The nociceptor ion channel TRPA1 is potentiated and inactivated by permeating calcium ions

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The transient receptor potential A1 (TRPA1) channel is the molecular target for environmental irritants and pungent chemicals such as cinnamaldehyde and mustard oil. Extracellular Ca2+ is a key regulator of TRPA1 activity, both potentiating and subsequently inactivating it. In this report we provide evidence that the effect of extracellular Ca2+ on these processes is indirect and can be entirely attributed to entry through TRPA1 and subsequent elevation of intracellular calcium. Specifically, we found that in a pore mutant of TRPA1, D918A, in which Ca2+ permeability was greatly reduced, extracellular Ca2+ produced neither potentiation nor inactivation. Both processes were restored by reducing intracellular Ca2+ buffering, which allowed intracellular Ca2+ levels to become elevated upon entry through D918A channels. Application of Ca2+ to the cytosolic face of excised patches was sufficient to produce both potentiation and inactivation of TRPA1 channels. Moreover, in whole cell recordings, elevation of intracellular Ca2+ by UV uncaging of DMNP-EDTA potentiated TRPA1 currents. In addition, our data show that potentiation and inactivation are independent processes. TRPA1 currents could be inactivated by Mg2+, Ba2+ and Ca2+, but potentiated only by Ba2+ and Ca2+. Saturating activation by cinnamaldehyde or mustard oil occluded potentiation but did not interfere with inactivation. Lastly, neither process was affected by mutation of a putative intracellular Ca2+-binding EF hand motif. In conclusion, we have further clarified the mechanisms of potentiation and inactivation of TRPA1 using the D918A pore mutant, an important tool for investigating the contribution of Ca2+ influx through TRPA1 to nociceptive signaling.

Members of the transient receptor potential (TRP) family of ion channels that are expressed by sensory neurons in dorsal root and trigeminal ganglia serve as sensors for temperature and noxious stimuli (1,2). Of these, TRPA1 is one of the key sensors for pungent chemicals and environmental irritants and is essential for behavioral responses of mice to conditions that evoke inflammatory pain (3-7). Inflammatory mediators, such as bradykinin, bind to G protein-coupled receptors on nociceptors, initiating a second messenger signaling cascade that leads to Ca2+ influx mediated in part by the opening of Ca2+ permeable TRPA1 channels (5,8,9). TRPA1 is also activated directly by a wide range of chemicals that cause painful sensations, including food additives such as mustard oil (MO), cinnamaldehyde (Cin), onion, raw garlic and thyme, environmental irritants such as formaldehyde and acrolein (a component of automobile exhaust) and products of oxidative stress (4,8,10-16). Many of these chemicals activate TRPA1 by binding covalently to reactive cysteine residues in the amino terminus of the protein (17,18), producing a modification of the channel that can last for more than an hour and which leads to persistent activation of TRPA1 currents (18,19).

Ca2+ plays at least two roles in regulating the activity of TRPA1 channels. TRPA1 currents that are activated by pungent chemicals are rapidly potentiated in the presence of extracellular Ca2+, an effect that may be mediated by Ca2+ entry through TRPA1 (4,20,21). This mechanism is attractive as it could also account for the observed activation of TRPA1 by some inflammatory...
mediators (5,8). For example, bradykinin, acting through its cognate receptor, stimulates a phospholipase C (PLC) based signaling pathway leading to elevation of intracellular Ca²⁺ and it has been proposed that Ca²⁺ is the proximate stimulus that gates TRPA1 (4,5) (but see also (8,22)). Activation of TRPA1 by intracellular Ca²⁺ has been observed in experiments where Ca²⁺ is dialyzed into cells or applied to excised patches (20,21,23), although it is not known whether this is the mechanism for Ca²⁺ potentiation or receptor-activation of TRPA1. The site for Ca²⁺-activation of TRPA1 has been proposed to be an EF hand present in the N terminus of the protein (20,21).

