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Requirement of extracellular Ca^{2+} binding to specific amino acids for heat-evoked activation of TRPA1

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Key points

- We found that extracellular Ca^{2+}, but not other divalent cations (Mg^{2+} and Ba^{2+}) or intracellular Ca^{2+}, is involved in heat-evoked activation of green anole (ga) TRPA1.
- Heat-evoked activation of chicken (ch) and rat snake (rs) TRPA1 does not depend solely on extracellular Ca^{2+}.
- Neutralization of acidic amino acids on the outer surface of TRPA1 by extracellular Ca^{2+} is important for heat-evoked large activation of gaTRPA1, chTRPA1 and rsTRPA1.

Abstract Transient receptor potential ankyrin 1 (TRPA1) is a homotetrameric non-selective cation-permeable channel that has six transmembrane domains and cytoplasmic N- and C-termini. The N-terminus is characterized by an unusually large number of ankyrin repeats. Although the 3-dimensional structure of human TRPA1 has been determined, and TRPA1 channels from insects to birds are known to be activated by heat stimulus, the mechanism for temperature-dependent TRPA1 activation is unclear. We previously reported that extracellular Ca^{2+}, but not intracellular Ca^{2+}, plays an important role in heat-evoked TRPA1 activation in green anole lizards (gaTRPA1). Here we focus on extracellular Ca^{2+}-dependent heat sensitivity of gaTRPA1 by comparing gaTRPA1 with heat-activated TRPA1 channels from rat snake (rsTRPA1) and chicken (chTRPA1). In the absence of extracellular Ca^{2+}, rsTRPA1 and chTRPA1 are activated by heat and generate small inward currents. A comparison of extracellular amino acids in TRPA1 identified three negatively charged amino acid residues (glutamate and aspartate) near the outer pore vestibule that are involved in heat-evoked TRPA1 activation in the presence of extracellular Ca^{2+}. These results suggest that neutralization of acidic amino acids by extracellular Ca^{2+} is important for heat-evoked activation of gaTRPA1, chTRPA1, and rsTRPA1, which could clarify mechanisms of heat-evoked channel activation.

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Abbreviations AITC, allyl isothiocyanate; chTRPA1, chicken TRPA1; gaTRPA1, green anole TRPA1; rsTRPA1, rat snake TRPA1; TRPA1, transient receptor potential ankyrin 1.

Introduction

Thermosensation is essential for survival and adaptation. The ability to sense ambient temperature mainly relies on sensory neurons (trigeminal and dorsal root ganglia) that project axonal processes to both peripheral termini (the outer layer of the skin) and central termini (spinal cord) (Woolf & Ma, 2007). In the last two decades, transient receptor potential (TRP) ion channels have been identified as molecular sensors for many different ambient stimuli, including temperature. The temperature sensitivity of TRP ion channel family members varies. TRP vanilloid (TRPV) 1–4 (Caterina *et al.* 1997, 1999; Guler *et al.* 2002; Peier *et al.* 2002*b*; Smith *et al.* 2002; Xu *et al.* 2002)

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and TRP melastatin (TRPM) 2–5 (Talavera *et al.* 2005; Togashi *et al.* 2006; Vriens *et al.* 2011) are activated by hot and warm temperatures. Meanwhile, TRPM8 and TRP canonical (TRPC) 5 in mouse and humans are activated by cold stimulus (McKemy *et al.* 2002; Peier *et al.* 2002*a*; Zimmermann *et al.* 2011).

TRPA1 is primarily expressed in dorsal root ganglia (DRG) as well as trigeminal ganglia (TG) and nodose ganglia in mammals, where it was reported to respond to noxious cold and some pungent chemicals (Story *et al.* 2003; Bautista *et al.* 2005). TRPA1 is a homo-tetrameric non-selective cation permeable channel with six transmembrane (TM) domains (Gaudet, 2008; Paulsen *et al.* 2015) that functions as a polymodal receptor to detect various noxious compounds (Bandell *et al.* 2004; Macpherson *et al.* 2005; Koo *et al.* 2007; Iwasaki *et al.* 2009; Okumura *et al.* 2010) and stimuli such as mechanical stimulus (Kwan *et al.* 2006), intracellular alkalization (Fujita *et al.* 2008) and hyperosmotic stress (Zhang *et al.* 2008). However, the response of TRPA1 to temperature shows species-specific differences. Several studies showed that mouse TRPA1 is a thermosensitive TRP channel that is activated by noxious cold both *in vitro* (Story *et al.* 2003) and *in vivo* (Karashima *et al.* 2009), while other studies found no cold activation of TRPA1 (Jordt *et al.* 2004; Zurborg *et al.* 2007). A comparison of mouse and *Drosophila* TRPA1s showed opposing TRPA1 thermosensitivity (Viswanath *et al.* 2003), suggesting that these two TRPA1s are not functional orthologues. Moreover, TRPA1 expressed by different snake species (Gracheva *et al.* 2010), fly (Kang *et al.* 2012), frog, lizard and chicken (Saito *et al.* 2012, 2014; Kurganov *et al.* 2014) are activated by heat, but not cold. TRPA1 activity in snakes (Cordero-Morales *et al.* 2011) and *Drosophila* (Kang *et al.* 2012) is thought to be modulated at the N-terminus, which is believed to be involved in thermosensation. On the other hand, it has been suggested that all thermosensitive TRP channels have a U-shaped activation profile (Clapham & Miller, 2011), which would explain the finding that several heat-activated TRP channels also show activation in response to cold. Several groups have provided experimental evidence for a U-shaped thermosensitive response of human TRPA1 (hTRPA1) (Moparthi *et al.* 2014, 2016), while other studies reported that hTRPA1 is not a thermosensitive channel (Chen *et al.* 2013). Meanwhile, some groups have proposed allosteric gating and coupling models to explain temperature-evoked activation of TRPA1 channels (Salazar *et al.* 2011; Voets, 2012; Jara-Oseguera & Islas, 2013; Qin, 2013). Thus, the mechanisms that mediate heat- or cold-evoked activation of TRPA1 channel activity remain unclear.

Ca^{2+} acts as a permeant ion and an intracellular modulator of various TRP channels. The importance of intracellular and extracellular Ca^{2+} for activation, as well as potentiation and inactivation, of TRPA1 has been reported (Jordt *et al.* 2004; Doerner *et al.* 2007; Zurborg *et al.* 2007; Wang *et al.* 2008; Karashima *et al.* 2009). However, whether extracellular Ca^{2+} plays a role in temperature-dependent gating of TRPA1 channels is unclear. Recently, we reported that heat-induced activation of green anole TRPA1 (gaTRPA1) requires extracellular Ca^{2+} (Kurganov *et al.* 2014). In this study, we identified several negatively charged amino acids near the opening of the ion channel pore that are necessary for heat-evoked activation of TRPA1, chicken TRPA1 (chTRPA1) and rat snake TRPA1 (rsTRPA1).

