Species-specific activation of human TRPA1 by protons

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Background: Extracellular acidosis mediates pain and inflammation by activating sensory afferent neurons.

Results: Protons activate and sensitize human TRPA1 in a strongly species-specific manner encoded by transmembrane domains 5 and 6.

Conclusion: Our data identify TRPA1 as an ion channel likely to mediate acid-induced pain in humans.

Significance: Protons are the first known endogenous agonists of TRPA1 with species-specificity for human TRPA1

SUMMARY

The surveillance of acid-base homeostasis is concerted by diverse mechanisms, including an activation of sensory afferents. Proton-evoked activation of rodent sensory neurons is mainly mediated by the capsaicin receptor TRPV1 and acid sensing ion channels. In this study we demonstrate that extracellular acidosis activates and sensitizes the human irritant receptor TRPA1 (hTRPA1). Proton-evoked membrane currents and calcium influx through hTRPA1 occurred at physiological acidic pH-values, were concentration-dependent, and blocked by the selective TRPA1 antagonist HC030031. Both rodent and rhesus monkey TRPA1 failed to respond to extracellular acidosis, and protons even inhibited rodent TRPA1. Accordingly, mouse dorsal root ganglion neurons lacking TRPV1 only responded to protons when hTRPA1 was heterologously expressed. This species specific activation of hTRPA1 by protons was reversed in both mouse and rhesus monkey TRPA1 by exchange of distinct residues within transmembrane domains 5 and 6. Furthermore, protons seem to interact with an extracellular interaction site to gate TRPA1 and not via a modification of intracellular N-terminal cysteines known as important interaction sites for electrophilic TRPA1-agonists. Our data suggest that hTRPA1 acts as a sensor for extracellular acidosis in human sensory neurons and should thus be taken into account as a yet unrecognized transduction molecule for proton-evoked pain and inflammation. The species specificity of this property is unique among known endogenous TRPA1-agonists, possibly indicating that evolutionary pressure enforced TRPA1 to inherit the role as an acid-sensor in human sensory neurons.
Species-specific activation of human TRPA1 by protons

Protons interact with and modulate numerous membrane proteins within the peripheral and central nervous system, and can act as excitatory or inhibitory co-transmitters regulating neuronal activity. Both ischemia and inflammation are often accompanied by pain, and the activation and sensitization of peripheral sensory neurons in both of these conditions are largely evoked by protons (1,2). Electrophysiological evidence for a direct proton-evoked activation of sensory neurons was presented decades ago (3), and meanwhile the identities of several membrane proteins acting as proton sensors have been identified (4). Acid sensing ion channels (ASIC) are expressed in a large population of sensory neurons and seem to contribute to acute and inflammatory pain (1,2,5,6). ASICs generate transient Na⁺ currents already upon modest extracellular acidifications (pH < 7.0), and this response is potentiated by lactate and adenosine tri-phosphate which are both released during ischemia (7,8). Stronger acidosis (pH< 6.3) also activates the capsaicin receptor TRPV1 (9), and more modest acidifications sensitize TRPV1 for activation by other TRPV1-agonists (10). Similar to ASICs, TRPV1 is required for nociceptive and inflammatory pain (1,9). Taking these properties of ASICs and TRPV1 into account, it is commonly assumed that they are key molecules for proton-evoked activation of sensory neurons and thus for proton-evoked pain (4). For obvious reasons, however, cellular studies exploring the mechanisms mediating proton-evoked activation in human sensory neurons are rare.

TRPA1 is known as a receptor for a large variety of exogenous and endogenous irritants and is crucially involved in several types of pain-related behaviour in rodents. It has been reported that both intracellular acidosis and alkalosis can activate rodent TRPA1 (13,14,15). While the study from Wang and colleagues claims that extracellular acidosis fails to activate rodent TRPA1 (14), a previous study suggested that extracellular protons can evoke a calcium influx through human TRPA1 expressed in HEK-293 cells (16) TRPA1 is indeed subject to a significant species specificity, and several exogenous agonists and antagonists have been shown to elicit different effects on human and rodent TRPA1 (17-22). We therefore asked if extracellular protons interact with TRPA1 in a species specific manner. By employing patch clamp and ratiometric calcium imaging in combination with site-directed mutagenesis, we obtained data revealing the molecular basis for an unambiguous species specificity of proton-evoked activation of human TRPA1.

EXPERIMENTAL PROCEDURES

cDNA and transfection procedures. The plasmids for human TRPA1 (hTRPA1) and hTRPA1-C621S/C641S/C665S (hTRPA1-3C) were kindly provided by Dr. Sven-Eric Jordt (New Haven, CT, U.S.A.). Mouse TRPA1 (mTRPA1), the chimeras mTRPA1-hTM5/6 and hTRPA1-mTM5/6, the mutants hTRPA1-FGATL1AM, hTRPA1-FATL, hTRPA1-IAM, hTRPA1-V875G and hTRPA1-S873L/T874L were kindly provided by Dr. Ardem Patapoutian (La Jolla, CA, USA). Rat TRPA1 (rTRPA1) was kindly provided by Dr. David Julius (San Francisco, CA, U.S.A.). Rhesus monkey TRPA1 (rhTRPA1) and the mutants were kindly provided by Dr. Jun Chen (Abbott Laboratories, Illinois, U.S.A.). All other mutants were generated by site-directed mutagenesis using the QuikChange II XL kit (Agilent Technologies, Santa Clara, CA) with modified primer design (23). Fidelity of mutagenesis was confirmed by dideoxynucleotide sequencing. Plasmids were transiently expressed in HEK293t cells by using a nanofectin transfection kit according to instructions of the manufacturer (PAA, Pasching, Austria). To visualize expression for patch clamp experiments, cells were cotransfected with pEGFP-N1 (0.5 µg, Clontech, Palo Alto, USA). After transfection, cells were replated into petri-dishes and used within 12-24 h for patch clamp recordings. Stably expressing hTRPA1-HEK293t cells were established by use of G418 (800 µg ml⁻¹). HEK-293t cells were cultured in Dulbecco’s modified Eagle’s medium (D-MEM, Gibco BRL, Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), 100 U ml⁻¹.
Species-specific activation of human TRPA1 by protons