A second and equally important effect of extracellular Ca²⁺ is to inactivate TRPA1 (e.g.(4,19,20,24) (but see also (19)). TRPA1 currents activated by pungent chemicals in the absence of extracellular Ca²⁺, decay within seconds when extracellular Ca²⁺ is introduced (24). Inactivation may be mediated by binding of Ca²⁺ to the outside of the channel or could be mediated by elevation of intracellular Ca²⁺, which is known to cause desensitization of other TRP channels (25-31). Alternatively, inactivation of TRPA1 might simply reflect the stochastic and obligatory entry of the channel into the inactivated state, following Ca²⁺-dependent activation, similar to inactivation of voltage-gated ion channels (32).

By studying mutant TRPA1 channels in which Ca²⁺ permeability is reduced, we show that both the potentiating and inactivating effects of extracellular Ca²⁺ can be attributed to entry of Ca²⁺ through TRPA1 and the subsequent elevation of intracellular Ca²⁺. We also provide evidence that potentiation and inactivation are mediated by independent mechanisms, each with a distinct sensitivity and specificity for divalent cations.

**EXPERIMENTAL PROCEDURES**

**CDNAs and expression in HEK-293 cells**

For most experiments we used an N terminal YFP fusion of rat TRPA1 generated by inserting a PCR fragment of YFP into an AgeI site at the 5’ prime end of a rTRPA cDNA. To confirm that the YFP fusion did not alter function, in some experiments we used an unaltered clone of rTRPA1 which was co-transfected with GFP (20:1). To assess whether some of our results could be attributed to species differences between rat and human variants of TRPA1, in some experiments we used hTRPA1, co-expressed at a ratio of 20:1 with GFP. Point mutations were generated by Quick Change mutagenesis (Stratagene, La Jolla, CA) and were verified by sequencing (Retrogen, San Diego, CA, or Macrogen, Rockville, MD). All constructs were transiently transfected into HEK-293 cells using TransIT-LT1 (Mirus Bio Corporation, Madison, WI) as suggested by the manufacturer and cells were cultured at 37 °C. Prior to recording, cells were treated with 0.05% trypsin and 4 mM EDTA in phosphate-buffered saline for 5 min at 37 °C, and replated in the recording chamber with Tyrode’s solution. Transfected cells were identified under epifluorescence. Recordings were performed approximately 24-48 hrs after transfection at room temperature.

**Patch clamp recording**

Patch clamp electrophysiology was performed as previously described (27,33). In brief, recordings were made with an Axopatch 200B amplifier, sampled at 5 kHz and filtered at 1 kHz. Data were digitized with a digidata 1322a, acquired with pClamp 8.2 and analyzed with Clampfit 8.2 (Axon Instruments, Union City, CA). Representative data shown in figures were exported into Origin (Microcal, Northampton, MA) and CorelDRAW (Corel Corporation, Eden Prairie, MN). Pipettes were fabricated from borosilicate glass, fire polished to a resistance of 2-3 mOhms. For whole-cell recordings, the membrane potential was ramped from -80 mV to +80 mV (1V/s). Excised patch recording was performed as previously described (27). Briefly, following formation of a gigaohm seal, the patch was excised into Ca²⁺-free solution and rapidly moved in front of a linear array of microperfusion pipes (Warner Instruments, Hamden, CT).