Methods

DNA

The entire coding regions of TRPA1 from green anole and chicken were previously cloned into pcDNA3.1 (Invitrogen Corp, Carlsbad, CA, USA) (Saito *et al.* 2012, 2014). Rat snake TRPA1 was a kind gift from David Julius (Gracheva *et al.* 2010) and was subcloned into the pcDNA3.1 vector for HEK293 cell expression.

Cell culture

Human embryonic kidney-derived 293T (HEK 293T) cells were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's Medium (WAKO Pure Chemical Industries, Osaka, Japan) containing 10% fetal bovine serum (Biowest SAS, Nuaillé, France), 100 units ml^{-1} penicillin (Invitrogen Corp.), 100 μg ml^{-1} streptomycin (Invitrogen Corp.), and 2 mM GlutaMAX (Invitrogen Corp.). For patch-clamp recordings, 1 μg green anole, chicken, or rat snake TRPA1 in pcDNA3.1 and 0.1 μg pGreen Lantern 1 cDNA were transfected to HEK293T cells cultured in 35 mm dishes using Lipofectamine Plus Reagent (Invitrogen Corp.). After incubating for 3–4 h, the cells were reseeded on coverslips and further incubated at 33°C in 5% CO_2. Patch-clamp recordings were performed 1 day after transfection.

Chemicals

Allyl isothiocyanate (AITC) was purchased from Kanto Chemical (Tokyo, Japan) and HC-030031 was purchased from Sigma-Aldrich (St. Louis, MO, USA). AITC was dissolved in methanol for stock solutions (1 M and 100 mM), and HC-030031 was dissolved in DMSO as a stock solution (10 mM) and diluted to the final concentration using bath solution.

Construction of mutant TRPA1 channels

TRPA1 point mutants were constructed according to the procedures described in the QuikChange site-directed

Table 1. Primer sets for making green anole, chicken and rat snake TRPA1 glutamate to glutamine, glutamine to glutamate, aspartate to asparagine and glutamate to lysine mutations (Small letters indicate mutated nucleotides)

	Mutation	Sense primer (5′→3′)	Antisense primer (5′→3′)
Green anole TRPA1	E894Q	CTTCTTGGTTCACAGcAAGCATATGGCACTCC	GGAGTGCCATATGCTTgCTGTGAACCAAGAAG
	E894K	TCACAGaAAGCATATGGCACTC	CATATGCTTtCTGTGAACCAAGAAG
	D918N	GATATCAATTATCATaATGCTTTCCTTGA	TCAAGGAAAGCATtATGATAATTGATATC
	E922Q	CATGATGCTTTCCTTcAACCAATGTTGAC	GTCAACATTGGTTgAAGGAAAGCATCATG
	E922Q (D918N)	CATaATGCTTTCCTTcAACCAATGTTGAC	GTCAACATTGGTTgAAGGAAAGCATtATG
Chicken TRPA1	Q897E	CCTTTTGGGTTCACAGgAAACATACAGCACAC	GTGTGCTGTATGTTTcCTGTGAACCCAAAAGG
	D921N	GACATAAATTATCACaATGCATTCCTTGA	TCAAGGAATGCATtGTGATAATTTATGTC
	D925N	CACGATGCATTCCTTaATCCATTACTGAG	CTCAGTAATGGATtAAGGAATGCATCGTG
	DD921-925NN	CACaATGCATTCCTTaATCCATTACTGAG	CTCAGTAATGGATtAAGGAATGCATtGTG
Rat snake TRPA1	Q895E	CTTCTTGGTTCACAGgAAACATATGGCACTCC	GGAGTGCCATATGTTTcCTGTGAACCAAGAAG
	E919Q	CCGTcAGGCTTTCCTTGAACCAATG	AAGGAAAGCCTgACGGTAGTTGTTATCTCC
	E923Q	TTCCTTcAACCAATGCTCGCTGATAAACTC	CATTGGTTgAAGGAAAGCCTCACGGTAG
	EE919-923QQ	CCGTcAGGCTTTCCTTcAACCAATG	CATTGGTTgAAGGAAAGCCTgACGGTAG

mutagenesis kit (Agilent Technologies inc, Santa Clara, CA, USA) with minor modifications. Point mutations were introduced by PCR using the gaTRPA1–pcDNA3.1, chTRPA1–pcDNA3.1 and rsTRPA1–pcDNA3.1 as templates with oligonucleotide primers (Table 1) containing the intended mutations. The amplified PCR products were transformed in *Escherichia coli*, and pcDNA3.1 vectors containing TRPA1 were then purified using standard procedures. The entire TRPA1 coding sequences were determined to confirm that only the intended mutations were introduced.

Electrophysiology

For whole-cell experiments, the experimental solutions were (1) bath solution: 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes and 10 mM glucose at pH 7.4 adjusted with NaOH (for Ca^{2+}-free experiments 5 mM EGTA was added instead of 2 mM $CaCl_2$); (2) pipette solution: 140 mM KCl, 5 mM EGTA and 10 mM Hepes at pH 7.4 adjusted with KOH. In some experiments, the free calcium concentration of the pipette solution was maintained at 1 μM as calculated with the MAXC program (Stanford University). Data from whole-cell voltage-clamp recordings were acquired at 10 kHz throughout the experiments and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software, Axon Instruments, Foster City, CA, USA). The membrane potential was clamped at −60 mV.

All experiments were performed at 25°C unless otherwise stated. Heat stimulation was induced by increasing the bath temperature using a pre-heated solution warmed in an inline heater (1°C s^{-1}, with a maximum of 62°C). The temperature was monitored using a thermocouple (TC-344; Warner Instruments, Hamden, CT, USA) placed within 100 μm of the patch-clamped cell. The heat stimulation was stopped immediately upon confirming that TRPA1 currents are inactivated or desensitized. Temperature profiles and Arrhenius plots for the data from whole-cell voltage-clamp recordings were calculated using Origin software (OriginLab, Northampton, MA, USA). Temperature sensitivity has been explained by a mechanism based on a fine balance of large changes in enthalpy and entropy (Voets *et al.* 2004). Alternatively, temperature gating could be driven by changes in heat capacity (Clapham & Miller, 2011). Temperature directionality could also be determined by the degree of allosteric coupling of a unidirectional temperature sensor to the channel gate (Jara-Oseguera & Islas, 2013). Although the above mechanisms of temperature activation differ substantially, we believe that thermal sensitivity (Q_{10}) is still a reliable marker to some extent. The absolute current values were plotted on a log scale against the reciprocal of the absolute temperature (T) (Arrhenius plot), and the temperature threshold for channel activation was determined by the temperature that caused a change in the slope. For current density analysis of TRPA1 channels, the peak currents induced by heat stimulation were measured and presented as pA pF^{-1}.

Statistical analysis

Data in all figures are shown as means ± standard error of the mean (S.E.M.). Statistical analysis was performed

by Student's *t* test or analysis of variance (ANOVA) followed by a two-tailed multiple *t* test with Bonferroni correction using Origin 8.5 software. Probability values (P) of <0.05 were considered statistically significant.