**Electrophysiology.** The pipette solution contained (in mM) KCl 140, MgCl2 2, EGTA 5 and HEPES 10; pH 7.4 was adjusted with KOH. The external calcium free solution contained (in mM) NaCl 140, KCl 5, MgCl2 2, EGTA 5, HEPES 10 and glucose 10; pH 7.4 was adjusted with tetramethylammonium hydroxide (TMA-OH). For calcium containing experiments we used NaCl 140, KCl 5, MgCl2 2, HEPES 10 (or MES 10), glucose 10 and CaCl2 2 (mM). The osmolarity of all solutions was adjusted with glucose to 290-300 mosmol/L. Patch pipettes were fabricated with borosilicate glass (Science Products, Hofheim, Germany) using a conventional puller (DMZ-Universal Puller, Zeitz Instrumente, Martinsried, Germany) and heat polished to give a pipette resistance of 3-5 MΩ. Only one EGFP co-transfected fluorescent cell per dish was used for experiments. Test solutions were applied via a gravity-driven perfusion system. Whole cell recordings were performed using a HEKA Electronics USB 10 amplifier combined with the Patchmaster Software (HEKA Electronics, Lambrecht, Germany). Currents were filtered at 1 kHz and sampled at 2 kHz. Off-line analysis were carried out using the Fitmaster Software (HEKA) and Origin Software (Origin 8.5.1 G, Origin Lab, Northampton, MA, U.S.A.). Mean values and data for dose response curves are shown as mean ± SEM. Statistical significance was assessed with Student’s t-test (*p< 0.05 and ** p< 0.01 and *** p< 0.001).

**Ca2+ Imaging.** For calcium imaging DRG- and HEK-cells were stained by the fluorescent dye fura-2 AM (5 µM) and 0.02% pluronic (both from Invitrogen, Grand Island NY, U.S.A.) for 30 min. After 15 min washout to allow ester hydrolysis, coverslips were placed in a glass chamber on an Olympus IX71 inverse microscope and constantly superfused with extracellular fluid (in mM: NaCl 145, KCl 5, CaCl2 1.25, MgCl2 1, Glucose 10, Hepes 10). Acetic solutions (pH 5, 6, 6.4), carvacrol (250 µM), AITC (100 µM) and capsaicin (0.3 µM) were applied using a control-unit operated 7-channel gravity-driven common-outlet superfusion system. A 60 mM potassium (DRGs) or 10 µM ionomycin stimulus (HEK cells) was applied as a control at the end of each experiment. Ca2+ influx was observed by Fura-2 excitation at 340 and 380 nm with a Polychrome V monochromator (Till Photonics), emission was detected with corresponding filter at 510 nm. Images were exposed for 200 µs at a rate of 1 Hz with a 12-bit CCD camera (Imago Sensicam QE, Till Photonics, Gräfeling, Germany). Fluorescence absorbance was analysed in the integrated TILLvisION 4.0.1.3 software (Till Photonics, Gräfeling, Germany). Ratios were calculated after background subtraction by setting regions of interest (ROIs). Averaged results are reported as mean (± SEM) of area under the curve (delta ratio F340/380 nm).

**DRG neuron cell culture.** Culturing of dorsal root ganglion (DRG) neurons from C57Bl/6 mice and from congenic TRPA1 as well as TRPV1 knockout mice was performed as described previously (24). Briefly, mice were killed by CO2-inhalation and DRGs from all levels were excised and transferred to D-MEM containing 50 µg ml-1 gentamicin (Sigma Aldrich, Germany). Following treatment with 1mg ml-1 collagenase and 0.1 mg ml-1 protease for 30 min (both from Sigma Aldrich, Germany) ganglia were dissociated using a fire-polished silicone coated Pasteur pipette. Isolated cells were transferred on poly-D-lysine-coated (200 µg/ml, Sigma Aldrich, Germany) cover slips and cultured in TNB 100- medium supplemented with TNB 100 lipid protein complex, 100 µg/ml streptomycin, penicillin (all from Biochrom, Berlin, Germany) and mouse NGF (100 ng/ml, Almone Labs, Tel Aviv, Israel) at 37 °C and 5% CO2. Cells were used for experiments within 24h after plating. TRPA1+/- mice were kindly provided by Drs. Kelvin Kwan and David Corey (Harvard, Boston, CT, U.S.A.), whereas TRPV1+/- mice were a gift from Dr. John Davis (fromlerly Glaxo-Smith-Kline, Harlow, UK). All procedures of this study were approved by the animal protection authorities (local district government, Ansbach, Germany). For transfection of DRG neurons with hTRPA1, DRGs from TRPA1/TRPV1 double knockout mice were
Species-specific activation of human TRPA1 by protons

used and prepared as described above. Following dissociation of DRGs, neurons were immediately transferred into electroporation cuvettes containing 100µl of nucleofector solution and 10µg of hTRPA1 cDNA. Cells were electroporated with an Amaju Nucleofector, program A-033 (Lonza), and plated on covers slips after a 5 min recovery period in Ca²⁺ free medium. Neurons which underwent the electroporation procedure without added cDNA were used as controls.

Chemicals. Stock solutions of 100 mM carvacrol, (Sigma-Aldrich, Germany), 100 mM HC-030031 (Tocris, Bristol, United Kingdom), were prepared in DMSO. 1 M L-Lactic acid, DL-Dithiothreitol 100 mM, 100 mM amiloride, 100 mM acrolein (Sigma-Aldrich, Germany) were dissolved in distilled water. All stock solutions were stored at -20°C.