**Solutions**

Tyrode’s solution contained 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM Dextrose, 10 mM HEPES (pH 7.4 with NaOH). Ca²⁺ free bath solution was: 150 mM NaCl, 10 mM HEPES, 2 mM HEDTA. Ca²⁺, Mg²⁺ or Ba²⁺-containing bath solutions contained 150 mM NaCl, 10 mM HEPES (pH 7.4 with NaOH) and 200 μM, 2 mM, 10 mM CaCl₂, 2 mM Mg²⁺, or 2 mM Ba²⁺. For lower Ca²⁺ concentrations, 1.5 mM
Ca²⁺ or 1.9 mM Ca²⁺ was added to Ca²⁺-free solution buffered by 2mM HEDTA, to produce 12 μM or 50 μM free Ca²⁺, respectively. Low buffer internal Cs⁺ based solution (LB-Cs⁺) contained: 0.02 mM EGTA, 145 mM CsCl, 2 mM MgATP, 10 mM HEPES, and high buffer internal Cs⁺ based solution (HB-Cs⁺) contained: 5 mM EGTA, 3 mM CaCl₂ (100 nM free Ca²⁺), 145 mM CsCl, 2 mM MgATP, 10 mM HEPES (pH 7.4 with CsOH). For excised inside-out patch experiments, the pipette solution contained: 150 mM NaCl, 10 mM HEPES with either 2 mM EGTA or 2 mM HEDTA (pH 7.4). Ca²⁺-free solution contained: 150 mM NaCl, 10 mM HEPES, with either 2 mM EGTA or 2 mM HEDTA. Cytosolic solutions with nanomolar or micromolar concentrations of free Ca²⁺ were obtained by adding Ca²⁺ (1.22 mM, 1.65 mM, 1.77 mM, 1.88 mM or 1.98 mM) to Ca²⁺-free solution buffered by 2 mM EGTA to obtain free Ca²⁺ concentrations of 100 nM, 300 nM, 500 nM, 1 μM and 5.2 μM, respectively. 1 mM pentasodium tripolyphosphate hexahydrate (polyP₅) was added to the cytoplasmic solution for excised patch experiments. All Ca²⁺ concentrations are reported as calculated with MaxChelator (www.stanford.edu/~cpatton/maxc.html). For cell-attached recordings, bath KCl was substituted for NaCl to zero the membrane potential. For flash photolysis experiments, caged Ca²⁺ solution contained: 120 mM CsAsp, 20 mM CsCl, 10 mM HEPES, 2.5 mM 2,6-dimethyl-4-nitropyridine (DMNP-EDTA; Invitrogen), 0.75 mM CaCl₂, pH 7.4. The bath solution in ion permeability experiments contained NaCl, KCl, CsCl, or NMDGC1 (150 mM), 10 mM HEPES and 2 mM HEDTA, or CaCl₂ (128 mM) with 10 mM HEPES (pH 7.4 with NaOH). For recordings during which intracellular Ca²⁺ concentration was measured the pipette solution contained 20 μM Fura-4F, 145 mM CsCl, 2 mM MgATP, 10 mM HEPES, with or without 5mM EGTA, or 10 μM Fluo-5F in caged Ca²⁺ solution (with 2 mM MgATP and 3 mM Na₂ATP).

Flash photolysis of caged compound

All experiments were performed on an Olympus IX71 microscope. To allow uniform dialysis of the cell with the caged compounds, we waited at least 100s after breaking into whole cell recording mode before uncaging. UV light from a mercury arc lamp that was then passed through a 350/50x bandpass filter (Chroma Technology Corp, San Diego, CA) was controlled by a uniblitz shutter (Vincent Associates, Rochester, NY). The shutter was opened for 100 ms to uncage Ca²⁺. For experiments in which Ca²⁺ levels were measured, UV light from a Xenon arc lamp was passed through a bandpass filter (380xv1, Chroma Technology Corp, San Diego, CA) that was part of a LEP filter wheel/shutter (Ludl, Hawthorne, NY) operated under computer control (Simple PCI, Compix Corp. Sewickley, PA). The shutter was opened for 1 s to uncage Ca²⁺.

Ca²⁺ imaging

Ca²⁺ imaging was performed as previously described (33). Images were acquired on an Olympus IX71 microscope equipped with an LEP filter wheel (Ludl Electronic Products, Hawthorne, NY) and ORCA ER camera (Hamamatsu Photonics) and were analyzed with simple PCI (Compix Corp.). The low affinity Ca²⁺ indicators Fluo-5F (10 μM) or Fura-4F (20 μM) were loaded through the patch pipette. The fluorescence emission from Fluo-5F was detected using an YFP filter set (Chroma Technology Corp., Rockingham, VT). The fluorescence emission from Fura-4F was detected with a Fura 2 filter set (Chroma) using excitation alternately at 340 nM and 380 nM. The ratio of the fluorescence emission (R = F340/F380) was used to calculate the intracellular Ca²⁺ concentration as follows:

\[
\text{Ca}^{2+} = K_d \times Q \times \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right)
\]