Results

Extracellular Ca^{2+}-dependent heat activation of gaTRPA1

We recently found that whole-cell and inside-out single-channel recordings in HEK293T cells expressing gaTRPA1 showed both heat and AITC-evoked current activation. We also found that temperature-evoked gaTRPA1 currents were not observed in the absence of extracellular Ca^{2+}, although these cells did respond to AITC stimulation (Kurganov *et al.* 2014). To explore how extracellular Ca^{2+} is involved in the heat-evoked activation of gaTRPA1, we first examined gaTRPA1 channel activation upon heat stimulation using a high concentration (1 μM) of intracellular free Ca^{2+} in the pipette solution because intracellular Ca^{2+} is known to affect TRPA1 channel function (Jordt *et al.* 2004; Doerner *et al.* 2007; Zurborg *et al.* 2007). In the whole-cell configuration, negligible inward currents were observed at −60 mV following heat stimulation (from 25°C to over 45°C) with 1 μM intracellular Ca^{2+} (Fig. 1*A*) or without intracellular Ca^{2+} (Fig. 1*C*) in the absence of extracellular Ca^{2+}, yet gaTRPA1 channels responded to AITC (400 μM) with large inward currents. This activation is known to occur through covalent modification of cytosolic cysteine residues of TRPA1 (Macpherson *et al.* 2005; Hinman *et al.* 2006) and was inhibited by the TRPA1-specific antagonist HC-030031 (50 μM) (Fig. 1*A*). Next, we tested whether other divalent cations are involved in heat-evoked activation of gaTRPA1. The absence of extracellular Mg^{2+} did not affect the heat-evoked activation of gaTRPA1 (Fig. 1*B*). Substitution of extracellular Ca^{2+} with another divalent cation, Mg^{2+} or Ba^{2+}, did not evoke gaTRPA1 currents upon heat stimulation, but AITC-evoked currents were induced (Fig. 1*C* and *D*), suggesting that heat-evoked activation of gaTRPA1 channel is solely dependent on extracellular Ca^{2+}. To gain more information on the dependency of TRPA1 heat activation on extracellular Ca^{2+}, we recorded channel activity in the presence of increasing extracellular Ca^{2+} concentrations (0 to 2 mM) under heat stimulation. The current densities of the heat-evoked gaTRPA1 currents increased with increasing extracellular Ca^{2+} concentrations (Fig. 1*E* and *F*). However, the temperature thresholds for heat-evoked gaTRPA1 activation did not differ between 1 and 2 mM extracellular Ca^{2+} (34.6 ± 1.5 °C, $n = 5$ and 35.8 ± 0.5 °C, $n = 6$, respectively) (Fig. 1*G* and *H*). These results suggest that extracellular, but not intracellular, Ca^{2+} ions are important for heat activation of gaTRPA1.

Extracellular Ca^{2+} is not essential for heat-evoked activation of chicken and rat snake TRPA1

To find the residue(s) responsible for heat-evoked gaTRPA1 activation in the presence of extracellular Ca^{2+}, we examined heat-evoked currents of TRPA1 from different species. Based on the finding that chTRPA1 and rsTRPA1 expressed in *Xenopus oocytes* are activated by heat (Gracheva *et al.* 2010; Saito *et al.* 2014), we examined whether heat-evoked activation of chTRPA1 and rsTRPA1 in HEK293T cells requires extracellular Ca^{2+}. We first compared chTRPA1 and rsTRPA1 activation in the presence and absence of extracellular Ca^{2+} in whole-cell configurations at −60 mV. Similar to gaTRPA1 (Fig. 1*B* and *E*), we observed large inward currents for both chTRPA1 (Fig. 2*A*) and rsTRPA1 (Fig. 2*E*) in the presence of extracellular Ca^{2+} which were inactivated/desensitized even during heat stimulation. Interestingly, small but significant inward currents were elicited upon heat stimulation even in the absence of extracellular Ca^{2+} for chTRPA1 (Fig. 2*B*) and rsTRPA1 channels (Fig. 2*F*), suggesting that heat-evoked TRPA1 activation in these species does not depend solely on extracellular Ca^{2+}. Although the heat-evoked currents were smaller in the absence of extracellular Ca^{2+}, temperature thresholds for heat-evoked activation of chTRPA1 and rsTRPA1 determined by constructing Arrhenius plots were unchanged in the presence of extracellular Ca^{2+} (chTRPA1, 39.3 ± 0.5°C, $n = 5$ and 39.6 ± 1.2°C, $n = 6$, Fig. 2*C* and *D*; and rsTRPA1, 37.4 ± 0.9°C, $n = 6$ and 39 ± 0.6°C, $n = 6$, Fig. 2*G* and *H*).

A single mutation within the pore vestibule participates in gaTRPA1 heat activation

Given the findings that gaTRPA1 heat activation requires extracellular, but not intracellular Ca^{2+} and that heat-evoked activation of chTRPA1 and rsTRPA1 occurred even in the absence of extracellular Ca^{2+}, we compared the TRPA1 amino acid sequences for the three species (Fig. 3*A*). Because extracellular Ca^{2+} is required for channel activation following heat stimulation, we hypothesized that negatively charged amino acids located on the extracellular face of gaTRPA1, but not chTRPA1 or rsTRPA1, are involved in this heat-evoked activation. Through an amino acid alignment, we found only one negatively charged glutamate at position 894 of gaTRPA1, whereas the equivalent residues in chTRPA1 (897) and rsTRPA1 (895) had neutral glutamines (Fig. 3*A* in red). To understand the location of gaTRPA1 Glu894 in greater

detail, we performed homology modelling of gaTRPA1 based on the cryo-EM structure of human TRPA1 (Paulsen *et al.* 2015). The resulting model suggests that Glu894 is exposed to the extracellular side of the gaTRPA1 channel at the pore vestibule (Fig. 3*B*). As such, we constructed a gaTRPA1 channel with Glu894 mutated to Gln (E894Q) and analysed the channel activity of this mutant expressed in HEK293T cells following heat and chemical stimulations. E894Q exhibited a large inward current upon heat application in the presence of extracellular Ca^{2+}, and a small but significant inward current was observed even in the absence of extracellular Ca^{2+} (Fig. 3*C* and *D*), suggesting that Glu894 of gaTRPA1 is involved in the heat-evoked activation of gaTRPA1 in the presence of extracellular Ca^{2+}. Although the sizes of the heat-evoked currents for E894Q differed in the presence and absence