RESULTS

Species specific activation by protons of human TRPA1. We first asked if protons evoke different effects on human (hTRPA1) and rodent TRPA1. HEK-293t cells expressing hTRPA1, mouse TRPA1 (mTRPA1) or rat TRPA1 (rTRPA1) were examined by means of whole-cell patch clamp recordings. Due to the voltage-dependency of TRPA1, currents evoked by most agonists typically exhibit an outward rectification (25). Therefore, the effects of protons on hTRPA1, mTRPA1, rTRPA1 and non-transfected HEK-293t cells were first explored with 500 ms long voltage ramps from -100 mV to 100 mV (Fig. 1). Acidic solutions with pH 7.4, 7.0, 6.4, 6.0 and 5.4 elicited a concentration-dependent activation of outwardly rectifying membrane currents on hTRPA1 with a threshold around pH 7.0 and a calculated EC₅₀-value of 6.5 ± 0.1 (n= 6, Fig. 1A, B). These currents were fully reversible and could be reproduced even after several applications of protons (data not shown). As is demonstrated in figure 1C, 100 µM of the selective TRPA1-blocker HC030031 completely and reversibly inhibited pH-evoked membrane currents in cells expressing hTRPA1 (n= 6). These proton-evoked membrane currents were never observed in control experiments on non-transfected HEK-293t cells at pH-values down to pH 5.4 (Fig. 1D). However, many non-transfected cells generated a prominent outward current when pH 5.0 was applied, i.e. an effect that did not require expression of TRPA1 (data not shown). This current was reported to originate from a constitutively expressed proton-activated outwardly rectifying anion channel activated by very low pH-values (26). Therefore, membrane currents were only investigated down to pH 5.4. In contrast to hTRPA1, both mTRPA1 (n= 12, Fig. 1E) and rTRPA1 (n= 6, Fig. 1F) failed to generate membrane currents when acidic solutions down to pH 5.4 were applied. In order to mimic a more physiological situation, we next examined proton-evoked effects in cells constantly held at -60 mV. In this configuration, protons evoked slowly activating and persistent inward currents in cells expressing hTRPA1 (Fig. 2A). These responses were concentration dependent (pH 7.0 43 ± 12 pA, n= 6; pH 6.0 130 ± 26 pA, n= 5; pH 5.4 231 ± 58 pA, n= 17), and inhibited by 100 µM HC-030031 (n= 6, Fig. 2A). Another typical property of TRPA1 is a pronounced potentiation by extracellular calcium (27). Accordingly, 2 mM Ca²⁺ induced an impressive increase of proton-evoked currents through hTRPA1 (615 ± 122 %, n= 6, Fig. 2B). Again, HEK-293t cells expressing mTRPA1 (Fig. 2C, n= 13) or rTRPA1 (data not shown) never generated persistent inward currents upon application of acidic solutions down to pH 5.4. While most non-transfected cells did not produce any proton-evoked inwards currents (Fig. 2D), some non-transfected and transfected cells generated transient inward currents when challenged by acidic solutions (Fig. 2E). These currents were blocked by amiloride (100 µM, Fig. 2E) and have previously been reported to originate from constitutively expressed ASIC1a channels in HEK-293 cells (28). Due to their distinct kinetic properties, ASIC-like currents could easily be isolated from the slow inward currents mediated by hTRPA1. In HEK-293t cells expressing mTRPA1, we noticed that application of pH 5.4 induced a decrease in leak currents (Fig.
Species-specific activation of human TRPA1 by protons

As this effect might be due to an inhibition of open TRPA1 channels, we further explored this effect by applying pH 5.4 on inward currents induced by acrolein or carvacrol on either hTRPA1 or mTRPA1. While protons potentiated acrolein-evoked currents of hTRPA1 (+107 ± 37%, n= 6, Fig. 2F), they inhibited currents generated by mTRPA1 (-41 ± 6%, n= 5, Fig. 2G). The same species specific differences were observed for carvacrol-evoked currents, i.e. potentiation of hTRPA1 (+210 ± 9%, n= 6, Fig. 2H) and inhibition of mTRPA1 (-91 ± 5%, n= 5, Fig. 2I). In order to substantiate the findings obtained by whole cell patch clamp recordings, we next performed Ca$^{2+}$ imaging experiments. As demonstrated in figure 3A, the acidic solutions pH 6.4, 6.0 and 5.0 evoked an immediate (< 30 s) and only partly reversible increase of (Ca$^{2+}$)$_i$ in a subset of cells responding to carvacrol (i.e. cells expressing hTRPA1; pH 6.4 21% (31/148), pH 6.0 45% (63/140) and pH 5.0 48% (72/150)). In contrast, non-transfected HEK-293t (n= 670) cells and cells expressing mTRPA1 (n= 107) or rTRPA1 (n= 184) never displayed an increase of (Ca$^{2+}$)$_i$ when challenged with pH 5.0 (Fig. 3B and C). The area under the curve (AUC) for proton-evoked increases of (Ca$^{2+}$)$_i$, calculated for the 60s long application of protons was significantly larger for hTRPA1 as compared to non-transfected cells and to cells expressing mTRPA1 or rTRPA1 (pH 5.0, all p< 0.001, ANOVA). In contrast, the AUC for pH 5.0-evoked responses on mTRPA1 and rTRPA1 expressing cells were not significantly different from the AUC calculated for untransfected cells (p> 0.6, ANOVA). When comparing the data obtained with patch clamp recordings as compared to those obtained with calcium imaging, the only relevant difference seems to be the finding that the majority of cells expressing hTRPA1 failed to evoked a detectable proton-evoked response in Ca$^{2+}$ imaging experiments. We believe that this discrepancy between the two methods is due to an inhomogeneous transfection efficacy and the fact that protons are rather weak TRPA1-agonists as compared to carvacrol. While patch clamp experiments only includes vital cells displaying a strong expression of EGFP (and thus also hTRPA1), calcium imaging experiments covering large populations of cells also include cells with a low expression of hTRPA1. Nevertheless, both methods strongly indicate that hTRPA1, but not rodent TRPA1 can be directly activated by extracellular acidosis. The lack of proton-evoked responses of mTRPA1 is supported by previous studies on mouse dorsal root ganglion (DRG) neurons, suggesting that TRPV1 is the predominant proton-receptor in mouse DRG neurons (29,30). We wanted to exclude the possibility that an improper functionality of recombinant mTRPA1 in HEK-293t cells is the reason for the lack of proton sensitivity observed in our experiments. We performed Ca$^{2+}$ imaging experiments on DRG neurons derived from wild type C56Bl/6, TRPV1-null, TRPA1-null and TRPV1/TRPA1-null mice. If mTRPA1 would mediate proton-evoked responses in DRG neurons, it should be best observed in DRG neurons lacking TRPV1. However, no increase of (Ca$^{2+}$)$_i$ was observed when pH 5.0 was applied for 5 minutes onto TRPV1-null DRG neurons (Fig. 4A, n= 181). As expected, application of pH 5.0 on wild type DRG neurons resulted in a large increase of (Ca$^{2+}$)$_i$ in 51% of all neurons (n= 35, Fig. 4B). When the TRPV1-antagonist BCTC was co-applied with pH 5.0, this TRPV1-mediated proton response was completely blocked in neurons derived from TRPA1-/- neurons (n= 118, Fig. 4C). In agreement with our patch clamp data on mTRPA1 in HEK-293t cells, pH 5.0 inhibited TRPA1-mediated responses evoked by both 50 µM allyl isothiocyanate (AITC, n= 83, Fig. 4D), and 300 µM carvacrol (n= 93, Fig. 4E). These data confirmed that extracellular protons rather inhibit than activate mTRPA1, and that this effect is independent of the expression system. A related question was whether proton-evoked activation of hTRPA1 was due to an overexpression in HEK-293t cells. Therefore, DRG neurons derived from TRPV1/TRPA1 double-knockout mice were transfected with hTRPA1 by electroporation. This procedure rendered 13% of all neurons sensitive to the TRPA1-agonist AITC (48/379). 21% (10/48)
of all AITC-sensitive DRG neurons also briskly responded to the application of pH 5.0 (Fig. 4F). Thus again only a fraction of cells expressing hTRPA1 responded to protons. Importantly, both AITC and pH 5.0 did not evoke any increase of \((\text{Ca}^{2+})_i\), in DRG neurons in which a sham transfection was performed without hTRPA1-cDNA (n= 108) (Fig. 4F). We also observed hTRPA1-mediated membrane currents activated by pH 5.4 in transfected neuroblastoma ND7/23 cells (n= 4) (Fig. 4G).

