressure-dependent single-channel current activity when reconstituted into artificial lipid bilayers. The hTRPA1 activity was abolished by the thiol reducing agent TCEP. Thus, depending on its redox state, hTRPA1 is an inherent mechanosensitive ion channel gated by force-from-lipids.

1. Introduction
The discovery of transient receptor potential (TRP) channels involved in mammalian sensory signaling has introduced a new class of polyomodal proteins as detectors of chemical, temperature and possibly mechanical stimuli [1–3]. A number of TRP channels has been suggested to be involved in mammalian mechanosensation under normal physiological conditions as well as in pathophysiology [1,2,–7]. The proposal that mouse TRPA1, with its large intracellular N-terminal ankyrin repeat domain (N-ARD), could be a mechanosensor involved in hearing [8] triggered a great interest in TRPA1 as a potential primary mechanosensitive calcium channel within the mammalian sensory nervous system [4,5]. Although, the transmembrane channel-like protein 1 and 2 (TM1C and TM2C) are most likely the mechanosensors responsible for hair-cell transduction [9], there are still many reports of TRPA1 being involved in mechanical sensory stimulation, and especially in noxious mechanotransduction e.g., related to nerve-injury, inflammation and anti-cancer treatment [4,5]. Furthermore, TRP channels may also be important mechanosensors in non-neuronal cell signaling including cellular migration and cancer development, in which calcium plays a critical role [6,7]. At a cellular level, activation of TRPA1 by membrane stress has been observed using chemical agents and hyperosmotic solutions but not by negative pressure applied to cell-attached patches [10–12]. Regardless, no evidence of TRPA1 or other mammalian TRP channel intrinsic mechanosensitivity has been provided [2,4–7,12].

2. Materials and methods
The purification of hTRPA1 proteins and patch-clamp experiments were performed as previously described in detail [13] and are briefly outlined as follows. Purified hTRPA1 and Δ1–688 hTRPA1 were reconstituted into preformed planar lipid bilayers composed of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids) and cholesterol (Sigma-Aldrich) in a 9:1 ratio and produced by using the Vesicle Prep Pro Station (Nanion Technologies). Ion channel activity was recorded using the Port-a-Patch (Nanion Technologies) either by 2-s voltage-ramps (-100 to +100 mV) or at a holding potential of +60 mV in a symmetrical K\textsuperscript{+} solution (50 mM KCl, 10 mM NaCl, 60 mM KF, 20 mM EGTA, and 10 mM HEPES; adjusted to pH 7.2 with KOH) and at room temperature (20–22 °C). Negative pressure was applied in a stepwise manner with the suction control pro (Nanion Technologies) to evoke TRPA1 currents. Signals were acquired with an EPC 10 amplifier and PatchMaster software (HEKA) at a sampling rate of 50 kHz. Electrophysiological data were analyzed using Clampfit 9 (Molecular Devices) and Igor Pro (WaveMetrics). The single-channel mean open probability (P\textsubscript{o}) was calculated from time constant values, which were obtained from exponential standard fits of dwell time histograms. Data were processed by a Gaussian low-pass filter at 1000 for analysis and 500 Hz for traces. GraphPad Prism 7.0. (GraphPad Software, La Jolla, CA) was used for curve fitting and drawing of graphs. Data are presented as the mean ± SEM of separate experiments, indicated by n.

Abbreviations: N-ARD, N-terminal ankyrin repeat domain; TRP, Transient receptor potential; TRPA1, transient receptor potential ankyrin 1; hTRPA1, human TRPA1; HO30031, 2-(13-Dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl)acetamide
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3. Results and discussion

In this study, we have explored the possibility that human TRPA1 (hTRPA1) is an inherent mechanosensitive ion channel when purified and reconstituted into artificial lipid bilayers, using the same experimental conditions as in our studies consolidating hTRPA1 as an inherent chemo- and thermosensitive ion channel \[13–16\]. Thus, purified hTRPA1 with and without its N-terminal ARD (Δ1 − 688 hTRPA1) were reconstituted into artificial lipid bilayers for electrophysiological recordings of single-channel activity in response to changes in bilayer pressure. Under these conditions, as shown by continuous recordings, either the membrane-impermeable electrophile maleimide-biotin (n = 2) or heat (n = 2) fully activated hTRPA1 although unresponsive to a pressure of 15 mmHg. (B) Traces out of 1-2 min recordings showing lack of channel activity in the absence of hTRPA1 proteins but in the presence of the detergent Fos-Choline-14 at various pressure and Vh + 60 mV (each n = 5). C = closed channel state. Open channel state = upward deflection.

Fig. 1. Human TRPA1 is intrinsically mechanosensitive. Purified hTRPA1 was reconstituted into planar lipid bilayers and single-channel currents were recorded with the patch-clamp technique in a symmetrical K+ solution. (A) A uniform protein orientation is favored with N- and C-termini facing the recording chamber (i.e., the “cytosolic compartment”) and changes in negative pipette pressure by suction evoked rapid single-channel responses at a holding potential (Vh) of +60 mV. Under these conditions, as shown by continuous recordings, either the membrane-impermeable electrophile maleimide-biotin (n = 2) or heat (n = 2) fully activated hTRPA1 although unresponsive to a pressure of 15 mmHg. (B) Traces out of 1-2 min recordings showing lack of channel activity in the absence of hTRPA1 proteins but in the presence of the detergent Fos-Choline-14 at various pressure and Vh + 60 mV (each n = 5). C = closed channel state. Open channel state = upward deflection.