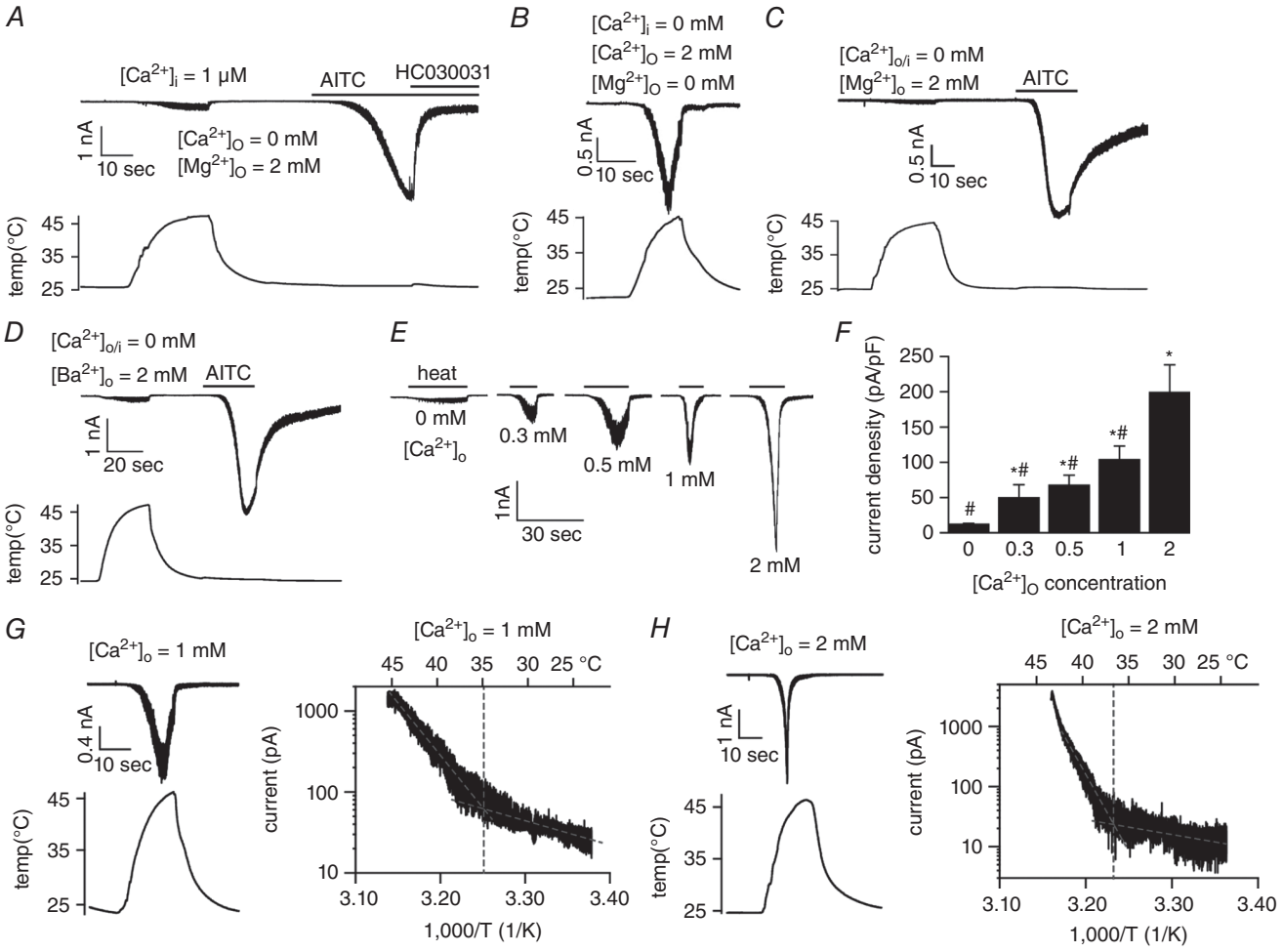

Figure 1. Heat-evoked gaTRPA1 currents in HEK293T cells in the absence and presence of extracellular Ca^{2+}

A, representative current (upper) and temperature (lower) traces in response to heat or AITC (400 μM) stimulation in the absence of extracellular Ca^{2+} in HEK293T cells expressing gaTRPA1. HC030031 (50 μM) was added after AITC stimulation. The pipette solution included 1 μM intracellular free Ca^{2+}. *B*, representative current (upper) and temperature (lower) traces in the absence of extracellular Mg^{2+} with extracellular Ca^{2+} (2 mM) in HEK293T cells expressing gaTRPA1. *C*, representative current (upper) and temperature (lower) traces for heat and AITC (400 μM) stimulation in the absence of extracellular and intracellular Ca^{2+}. *D*, representative current (upper) and temperature (lower) traces in response to heat or AITC (400 μM) simulation in the presence of extracellular Ba^{2+} (2 mM) and absence of extracellular Ca^{2+} in HEK293T cells expressing gaTRPA1. *E*, representative gaTRPA1-mediated heat-evoked whole-cell current traces. Lines above the traces show heat stimulation duration. Extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) are shown below the traces. *F*, heat-evoked gaTRPA1 current densities at different extracellular Ca^{2+} concentrations. *G* and *H*, representative current (upper) and temperature (lower) traces for gaTRPA1 at 1 mM (*G*, left) and 2 mM (*H*, left) extracellular Ca^{2+}, and Arrhenius plots of currents elicited by heat stimulation from 25°C at 1 mM (*G*, right) and 2 mM (*H*, right) extracellular Ca^{2+}. Data represent means plus/minus S.E.M. (n = 5–8). $^{*}P < 0.05$ *vs*. 0 mM; $^{\#}P < 0.05$ *vs*. 2 mM.

of extracellular Ca^{2+}, AITC-evoked currents were similar among wild-type (WT) TRPA1 (420.2 ± 50.6 pA pF^{-1}, $n = 6$) in the presence of extracellular Ca^{2+} and TRPA1 E894Q in the presence (425.3 ± 67.9 pA pF^{-1}, $n = 5$) and absence (415.2 ± 81.5 pA pF^{-1}, $n = 6$) of extracellular Ca^{2+} (Fig. 3*E*). Moreover, changing Glu894 to positively charged Lys (E894K) conferred heat-evoked current activation in the presence (219.4 ± 26.5 pA pF^{-1}, $n = 5$) and absence (87.7 ± 15.3 pA pF^{-1}, $n = 6$) of extracellular Ca^{2+} (Fig. 3*F* and *G*), which was similar to that seen for E894Q, suggesting that neutralization of the negative charge is important for heat-evoked activation of gaTRPA1 in the absence of extracellular Ca^{2+}. To further confirm the importance of the negative charge at position 894 of gaTRPA1 for heat-evoked activation in the presence of extracellular Ca^{2+}, we mutated Gln897 in chTRPA1 and Gln895 in rsTRPA1 to Glu. Interestingly, both Q897E (chTRPA1) and Q895E (rsTRPA1) mutants showed significantly smaller inward currents upon heat stimulation in the absence of extracellular Ca^{2+} (20.9 ± 6.3 pA pF^{-1}, $n = 4$ and 50.8 ± 11.9 pA pF^{-1}, $n = 5$, respectively) than in WT channels (Fig. 3*I*, *J*, *L* and *M*), while Q897E (chTRPA1) and Q895E (rsTRPA1) mutants showed large inward currents upon heat stimulation in the presence of extracellular Ca^{2+} (97.1 ± 16.5 pA pF^{-1}, $n = 5$ and 335.7 ± 22.2 pA pF^{-1}, $n = 5$, respectively) (Fig. 3*H*, *J*, *K* and *M*), similar to that seen for WT channels. These data suggest that more negative charges on the surface increase Ca^{2+} sensitivity of the channels to heat stimulation as in gaTRPA1. Together, these results indicate that AITC-induced activation of gaTRPA1 is different from activation induced by temperature and that a negatively charged amino acid is important for heat-evoked activation of TRPA1 channels in the presence of extracellular Ca^{2+}.