**Determinants of species specific actions of protons on TRPA1.** We next asked how the species specificity of proton-evoked effects on TRPA1 is determined. Previous studies showed that the transmembrane domains (TM) 5 and 6 contain important residues for distinct effects of agonists and antagonists on human versus rodent TRPA1 (18,21). While rat and mouse TRPA1 share 100% sequence homology within TM5/6, this region contains 17 amino acids which are not identical in human versus rodent TRPA1 (Fig. 5A). We therefore examined the effects of protons on the chimeras hTRPA1-mTM5/6 and mTRPA1-hTM5/6, in which the segments encompassing TM5/6 were swapped between hTRPA1 and mTRPA1 (21). The chimera mTRPA1-hTM5/6 generated robust membrane currents evoked by pH 5.4, and these currents were blocked by HC030031 (at -60 mV: 411 ± 159 pA, n= 11, Fig. 5B). When the same cells were investigated with \((\text{Ca}^{2+})_i\) imaging, 14% (164/1169) of all carvacrol-sensitive cells generated a large increase of \((\text{Ca}^{2+})_i\) following application of pH 5.0 (Fig. 5C). Although the percentage of mTRPA1-hTM5/6 expressing cells responding to protons was considerably lower as compared to wild type hTRPA1, these data indicate that the insertion of human TM5/6 into mTRPA1 resulted in a de novo proton sensitivity. Furthermore, mTRPA1-hTM5/6 generated acrolein-evoked inward currents which were not inhibited by pH 5.4 (n= 6, Fig. 5D). In contrast to mTRPA1-hTM5/6, the reverse chimera (hTRPA1-mTM5/6) failed to generate proton-evoked membrane currents (n= 8, Fig. 5e) and an increase of \((\text{Ca}^{2+})_i\) (0/228, Fig. 5F). Moreover, potentiation of acrolein-evoked inward currents by pH 5.4 was only marginal (n= 8, Fig. 5G). Thus the effects elicited by extracellularly applied protons could almost perfectly be reversed by the exchange of TM5/6 between hTRPA1 and mTRPA1. Interestingly, Xiao and colleagues identified 9 out of the 17 non-identical amino acids in human and mouse TM5/6 to be collectively relevant for the species specific effects of menthol on hTRPA1 (activation) and mTRPA1 (inhibition and activation) (21). As we observed similar differences between hTRPA1 (activation) and mTRPA1 (inhibition) in regard to proton sensitivity, we asked if these 9 amino acids have the same impact on proton sensitivity. The mutant construct hTRPA1-FGFATLIAM, in which these 9 amino acids were exchanged to the corresponding residues in mTRPA1, did not generate any proton-evoked membrane currents or proton-evoked increase of \((\text{Ca}^{2+})_i\), (data not shown). Consequently, we tried to identify the amino acids rendering hTRPA1-FGFATLIAM proton insensitive by exploring the mutant constructs hTRPA1-FATL and hTRPA1-IAM. Both mutants were completely proton insensitive in \((\text{Ca}^{2+})_i\) imaging experiments (data not shown), suggesting that more than one residue encodes for the proton-insensitive phenotype of hTRPA1-FGFATLIAM and thus also for wild type mTRPA1. On the other hand, it did not exclude the possibility that other residues than those exchanged in hTRPA1-FGFATLIAM might as well be required for proton sensitivity. To test this possibility, we examined the relevance of two amino acids which typically interact with protons in other proton sensitive ion channels. When screening the non-identical amino acids in TM5/6 of hTRPA1 and mTRPA1, we identified Glu 920 (E920) and His 933 (H933) as possible interaction sites for protons in hTRPA1. We constructed the mutations hTRPA1-E920D and hTRPA1-H933Y, but also the reverse mutant constructs mTRPA1-D923E and mTRPA1-Y936H. Both hTRPA1-E920D and hTRPA1-H933Y displayed a preserved proton sensitivity, i.e. protons evoked membrane currents and an increase in \((\text{Ca}^{2+})_i\), (data not shown). The mutant mTRPA1-D923E failed to
Species-specific activation of human TRPA1 by protons

produce an increase of \((\text{Ca}^{2+})\), when challenged by pH 5.0 (data not shown). The mutant mTRPA1-Y936H was non-functional.

As an alternative approach to identify residues being relevant for proton sensitivity of TRPA1 and to further explore how specific proton-gated activation is for hTRPA1, we next investigated the proton sensitivity of TRPA1 from rhesus monkey (rhTRPA1). The sequence homology between rhTRPA1 and hTRPA1 is 98%, and a recent report suggests that they display almost identical functional and pharmacological properties (22).