Where \(K_d\) is the Ca²⁺ dissociation constant of the indicator Fura-4F (770 nM), \(Q\) is emission ratio at 380 nM recorded at 0 Ca²⁺ and at a saturated Ca²⁺ concentration, and \(R_{\text{max}}\) and \(R_{\text{min}}\) are the ratios under saturating Ca²⁺ or 0 Ca²⁺ conditions, respectively. \(R_{\text{min}}\) was measured from a separate set of cells in which intracellular solution was heavily buffered by 5 mM EGTA, and the extracellular solution was Ca²⁺-free (buffered with 2 mM HEDTA). To obtain \(R_{\text{max}}\) and \(Q\), at the end of each experiment, ionomycin was added to the bath, and the emission with 2 mM Ca²⁺ and 0 Ca²⁺ in the bath was measured.
Ion selectivity measurements

TRPA1 currents were activated by brief exposure to cinnamaldehyde, and the current in the presence of different external cations was measured by rapidly exchanging the extracellular solution using a linear array of microperfusion pipes. The internal solution contained Cs+ (HB-Cs+) and the ion permeability relative to Cs+ was calculated from the reversal potential under bi-ionic conditions according to:

\[
\frac{P_{Ca}}{P_{Cs}} = \frac{[Ca^2+]_o}{4[Ca^2+]_o} \times \exp \left( \frac{E_{rev} \times F}{RT} \right) \times \left\{ \exp \left( \frac{E_{rev} \times F}{RT} \right) + 1 \right\}
\]

where \(P_{Ca}/P_{Cs}\) and \(P_X/P_{Cs}\) are the Ca\(^{2+}\) and monovalent cation permeability relative to Cs\(^+\), respectively, \(E_{rev}\) is the reversal potential, \(F\) is Faraday’s constant, \(R\) is the universal gas constant, and \(T\) is the absolute temperature (34). \(E_{rev}\) was corrected for the liquid junction potentials measured under the conditions of our experiments.

Chemicals

Cinnamaldehyde, menthol, and pentasodium tripolyphosphate hexahydrate (polyP\(_3\)) were purchased from Sigma (St. Louis, MO). Fluo-5F, Fura-4F and ionomycin were from Invitrogen Molecular probes (Eugene, OR). HC-030031 was from ChemBridge (San Diego, CA). For stock solutions, cinnamaldehyde, HC-030031 and ionomycin were dissolved in DMSO, menthol was in ethanol and other chemicals were in water. Prior to use, the stock solution was diluted in the appropriate bath solution. The final DMSO or ethanol concentration was < 0.2%.

RESULTS

A dual effect of extracellular Ca\(^{2+}\): potentiation and inactivation of TRPA1