Conserved mutations near the extracellular face of pore helix 2 are needed for large activation by heat

Since the gaTRPA1 E894Q mutant is similar to chTRPA1 and rsTRPA1 in that heat-evoked currents are smaller in the absence of extracellular Ca^{2+} than in the

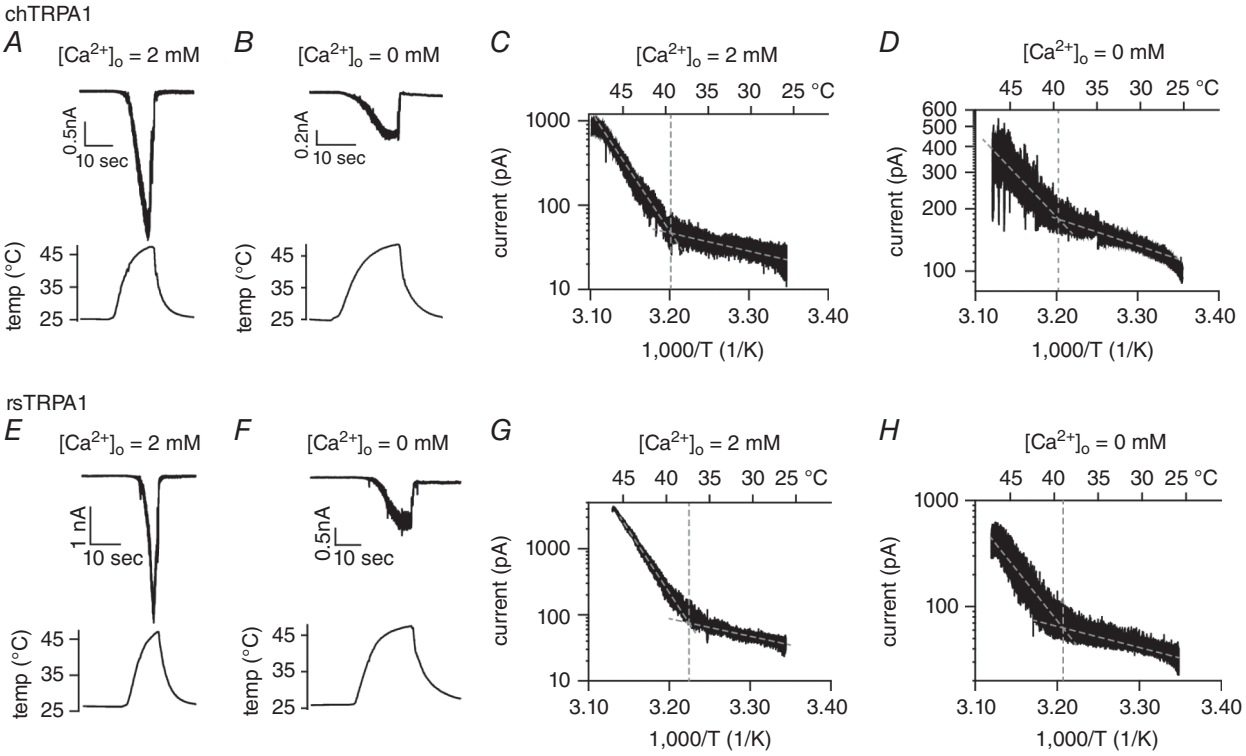

Figure 2. Heat-evoked currents of chTRPA1 and rsTRPA1 expressed in HEK293T cells in the presence and absence of extracellular Ca^{2+}
A and *B*, representative current (upper) and temperature (lower) traces for chTRPA1 in the presence (*A*) and absence (*B*) of extracellular Ca^{2+}. *C* and *D*, Arrhenius plots of currents elicited by heat stimulation from 25°C in the presence (*C*) and absence (*D*) of extracellular Ca^{2+}. The average temperature thresholds for heat-evoked activation were 39.3 ± 0.5°C, ($n = 5$) and 39.6 ± 1.2°C ($n = 6$) in the presence and absence, respectively, of extracellular Ca^{2+}. *E* and *F*, representative current (upper) and temperature (lower) traces for rsTRPA1 in the presence (*E*) and absence (*F*) of extracellular Ca^{2+}. *G* and *H*, Arrhenius plots of currents elicited by heat stimulation from 25°C in the presence (*G*) and absence (*H*) of extracellular Ca^{2+}. Temperature thresholds for heat-evoked activation were 37.4 ± 0.9°C ($n = 6$) and 39 ± 0.6°C ($n = 6$) in the presence and absence, respectively, of extracellular Ca^{2+}.