Surprisingly, we found that rhTRPA1 failed to produce an increase of \((\text{Ca}^{2+})\) when challenged by pH 5.0 (0/184, Fig. 6A) and to generate proton-evoked ramp currents or inward currents (n= 9, Fig. 6B and C). An alignment of TM5/6 from rhTRPA1 and hTRPA1 revealed 4 non-identical amino acids (Fig. 6D). The exchange of any of these four amino acids in hTRPA1 resulted in a decrease in proton sensitivity as examined with \(\text{Ca}^{2+}\) imaging (Fig. 7E). In wild type hTRPA1 the magnitude of the pH 5.0-evoked calcium response was 21 ± 1% of that observed for the response evoked by carvacrol. This normalized response was reduced to 10 ± 3% for hTRPA1-V942I, 2 ± 1% for hTRPA1-V935F, 14 ± 2% for hTRPA1-S943A and 11 ± 2% for hTRPA1-T945A (n = 117-224, ANOVA F\((4,1168)\)=17, HSD post-hoc tests p < 0.001 vs. wild type, Fig. 6F). While patch clamp experiments on these mutants still revealed small proton-evoked inward currents, we found that the functional expression of hTRPA1-V935F was very poor (data not shown). Therefore, the apparent loss of proton sensitivity found for hTRPA1-V935F might not be specific. In order to verify that these amino acids indeed encode for proton sensitivity, we next examined if the reverse exchanges in rhTRPA1 results in a gain of proton sensitivity. Among the four single amino acid mutants, \(\text{Ca}^{2+}\) imaging experiments revealed that rhTRPA1-I942V conveyed the highest sensitivity to pH 5.0 (5 ± 2% of the carvacrol response, p= 0.003, n= 212, t-test compared to rhTRPA1 wild type, Fig. 6F). Although rhTRPA1-C943S did not display a relevant proton sensitivity in these experiments, the combination of both mutations in rhTRPA1-I942V/C943S displayed the most robust proton sensitivity of all investigated rhTRPA1 mutants (8 ± 2% of the carvacrol response, p= 0.002, n= 270, t-test compared to rhTRPA1 wild type, Fig. 7F, G). This finding fits the results of the reverse mutants in hTRPA1, where the exchange of both V942 and S943 resulted in a significant reduction of proton sensitivity. Even if the percentage of cells expressing rhTRPA1-I942V/C943S displaying proton sensitivity (7%, 20/270) was lower than that found for wild type hTRPA1, it displayed a clearly changed functional phenotype in regard to proton sensitivity when compared to the completely proton insensitive wild type rhTRPA1 (Fig. 7G). This finding was further supported by patch clamp experiments. In contrast to wild type rhTRPA1, rhTRPA1-I942V/C943S generated prominent membrane currents when voltage ramps were applied (Fig. 7I, n= 7)). Furthermore, rhTRPA1-I942V/C943S produced prominent inward currents evoked by pH 5.4 in presence of 2 mM Ca\(^{2+}\) in cell held at -60 mV (Fig. 7J, n= 5). Taken together, these data prove that the poorly conserved amino acids 942 and 943 are of significant importance for the proton sensitivity of hTRPA1.

Protons activate hTRPA1 via an extracellular mechanism. Now we had identified TM5/6 as an important region, and the amino acids V942 and S943 as important determinants for proton-sensitivity of hTRPA1. However, the data did not allow us to postulate a molecular mechanism for proton-evoked activation of hTRPA1. Studies on both ASICs and TRPV1 have shown that the mechanism for proton-evoked activation can be complex and involve several residues throughout the channel protein (31-34). Thus, an unbiased screen for putative interaction sites for protons on hTRPA1 would go beyond the scope of this study. However, it was recently demonstrated that weak acids activate rodent TRPA1 by inducing an intracellular acidosis and that protons directly activate rodent TRPA1 when applied onto the cytosolic side (14,15). On the other hand, Takahashi and co-workers demonstrated that hTRPA1
Species-specific activation of human TRPA1 by protons

produces a proton-evoked increase of (Ca\textsuperscript{2+}), and suggested that the N-terminal cysteines 414 and 421 are required for this effect (16). As extracellularly applied protons can cross the membrane to induce intracellular acidosis (36), we explored the possibility that the exclusive activation of hTRPA1 by protons is due to an intracellular acidification and a successive modification of N-terminal cysteines. We first performed cell-attached patch clamp recordings on HEK-293t cells expressing hTRPA1. This configuration allows to record channels which are isolated within the pipette orifice, and thus not confronted with the acidic solution from the extracellular side. While 300 µM carvacrol evoked a robust response, extracellular protons failed to gate hTRPA1 in this configuration (n= 5, Fig. 7A). Moreover, carvacrol-evoked responses were even inhibited by protons in this configuration (Fig. 7B, n= 8). If an intracellular acidification would be decisive for activation of hTRPA1 by protons applied from the extracellular site, a pre-existing intracellular acidosis might inhibit this effect by desensitization. Therefore, whole-cell recordings with an acidic pipette solution (pH 5.4) were performed. Extracellularly applied protons (pH 5.4) still evoked small but reproducible currents in these experiments (n= 6, Fig. 7C). We also explored the proton-sensitivity of the mutant construct hTRPA1-C621S/C641S/C665S (hTRPA1-3C), which is known to be insensitive to several electrophilic agonists (36,37). hTRPA1-3C displayed a preserved proton-sensitivity in patch clamp experiments (pH 5.4: 235 ± 52 pA, n= 7, Fig. 7D). Cells expressing hTRPA1-3C also responded to protons when examined with Ca\textsuperscript{2+} imaging (Fig. 7E). However, the percentage of hTRPA1-3C (6%, 117/1944) expressing cells responding to pH 5.0 was considerably than for cells expressing wild type hTRPA1. Finally, the reducing agent dithiothreitol (DTT, 5 mM) which is able to reverse electrophilic cysteine interactions, did not inhibit proton-evoked currents of hTRPA1 (n= 6, Fig. 7F). Taken together, these experiments did not support the possibility that proton-evoked activation of hTRPA1 is mediated by an intracellular mechanism involving oxidation or modification of N-terminal cysteines.