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The deletion of the N-ARD does not allow heterologous cell membrane expression of functional mammalian TRPA1 \[19\], and thus our strategy using purified Δ1 − 688 hTRPA1 offers a unique possibility to explore TRPA1 gating by bilayer pressure independent of the N-ARD as well as avoiding potential artificial effects on channel gating caused by N-ARD mutational and chimeric strategies \[5\]. We found that hTRPA1 without its N-ARD was at least equally responsive to pressure as intact hTRPA1, and thus the many N-terminal ankyrin repeats are not needed for TRPA1 to respond to mechanical stimuli as generally believed \[4,5\].
However, this does not necessarily exclude a role of the N-ARD in co-
ordinating mechanical stimuli and channel activity in a native en-
vironment by interacting with lipids of the cell membrane as well as
tethered with the cytoskeleton. The N-ARD may also intramolecularly
modulate the mechano-gating of hTRPA1 as indicated by the
flickering behavior of hTRPA1 compared to Δ1 – 688 hTRPA1 (Fig. 2B).
Furthermore, any interaction with N-ARD cysteines by electrophilic com-
 pounds and redox agents could easily change the overall protein
conformation [4,5] possibly affecting the mechsensivity of TRPA1.
Indeed, the single-channel activity of purified hTRPA1, which is par-
tially oxidized [15], evoked by 45 mmHg was abolished by the thiol
reducing agent TCEP (Fig. 2E) at a concentration that abolished pur-
i fi ed hTRPA1 single-channel activity evoked by H2O2 and cold/warm
temperatures [15]. Oxidants including H2O2 also sensitized TRPA1 heat
responses in cells and isolated tissues [15,20], and a recent study
showed H2O2 sensitization of TRPA1-mediated mechanical responses in

Fig. 2. Human TRPA1 is intrinsically mechsensitive without its N-terminal ankyrin repeat domain and depending on its redox state. Purified hTRPA1 and Δ1-688
hTRPA1 were reconstituted into planar lipid bilayers and single-channel currents were recorded with the patch-clamp technique in a symmetrical K+ solution. (A)
Exposure to a negative pressure of 52.5 mmHg evoked channel currents at both negative and positive potentials (black trace) when recorded repeatedly in 2-s voltage
ramps from -100 to +100 mV (each n = 5). Red dotted line shows zero channel current level. (B) As shown by representative traces, exposure to a change in negative
pressure evoked outward single-channel currents at a holding potential (Vh) of +60 mV. (C) The graphs show single-channel mean open probability values as a
function of applied negative pressure. Data were fitted with a Boltzmann equation and each data point is the mean ± SEM of 3-9 observations from independent
experiments (shown within parentheses). (D and E) Traces showing inhibition of hTRPA1 mechanical activity at 45 mmHg by (D) the TRPA1 antagonist HC030031
and (E) the thiol reducing agent TCEP at Vh +60 mV (each n = 3). C = closed channel state. Open channel state = upward or downward de
flection (A) and upward
deflection (B, D and E).
mouse trigeminal sensory neurons when exposed to a hyperosmotic solution [11]. Thus, it is suggested that oxidative stress can shape the response to mechanical stimuli by shifting hTRPA1 into a force-to-lipid sensitive protein conformation. Further studies are needed to investigate whether differences in TRPA1 redox environment and other environmental factors (e.g., pH, Ca²⁺, polyphosphates) known to modulate TRPA1 activity [4,5] can explain why TRPA1 and other TRP channel mechanical activity cannot always be detected at a cellular level or in artificial lipid bilayers [12]. Furthermore, if purified hTRPA1 responds to pressure in a voltage- and N-ARD-dependent manner as shown for electrophilic and non-electrophilic activators [13] as well as how the lipid composition influences TRPA1 stimulus modalities such as pressure, temperature and voltage, each or combinations thereof, are other interesting topics for future studies [21–23].

It has been suggested that the effect of non-electrophilic TRPA1 ligands may be indirect by changing the lipid tension stress on TRPA1 within the cell membrane [10,24–26]. The ability of primary and secondary alcohols as well as different alkyl-substituted phenol derivatives including carvacrol to activate hTRPA1 increased with increasing lipophilicity [10,27,28]. Our observation that carvacrol induced conformational changes of purified hTRPA1 in a bilayer-free environment indicates, however, a direct interaction with TRPA1 [15]. In another study on the structure-activity relationship of cannabinoids and TRPA1 from mouse and human, we found that methylation of the Δ⁶-tetrahydrocannabinol C-1 hydroxyl group removed its ability to activate TRPA1 although both compounds have very similar lipophilicity [29]. Furthermore, the synthetic cannabinoid and very potent cannabinoid Δ9-tetrahydrocannabinol alicyclic C-9 methyl group and the C-3 carbon tail further suggested a specific cannabinoid binding site on TRPA1 [29]. Thus, future studies are needed to understand how membrane perturbation properties including lipophilicity and amphipathicity contribute to the pharmacology of non-electrophilic TRPA1 ligands including cannabinoids.

In summary, hTRPA1 is an inherent mechanosensitive channel that like the other mammalian mechanosensitive channels TREK-1, TREK-2, TRAAK and Piezo are gated by force-from-lipids [3,2,10]. The hTRPA1 mechanosensitivity is dependent on its redox state, and it is suggested that oxidative stress shifts hTRPA1 into a protein conformation sensitive to mechanical stimuli. Future studies of TRPA1 from both vertebrates and invertebrates could help to obtain an evolutionary detailed mechanistic understanding of the intrinsic mechanosensitive properties of TRPA1 and perhaps other TRP channels.

Author contributions
L.M. and P.M.Z. designed research; L.M. performed research; L.M. and P.M.Z. analyzed data; L.M. and P.M.Z. wrote the paper.

Declaration of Competing Interest
The authors declare no conflict of interest.

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