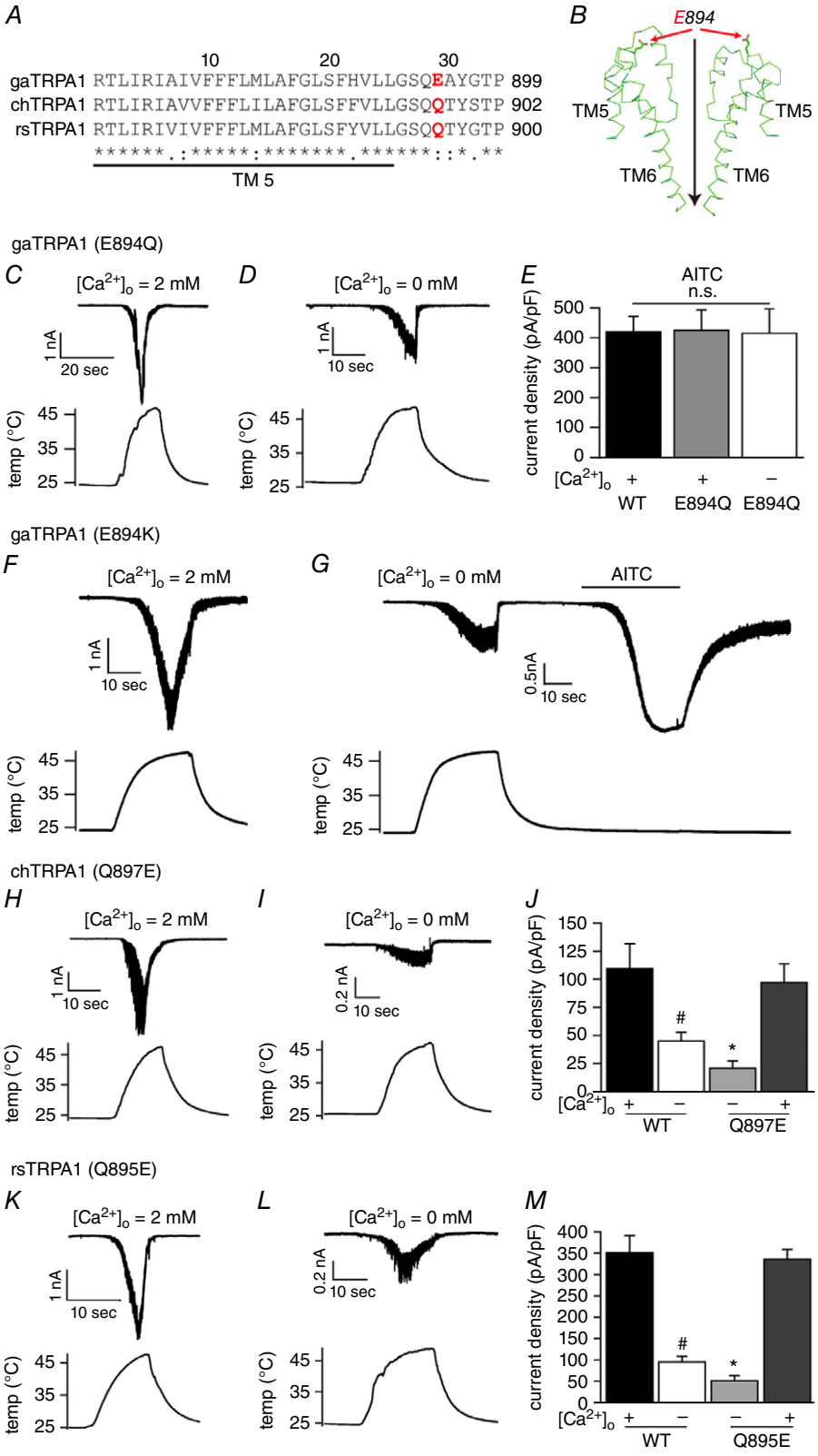

Figure 3. Location of amino acid residues in gaTRPA1 that are required for heat-evoked activation
A, alignment of TM5 amino acids in gaTRPA1, chTRPA1 and rsTRPA1. The location of amino acids that differ from gaTRPA1 (E894) is indicated in red. Numbering is based on the gaTRPA1 sequence. *, : and . indicate identical, similar and different, respectively. *B*, a side view of the homology model structure for two gaTRPA1 subunits based

on hTRPA1. Red and black arrow indicates the E894 side chain and direction of ion flow, respectively. *C* and *D*, representative current (upper) and temperature (lower) traces for gaTRPA1 (E894Q) in the presence (*C*) and absence (*D*) of extracellular Ca^{2+}. *E*, comparison of AITC-evoked currents for wild-type (WT; black) and E894Q (grey and white) gaTRPA1 in the presence and absence of extracellular Ca^{2+}. *F* and *G*, representative current (upper) and temperature (lower) traces for heat and AITC (400 μM) stimulation in the presence (*F*) and absence (*G*) of extracellular and intracellular Ca^{2+} in HEK293T cells expressing gaTRPA1 mutant (E894K). *H*, *I*, *K* and *L*, representative current (upper) and temperature (lower) traces for chTRPA1 (Q897E) (*H*, *I*) and rsTRPA1 (Q895E) (*K*, *L*) in the presence (*H*, *K*) and absence (*I*, *L*) of extracellular Ca^{2+}. *J* and *M*, comparison of heat-evoked currents for wild type (black and white) and Q to E mutants (light and dark grey) of chTRPA1 (*J*) and rsTRPA1 (*M*) in the presence (+) and absence (−) of extracellular Ca^{2+}. Data represent the means ± S.E.M. (n = 4–6). n.s., no significance; $^{*}P < 0.05$ *vs.* WT (+ and −), Q897E and Q895E (+); $^{\#}P < 0.05$ *vs.* WT (+), Q897E and Q895E (+).

presence of extracellular Ca^{2+} (Figs 2 and 3), we hypothesized that negatively charged amino acids at the extracellular side of the channel could be conserved across all three species (gaTRPA1, chTRPA1 and rsTRPA1). Further amino acid sequence alignment identified two candidate amino acids, Glu and Asp, before (green) and within (cyan) the pore helix 2 (Fig. 4*A*). To neutralize these charged amino acids, we mutated the Glu and Asp to Gln and Asn, respectively, and made single, double and triple mutants of the channels. As mentioned above (Fig. 3*D*), gaTRPA1 (E894Q) exhibited significantly larger heat-evoked currents at −60 mV in the absence of extracellular Ca^{2+} than wild-type gaTRPA1 (97.8 ± 14.3 pA pF^{-1}, n = 6 *vs.* 11.9 ± 1.2 pA pF^{-1}, n = 5) (Fig. 4*C*). We therefore made two double mutations (E894Q–D918N and E894Q–E922Q) and one triple mutation (E894Q–D918N–E922Q) in gaTRPA1. Although the two double mutations showed heat-evoked currents that were similar in size to the single mutation E894Q (E894Q–D918N, green, 125.6 ± 40.9 pA pF^{-1}, n = 6; E894Q–E922Q, cyan, 120.5 ± 38.7 pA pF^{-1}, n = 5) in the absence of extracellular Ca^{2+}, the triple mutant showed large inward currents upon heat stimulation in the absence (197.2 ± 41.1 pA pF^{-1}, n = 5, navy) and presence (200.4 ± 45.2 pA pF^{-1}, n = 6, pink) of extracellular Ca^{2+}, and the currents were similar in size to those of wild-type gaTRPA1 currents in the presence of extracellular Ca^{2+} (199.1 ± 39.1 pA pF^{-1}, n = 5, black) (Fig. 4*B* and *C*). Unlike gaTRPA1, in the absence of extracellular Ca^{2+}, chTRPA1 and rsTRPA1 channels were activated by heat with small inward currents (Fig. 2*B* and *F*). Thus, we made two single point mutants in chTRPA1 and rsTRPA1 (D921N and D925N for chTRPA1; E919Q and E923Q for rsTRPA1), as well as double mutants (D921N–D925N for chTRPA1; E919Q–E923Q for rsTRPA1) and examined the channel activity of these mutants after heat stimulation in the absence or presence of extracellular Ca^{2+}. Single point mutations in chTRPA1 (D921N and D925N) had inward currents (D921N, green, 48.6 ± 14 pA pF^{-1}, n = 6; D925N, cyan, 44.8 ± 10.4 pA pF^{-1}, n = 6) that were similar to wild type (white, 38.5 ± 7.6 pA pF^{-1}, n = 6) (Fig. 4*D* and *E*). A double chTRPA1 mutant (D921N/D925N) showed large inward currents (104 ± 16 pA pF^{-1}, n = 6, navy), which were not affected by the presence of extracellular Ca^{2+} (113 ± 26 pA pF^{-1}, n = 6, pink) and were similar to wild-type chTRPA1 currents in the presence of extracellular Ca^{2+} (109.3 ± 22.2 pA pF^{-1}, n = 6, black). For rsTRPA1, the single mutant E919Q (141.6 ± 37.5 pA pF^{-1}, n = 5, green) had similar inward currents in response to heat stimulation compared to wild type (95.1 ± 25.4 pA pF^{-1}, n = 6, white), whereas the E923Q single mutant showed significantly larger heat-evoked currents (189.9 ± 24.9 pA pF^{-1}, n = 5, cyan) than wild type (Fig. 4*F* and *G*). Meanwhile, the E919Q–E923Q rsTRPA1 double mutant showed large inward currents (298.4 ± 56.5 pA pF^{-1}, n = 5, navy) that were not affected by extracellular Ca^{2+} (330.2 ± 51.4 pA pF^{-1}, n = 5, pink) and were similar to the wild-type rsTRPA1 currents in the presence of extracellular Ca^{2+} (340.3 ± 40.9 pA pF^{-1}, n = 7, black); this pattern was similar to that of chTRPA1. These results suggest that neutralization of the two charged amino acids by extracellular Ca^{2+} is important for a heat-evoked large activation of gaTRPA1, chTRPA1, and rsTRPA1.