DISCUSSION

In this study we identified TRPA1 as a transduction molecule for activation of human sensory neurons by protons. This property of hTRPA1 is subject to a striking species specificity, and our data indicate that several residues within transmembrane domains 5 and 6 encode for this specificity. To our knowledge, protons are the first identified endogenous TRPA1-agonists which display this exclusive species specificity. Distinct properties of TRPA1 from different species have already been acknowledged to complicate the development of TRPA1-modifying therapeutics (17,22). Thus our data not only unravel the yet unrecognized property of hTRPA1 as a sensor for extracellular acidosis, but also strengthen the notion that the species of TRPA1 matters.

Early electrophysiological experiments document excitatory effects of protons on rodent DRG neurons (3,38), and C-fibers (39). In rat and mouse DRG neurons, protons evoke a fast transient current followed by a slow sustained current (30,38). While the transient current is generated by ASICs, TRPV1 generates the sustained current (29,30). Moreover, rodent ASIC3 generates a sustained current which was suggested to contribute to persistent activation of sensory neurons (40). Although several other membrane molecules in sensory neurons are modulated by protons, ASICs and TRPV1 are regarded as the principle transducers driving proton-evoked activation of sensory neurons (4). Interestingly, Baumann and colleagues identified a delayed, slowly activating and inactivating proton-evoked inward current in about 50% of cultured human DRG neurons (41,42). This current has never been reported in rodent sensory neurons, but its properties accurately describe the proton-evoked currents we observe on hTRPA1. As to the suggested ability of ASIC3 to generate a sustained current mediating persistent pain (40), Delaunay and colleagues recently demonstrated that human ASIC3 does not
Species-specific activation of human TRPA1 by protons

Proton-evoked membrane currents through hTRPA1 displayed a pronounced outward rectification, were blocked by HC030031 and potentiated by extracellular calcium, i.e. current properties which are commonly observed for many TRPA1-agonists. Unlike electrophilic TRPA1 agonists, protons do not seem to interact with the N-terminal cysteines C621, C641 and C665 in order to gate hTRPA1 (36,37). As a specific interaction between protons and cysteines cannot be expected, this finding might seem trivial. However, Takahashi and co-workers suggested that the cytoplasmic cysteines 414 and 421 are required for activation of hTRPA1 by protons (16) and thus we had good reasons to investigate this possibility. It is also important to note that all of the above mentioned N-terminal cysteines are conserved in human, rodent and rhesus monkey TRPA1.

Taking into account the exclusive activation of hTRPA1 by extracellular protons, it seems reasonable that protons target other residues than the N-terminal cysteines to gate TRPA1. Although protons can rapidly permeate through the membrane to induce an intracellular acidosis (35), we also argue that protons are likely to target extracellularly located residues to activate hTRPA1. This notion is in part based on our experiments in the cell-attached mode, by which protons completely failed to activate or potentiate hTRPA1. Furthermore, both human and rodent TRPA1 are activated by intracellular protons (14,15). This property more or less excludes an intracellular mechanism for the specific activation of hTRPA1 by extracellular protons. When it comes to identifying putative binding sites for protons on TRPA1, studies on ASICs and TRPV1 have shown that more than one amino residue can be involved. Although several interaction sites for protons have been identified on both ASICs and TRPV1, the exact mechanisms for activation by protons are still to be defined (31-34). Our data strongly suggest that the architecture of the transmembrane domains 5 and 6 dictate the pronounced species specificity of proton-sensitivity of TRPA1. The complete exchange of TM5/6 or mutations within this region lead to a sustained current during acidosis (43). Unfortunately, we do not have access to vital human DRG neurons which would allow us to verify our findings. However, we show that hTRPA1 generates proton-evoked responses when expressed in mouse DRG neurons lacking both mTRPA1 and TRPV1. As was already described in the first study on TRPV1-null mice, we could also confirm that mouse DRG neurons lacking TRPV1 fail to respond to pH 5.0 when examined with Ca²⁺ imaging (29). Thus mTRPA1 fails to respond to protons even if expressed in native DRG neurons and the proton sensitivity of hTRPA1 observed in HEK-293t cells is not due to the non-neuronal expression system.

It is important to note that the relevance of proton-evoked activation of hTRPA1 found in our study is yet to be determined in more physiological models on human volunteers. However, such studies would require a specific TRPA1-blocker permitted for human use and we are not aware of any such substances. Interestingly, a continuous infusion of acidic buffer (pH 5.2) into the forearm skin of healthy volunteers evokes a sustained pain driven by a non-adapting excitation of nociceptors (44,45). ASICs were shown to undergo inactivation during sustained acidosis (46). Similarly, TRPV1 displays a strong tachyphylaxis upon prolonged activation (47). Thus it is possible that TRPA1 drives this proton-evoked non-adapting activation of nociceptors in human skin. In similar human pain models, pain induced by moderate acidosis was found to be reduced by amiloride, but enhanced by the NO-donor glyceryl trinitrate (48,49,50). As ASICs are blocked by amiloride and potentiated by NO, the authors of these studies concluded that ASICs are likely to be predominant transducers for acid-evoked pain in human skin. More recent reports demonstrated that the actions of both these substances are not specific for ASICs, i.e. high concentrations of amiloride inhibits TRPA1 (51) and NO activates TRPA1 (52). Together with these reports, our cellular data suggest that TRPA1 might also act as a transducer for proton-evoked pain in humans.
rendered both mTRPA1 and rhTRPA1 proton sensitive, or hTRPA1 proton insensitive. It is clear that more than one of the non-identical amino acids within TM5/6 of mouse, rhesus monkey and human TRPA1 encode for these effects. In case of hTRPA1, the reduced or abolished proton-sensitivity of the constructs hTRPA1-mTM5/6, -FGFATL, -IAM, -S943A and -V942I demonstrates that the mechanisms enabling activation by protons is rather fragile. On the other hand, our data do not allow us to postulate a defined motif other than TM5/6 which renders rodent TRPA1 proton sensitive. The characterization of rhTRPA1 allowed us to draw more definitive conclusions in regard to which amino acids are crucial for proton sensitivity. As rhTRPA1 and hTRPA1 share 97% sequence homology and display almost identical pharmacological properties (22), we were surprised to observe that wild type rhTRPA1 is completely proton-insensitive. We can conclude that the identities of the residues 942 and 943 can essentially dictate whether or not rhTRPA1 is activated by extracellular acidosis. The double mutant rhTRPA1-I942V/C943S displayed a robust proton-sensitivity as compared to an absolute lack of proton-sensitivity found for wild type rhTRPA1. However, no rhTRPA1 mutant construct displayed a proton-sensitivity that was comparable to that of hTRPA1. This finding might reflect a generally smaller chemical sensitivity of rhTRPA1 as compared to hTRPA1 (32), but might as well indicate that further residues within or outside TM5/6 also have an impact on proton sensitivity of hTRPA1.