Discussion

Our findings showed that heat-evoked activation of gaTRPA1 is dependent on extracellular Ca^{2+} and requires Ca^{2+} binding to negatively charged amino acids near the extracellular face of the channel pore. Together, extracellular Ca^{2+} binding and heat could activate the TRPA1 channel, whereas the absence or substitution of other divalent cations for extracellular Ca^{2+} abrogated heat-evoked currents (Fig. 1 *A*–*D*). The inability of intracellular Ca^{2+} to activate TRPA1 following heat stimulation in our experiments could support our hypothesis that extracellular Ca^{2+} is involved only in heat-evoked activation of gaTRPA1, as opposed to previous reports showing that intracellular Ca^{2+} is important for TRPA1 activation (Jordt *et al.* 2004; Doerner *et al.* 2007; Zurborg *et al.* 2007), as well as potentiation/activation and secondary inactivation after Ca^{2+} influx through the TRPA1 channel following chemical stimulation (Nagata *et al.* 2005; Karashima *et al.* 2008; Wang *et al.* 2008). As we previously reported, the gaTRPA1 channel kinetics were quite different between AITC- and heat-mediated stimulation (Kurganov *et al.* 2014), suggesting that the

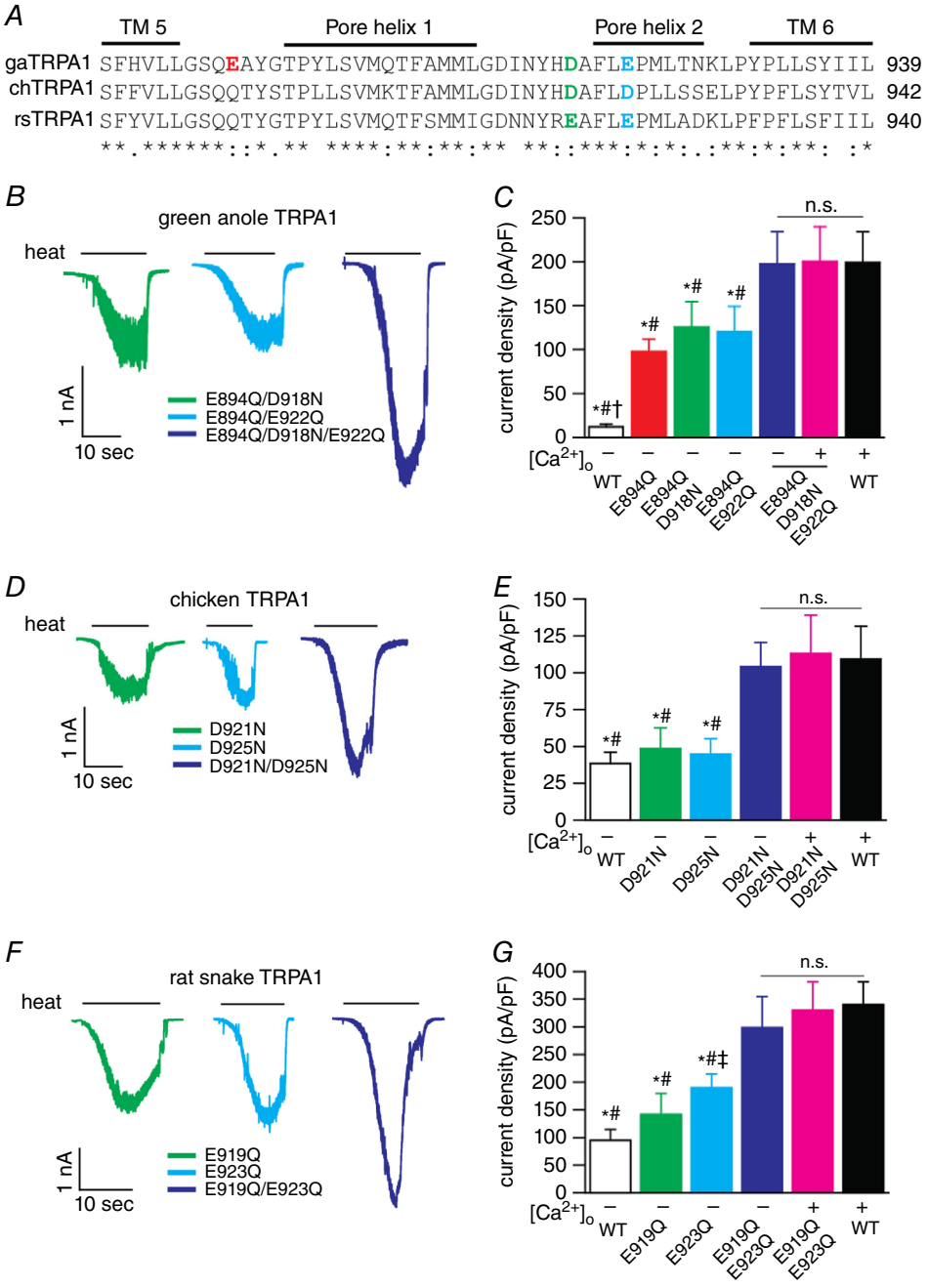

Figure 4. Heat-evoked activation of wild-type and mutant TRPA1 channels from three species
A, amino acid sequence alignment of gaTRPA1, chTRPA1, and rsTRPA1. The locations of TM5, pore helix 1, pore helix 2, and TM6 are indicated by lines above the alignment. The negatively charged amino acids examined are shown in red (gaTRPA1), green and cyan (all three species). Numbering on the right of alignment is based on gaTRPA1. *, : and . indicate identical, similar and different, respectively. *B*, *D* and *F*, representative heat-evoked whole-cell current traces recorded at −60 mV for gaTRPA1 (*B*), chTRPA1 (*D*), and rsTRPA1 (*F*) having the indicated mutations. *C*, *E* and *G*, comparison of heat-evoked activation of wild-type and mutated gaTRPA1 channels (*C*), chTRPA1 (*E*) and rsTRPA1 (*G*) in the presence (+) and absence (−) of extracellular Ca^{2+}. Data represent the means ± S.E.M. ($n = 5$–7). n.s., no significance. $^{*}P < 0.05$ *vs*. WT (+). $^{\#}P < 0.05$ *vs*. E894Q/D918N/E922Q (−,+); D921N/D925N (−,+); E919Q/E923Q (−,+). $^{\dagger}P < 0.05$ *vs*. E894Q; E894Q/D918N; E894Q/E922Q (−). $^{\ddagger}P < 0.05$ *vs*. WT (−).

gating mechanisms differed between the two stimuli. This result could indicate that the requirement for intracellular Ca^{2+} also differs in the different stimulation. Meanwhile, heat-evoked activation of chTRPA1 and rsTRPA1 was observed both in the absence and presence of extracellular Ca^{2+}, although the inward currents were significantly smaller in the absence of extracellular Ca^{2+} (Figs 2 and 4). An alignment of gaTRPA1, chTRPA1, and rsTRPA1 amino acid sequences showed that the residues corresponding to Glu894 in gaTRPA1, 897 and 895, in chTRPA1 and rsTRPA1, respectively, were both Gln (Fig. 3*A*). Mutation of Glu894 to Gln in the gaTRPA1 channel led to a small, but significant heat-evoked activation even in the absence of extracellular Ca^{2+}, whereas currents for the chTRPA1 (Q897E) and rsTRPA1 (Q895E) mutants were reduced in the absence of extracellular Ca^{2+} (Figs 3 and 4). Moreover, neutralization of acidic amino acids in TRPA1 that are common to all three species may mimic the effect of extracellular Ca^{2+}, since TRPA1 with Gln showed activation of large heat-evoked inward currents even in the absence of extracellular Ca^{2+}, and the current sizes were comparable to those seen for wild-type channels in the presence of extracellular Ca^{2+} (Fig. 4). Thus, binding of extracellular Ca^{2+} to acidic amino acids is likely to be important for heat-evoked activation of TRPA1 expressed in green anole, chicken and rat snake. As shown in this study and in previous studies (Gracheva *et al.* 2010; Kurganov *et al.* 2014; Saito *et al.* 2014), the activation thresholds of TRPA1 from the three species are between 36°C and 40°C, suggesting that these TRPA1 channels would not be activated at 25°C, the temperature at which we performed patch-clamp recordings. However, as we reported previously (Kurganov *et al.* 2014), there is a synergism between temperature and chemical agonists such as AITC, which indicates that in the presence of other stimulations, temperatures lower than the threshold can theoretically activate TRPA1 channels. Indeed, in an earlier study (Fujita *et al.* 2008), we observed single-channel opening of human TRPA1 in HEK293T cells at 25°C in the absence of a possible agonist, suggesting that TRPA1 channels can be open under specific conditions.

Atomic level structures of TRPV1, a capsaicin- and heat-activated TRP channel, have recently been determined by single-particle analysis with cryo-EM under conditions wherein the purified protein was in complex with amphipathic polymers or in nanodiscs (Caterina *et al.* 1997; Cao *et al.* 2013; Liao *et al.* 2013; Gao *et al.* 2016). The structural data suggest that the presence of phosphatidylinositides supports a closed state of TRPV1 and removal of endogenous phosphatidylinositides upon heat stimulation could contribute to channel gating and further cation influx through the TRPV1 pore (Gao *et al.* 2016). Thus, we propose a new model for extracellular Ca^{2+}-dependent heat-evoked activation of TRPA1 wherein binding of extracellular Ca^{2+} to acidic amino acids near the extracellular side of the TRPA1 channel contributes to channel gating and cation influx upon heat stimulation (Fig. 5). Support for this model is provided by the involvement of Glu at position 920 of human TRPA1 in collecting cations into the mouth of the pore and changing the surface potential by ~16 mV (Christensen *et al.* 2016). Our model is also supported by the channel gating and ion permeation of the highly Ca^{2+}-selective channel TRPV6, which involves extracellular cation binding to the channel (Saotome *et al.* 2016). Although the Ca^{2+} sensitivity and permeability of TRPV6 (>100) (Yue *et al.* 2001) and TRPA1 (>5) (Karashima *et al.* 2010) differ, the overall locations of the acidic amino acids in both channels are similar, suggesting that a comparison of the characteristics of the two channels could clarify the significance of Ca^{2+} binding in heat-evoked gating of TRPA1 channels.

Since extracellular Ca^{2+} dependency has not been clearly shown in heat-activated TRPA1 channels from various species (Hamada *et al.* 2008; Gracheva *et al.* 2010; Kang *et al.* 2012; Saito *et al.* 2012, 2014), we emphasize that, in addition to potentiation/activation or secondary inactivation (Nagata *et al.* 2005; Karashima *et al.* 2008; Wang *et al.* 2008) by intracellular Ca^{2+}, binding of extracellular Ca^{2+} to acidic amino acids in the TRPA1 channel vestibule is important for heat-evoked channel gating. Ca^{2+} concentrations vary in extracellular spaces during development (Brown *et al.* 1995) and proliferation of various cells such as skin keratinocytes (Pillai *et al.*

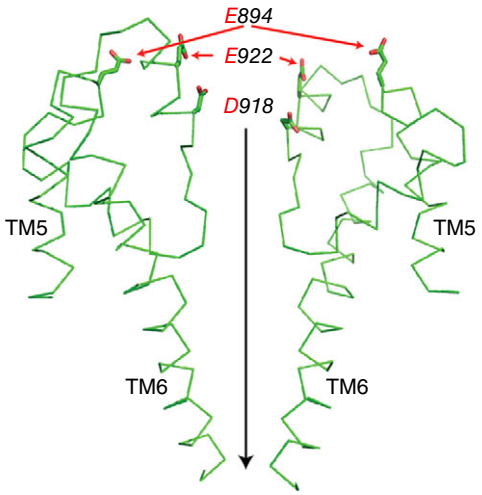

Figure 5. Possible external Ca^{2+} binding sites on the gaTRPA1 channel
A ribbon representation showing a side view of the transmembrane domains of two opposing gaTRPA1 model subunits based on hTRPA1 is shown. The side chains of mutated amino acids are indicated by red arrows and the black downward vertical arrow indicates the direction of ion flow.

1990), although TRPA1 is not strongly expressed in epithelial cells. In the sensory neurons of vertebrates where TRPA1 is well expressed, physiological extracellular Ca^{2+} concentrations generally fluctuate between 1.8 and 2 mM, but do not fall below 1 mM. Thus, the physiological significance of the extracellular Ca^{2+} for heat-evoked activation of TRPA1 from several species is not known. Nonetheless, the dependence of TRPA1 heat-induced activation on extracellular Ca^{2+} in some species could be involved in physiological phenomena that are affected by extracellular Ca^{2+} levels.

In summary, we have identified important negatively charged amino acid residues located at the outer vestibule of the gaTRPA1 channel that play an important role in heat-induced channel activation, but do not participate in responses to other stimuli such as AITC. Moreover, the observation that several acidic amino acids in TRPA1 from three species (green anole, chicken and rat snake) contribute to large channel activity following heat stimuli suggests that allosteric coupling may be involved in this type of channel activation.

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Additional information

Competing interests

The authors declare no competing financial interests.

Author contributions

E.K. and M.T. designed the experiments and wrote the manuscript. E.K. and C.T.S. performed the experiments. E.K., C.T.S., S.S. and M.T. discussed and interpreted the data. M.T. supervised this work. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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