In this study we identified human TRPA1 as a receptor for extracellular protons and argue that TRPA1 is likely to play a significant role in the surveillance system which monitors the acid-base homeostasis in humans. TRPA1 is the principal receptor for numerous endogenous algogenic substances which accumulate during tissue injury, inflammation and metabolic dysregulation. An interstitial acidosis is regarded to contribute to several types of pain, thus activation and sensitization of TRPA1 by protons might be an important event in peripheral pain processing. Future studies which circumvent the challenges arising from the species specificity of this property are required to further elucidate the relevance of the acid-sensitivity of hTRPA1.
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Species-specific activation of human TRPA1 by protons


Species-specific activation of human TRPA1 by protons

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FOOTNOTES

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The abbreviations used are: ASIC, acid sensing ion channel; DRG, dorsal root ganglion; DTT, dithiothreitol; HEK 293, human embryonic kidney 293; TM, transmembrane; TRP, transient receptor potential.

FIGURE LEGENDS

FIGURE 1. Protons activate hTRPA1. Currents were recorded during a 500 ms voltage ramp from -100 mV to 100 mV in cells held at -60 mV. A. Membrane currents evoked by acidic solutions at pH 7.4, 7.0, 6.4, 6.0 and 5.4, and by 300 µM carvacrol on hTRPA1. B. Concentration-response curve for proton-evoked outward currents through hTRPA1. Peak current amplitudes at 100 mV were determined for all pH-values and normalized to the amplitude evoked by pH 5.4. The line represents a fit of the data to the Hill equation. C. Proton-evoked currents through hTRPA1 were blocked by the TRPA1-antagonist HC030031 (100 µM). Cells we challenged with pH 6.0 with and without HC030031. D. Membrane currents in non-transfected HEK-293t cells challenged with acidic solutions. E, F. Membrane currents evoked by acidic solutions and by 300 µM carvacrol in cells expressing mTRPA1 (E) or rTRPA1 (F).

FIGURE 2. Protons activate inward currents through hTRPA1. Current were recorded in cells held at -60 mV. A. Typical inward current evoked by pH 5.4 in a cell expressing hTRPA1. Note that co-application of pH 5.4 with 100 µM HC-030031 resulted in a complete inhibition of the proton-evoked inward current. B. Typical current trace displaying a proton-evoked current through hTRPA1 which is potentiated by 2 mM Ca^{2+}. The column bars displays the average peak current amplitudes of currents evoked by pH 6.0 in presence of 0 and 2 mM Ca^{2+}. Data are expressed as mean ± S.E.M. C. Typical responses evoked by application of pH 5.4 in cells expressing mTRPA1 (C) or in non-transfected HEK 293t cells (D). E. ASIC-like transient inward currents evoked by pH 5.4 in a non-transfected cell. Note that co-application of pH 5.4 with 100 µM amiloride resulted in an almost complete inhibition of the ASIC-like inward current. F-I. Current traces displaying the effects of pH 5.4 on acrolein- and carvacrol-evoked inward currents through hTRPA1 (F, F), and mTRPA1 (G, I). pH 5.4 was co-applied with acrolein or carvacrol when inward currents evoked by these agonists had reached a steady-state.

FIGURE 3. Protons evoke calcium influx through hTRPA1, but not through rodent TRPA1. A. Upper panel: Representative snapshots of hTRPA1-expressing cells loaded with FURA-2 during application of control solution (basal), pH 5.0 and 300 µM carvacrol. Lower panel: Average effects of pH 6.4 (n= 148), 6.0 (n= 140) and 5.0 (n= 150) as well as 300 µM carvacrol on intracellular calcium in cells expressing hTRPA1 as determined by ratiometric imaging. Protons and carvacrol were both applied for 30 s. B. Average effects of pH 5.0 on non-transfected HEK-293t cells (B), and on cells expressing mTRPA1 (C) or rTRPA1 (C) as determined by ratiometric imaging. Note that the minimal effect caused by application of protons and carvacrol in HEK-293t cells is due to an application artefact. D. Areas under the curve for the average responses evoked by protons in hTRPA1, mTRPA1, rTRPA1 and non-transfected HEK-293t cells. All data are displayed as mean ± S.E.M.
FIGURE 4. Proton-evoked calcium influx in mouse DRG neurons depends on TRPV1. A-C. Ratiometric imaging of intracellular Ca\(^{2+}\) examined on DRG neurons derived from TRPV1-/- mice (A) (n = 181), wild type C57Bl/6 mice (B) and TRPA1-/- mice (C). Protons were applied for 5 minutes. While protons completely failed to evoke a calcium influx in neurons lacking TRPV1 (A), protons evoked a fast increase of intracellular calcium in wild type neurons (B). This TRPV1-mediated calcium influx in neurons lacking TRPA1 could be completely blocked by the TRPV1-antagonist BCTC (10 µM) (C). D, E. Protons inhibit calcium influx mediated by TRPA1 in TRPV1-/- DRG neurons. Co-application of pH 5.0 and 100 µM AITC resulted in a suppression of calcium influx, as became obvious by a rebound effect following washout of protons (D). Protons were applied for 5 minutes, and AITC for 30 s. Co-application of pH 5.0 and 300 µM carvacrol resulted in a suppression of calcium influx (E). Protons were applied for 5 minutes, and carvacrol for 30 s. Data in a to e are displayed as mean ± S.E.M. F. Original Ca\(^{2+}\) imaging recordings on DRG neurons derived from TRPV1/TRA1-/- mice. Protons were applied for 60 s, AITC for 30 s and capsaicin for 20 s. **Upper panel:** Recordings on neurons transfected with hTRPA1. Note that a subpopulation of neurons responding to both protons and to AITC. G. Representative membrane currents evoked by pH 5.4 and 600 µM carvacrol in ND7/23 cells expressing hTRPA1. Currents were recorded as described under figure 1.

FIGURE 5. Transmembrane domains 5 and 6 dictate the species specific effects of protons on TRPA1. A. Alignment of TM5/6 from hTRPA1 and mTRPA1. Non-identical amino acids are marked by colored letters. Amino acids involved in the multiple mutant hTRPA1-FGFATLIAM are underlined, and putative binding sites for protons are marked by bold letters. B, E. Membrane currents of mTRPA1-hTM5/6 (B) and hTRPA1-mTM5/6 (E), examined as described under figure 1. mTRPA1-hTM5/6 generated prominent proton-evoked inward currents at -60 mV (insert), and ramp currents upon application of pH 6.4 and 5.4. 100 µM HC030031 (HC) blocked outward currents evoked by pH 5.4. In contrast, hTRPA1-mTM5/6 failed to generate both inward currents (inset) and prominent outward currents when challenged by pH 6.4 and 5.4. C, F. Typical acrolein-evoked inward currents through mTRPA1-hTM5/6 (C) and hTRPA1-mTM5/6 (F) (n = 8) modified by pH 5.4. C. Co-application of pH 5.4 with 100 µM acrolein resulted in a small potentiation of the acrolein-evoked current in mTRPA1-hTM5/6, and this effect was even smaller in hTRPA1-mTM5/6 (F). Cells were held at -60 mV and protons were co-applied with acrolein once the inward current evoked by acrolein alone had reached a steady state. D, G. Ratiometric calcium imaging on mTRPA1-hTM5/6 (D) and hTRPA1-mTM5/6 (G) expressed in HEK-293t cells. Protons and carvacrol were both applied for 30 s. Note that only a small fraction (14%) of all recorded carvacrol-sensitive cells also responded to pH 5.0 (average trace with the large peak following application of protons). When the mean response of all investigated cells was depicted, no significant proton-evoked effect could be observed (average trace lacking an effect following application of protons). hTRPA1-mTM5/6 completely failed to produce a calcium influx when pH 5.0 was applied, but responded briskly upon application of 300 µM carvacrol.

FIGURE 6. Specific residues within TM5/6 dictate the effects of protons on hTRPA1 and rhTRPA1. A. Calcium imaging on cells expressing wild type rhTRPA1 challenged with pH 5.0 and 300 µM carvacrol. B. Typical ramp currents elicited in presence of pH 7.4, pH 5.4 and 300 µM carvacrol in cells expressing rhTRPA1. Currents were examined as described under figure 1. C. Current trace on a cell expressing rhTRPA1 examined at -60 mV. Note that rhTRPA1 completely failed to respond to pH 5.4, whereas 300 µM carvacrol elicited large inward currents. D. Alignment of TM6 from hTRPA1, rhTRPA1 and mTRPA1. Non-identical amino acids are marked by colored letters. E. Average calcium imaging responses of cells expressing hTRPA1-wild type, -V935F, -V942I, -S943A and -T945A, or rhTRPA1-wild type, -I942V, -C943S and -942V/C943S. Cells were challenged by pH 5.0 and 300 µM carvacrol. F. Proton-evoked responses of hTRPA1 mutant constructs, normalized to the response obtained by carvacrol. G. Original calcium imaging traces on cells expressing rhTRPA1-wild type (black) or -942V/C943S (red), again challenged by protons and carvacrol as described for E. I. Representative membrane currents of the mutant rhTRPA1-I942V/C943S, examined as described under figure 1. Note that this mutant produces outwardly rectifying current evoked by protons. J. Representative proton-evoked (pH 5.4) inward currents in cells expressing wild rhTRPA1-I942V/C943S, -I942V and –C943S. Recordings were performed in an extracellular solution containing 2 mM Ca\(^{2+}\). The inward current as blocked by co-application of 100 µM HC030031.
FIGURE 7. Activation of hTRPA1 by protons is not mediated by an interaction with intracellular cysteines. A, B. Protons (pH 5.4) fail to activate and to sensitize membrane currents through hTRPA1 when examined in cell-attached recordings. Ramp currents evoked by 500 ms long voltage ramps from -100 to 100 mV revealed outwardly rectifying currents evoked by 300 µM carvacrol, but not by protons (A). Protons also failed to potentiate carvacrol-evoked currents recorded in the cell-attached mode (B). Protons were co-applied with carvacrol once the current evoked by carvacrol alone had reached a steady state. C. Typical ramp current on hTRPA1 recorded in the whole cell mode with an intracellular pH 5.4. Ramp currents were activated as described under figure 1. Note that pH 5.4 gates hTRPA1 although an intracellular acidosis is already established. D, E. Current traces of hTRPA1-C621S/C641S/C665S (hTRP1-3C) evoked by pH 5.4 and 300 µM carvacrol. Ramp currents and inward currents (inset) through hTRPA1-3C could be evoked by pH 5.4. Note that 100 µM HC030031 completely blocked the membrane currents induced by pH 5.4 (D). Average responses of hTRPA1-3C to application of pH 5.0 (30 s) and carvacrol (100 µM, 30 s) as examined by calcium imaging (E). Note that only a small fraction (6%) of all recorded carvacrol-sensitive cells also responded to pH 5.0 (average trace with the large peak following application of protons). When the mean response of investigated cells was depicted, no significant proton-evoked effect could be observed (average trace lacking an effect following application of protons). F. Proton-evoked inward current which is not reduced by 5 mM DTT. DTT was applied together with pH 6.0 once inward currents evoked by pH 6.0 alone had reached a steady-state.
Species-specific activation of human TRPA1 by protons

Figure 1
Figure 2
Species-specific activation of human TRPA1 by protons

**Figure 3**

(A) hTRPA1

(B) HEK-293t

(C) mTRPA1 / rTRPA1

(D) AUC (F 340/380 nm)

- pH 6.4
- pH 6.0
- pH 5.0
- carvacrol

*Note: The figures illustrate the activation of TRPA1 by protons and carvacrol across different cell types and pH conditions.*
Species-specific activation of human TRPA1 by protons

**Figure 4**
Species-specific activation of human TRPA1 by protons

Figure 5
Species-specific activation of human TRPA1 by protons

Figure 6
Species-specific activation of human TRPA1 by protons

Figure 7
The molecular basis for species-specific activation of human TRPA1 by protons involves poorly conserved residues within transmembrane domains 5 and 6
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