To study Ca\(^{2+}\)-regulation of TRPA1 channels, we expressed rTRPA1 fused to YFP in HEK 293 cells and recorded responses to TRPA1 agonists with patch clamp electrophysiology. Bath perfusion of cinnamaldehyde (Cin; 100 \(\mu\)M) for \(~40\) s in the absence of extracellular Ca\(^{2+}\) strongly activated a non-selective cation current of 3.9 ± 0.4 nA at +80 mV (n=10) in TRPA1 expressing cells (Fig. 1A), but not in untransfected cells. The TRPA1 current persisted following washout of the agonist (Fig. 1A) consistent with the observation that Cin binds covalently to cysteine residues in the N terminus of the channel and therefore its binding is not readily reversible (17,18). Addition of 2 mM Ca\(^{2+}\) to the bath caused a strong potentiation which was followed by a rapid decay of the current as previously noted (4,24) (Fig. 1A). We quantitated the degree of potentiation by the fold increase in the current after introduction of Ca\(^{2+}\), where “1” indicates no increase and “2” indicates a doubling of the current. Note that this analysis underestimates the full degree of potentiation, which is partly masked by the concomitant process of inactivation. In these experiments, addition of 2 mM Ca\(^{2+}\) potentiated the currents by 1.85 ± 0.06 fold at +80 mV and 3.98 ± 0.50 fold at -80 mV (n=10). The greater potentiation of the currents at negative voltages reflects a decrease in the outward rectification of the currents (from 6.56 ± 0.79 to 3.58 ± 0.76, measured as the current at +80mV divided by the current at -80mV) as is commonly observed during strong activation of many TRP channels (35). The currents subsequently decayed to 50% of their peak value (T50) in 4.8 ± 0.5 seconds (measured at +80 mV; n=10). Similar results were obtained with mustard oil as the agonist, indicating that as previously noted, Ca\(^{2+}\) potentiation and inactivation are independent of the specific agonist used to activate TRPA1 (21). Ca\(^{2+}\) potentiation and inactivation appeared to be unaffected by fusion of YFP to the N terminus of TRPA1, as there was no difference in the degree of potentiation or rate of inactivation when an unfused rTRPA1 cDNA was used (2.52 ± 0.45 fold and 4.85 ± 1.29 fold potentiation at +80 and -80 mV respectively; T50 = 4.7 ± 0.9 s, n=3; see supplemental Fig. 1)

To confirm that the decay of TRPA1 currents upon addition of external Ca\(^{2+}\) represents inactivation rather than a return to the resting state, we determined whether TRPA1 currents could be re-activated following exposure to external Ca\(^{2+}\). For these experiments we used a low concentration of menthol which does not bind covalently to TRPA1 and can be used to repeatedly activate TRPA1 currents in the absence of external Ca\(^{2+}\) (36). Following activation of TRPA1 by menthol, addition of external Ca\(^{2+}\) caused a rapid potentiation and subsequent decay.
of the current similar to that observed when the channels had been activated by Cin (Fig. 1B). Following decay of the current, no additional responses to menthol could be evoked for a period up to 20 minutes (Fig. 1B; n = 5). Thus external Ca\(^{2+}\) promotes the entry of TRPA1 channels into a long lasting inactivated state.

**Complete activation of TRPA1 by cinnamaldehyde occludes Ca\(^{2+}\) potentiation**

For both practical and theoretical reasons it was important to understand whether extracellular Ca\(^{2+}\) and Cin act independently or in concert to regulate gating of TRPA1. Towards this end, we determined whether TRPA1 currents could be potentiated by Ca\(^{2+}\) when the currents had been maximally activated by Cin. Because Cin binds covalently to TRPA1, the fraction of channels activated by a fixed concentration of the agonist is expected to be sensitive to the duration of the exposure. Consistent with this prediction, we found that a prolonged exposure (156 ± 17 s) to 100 μM Cin in the absence of external Ca\(^{2+}\) activated an average current of 7.7 ± 1.1 nA (at +80 mV, n=4), which was twice as large as the average current measured in response to an exposure of ~ 40 s (Fig 1C,D, see above). After exposure to Cin for ~ 120 s, the currents reached a plateau, presumably reflecting the full activation of TRPA1 channels (Fig. 1C). Interestingly, when fully activated the currents were significantly less rectifying than when they were partially activated (Fig. 1C; rectification ratio was 1.32 ± 0.20, n=4).

At this point, addition of extracellular Ca\(^{2+}\) only slightly potentiated the currents (Fig 1C,D; fold increase in current was 1.07 ± 0.04 and 1.12 ± 0.05 at +80 and -80 mV, respectively, n=4), while inactivation was slightly slowed (T50 = 10.5 ± 3.0 s; p<0.05 compared with brief exposure). Therefore, Ca\(^{2+}\) potentiation and Cin activation are not independent and may share a final common mechanism (23). Similar results were obtained for mustard oil activation of hTRPA1 (Supplemental Fig. 6). In the following experiments, we took care to only partially activate the channels with Cin or MO, so that Ca\(^{2+}\) potentiation would not be occluded.

The dual effects of Ca\(^{2+}\) on TRPA1 currents could be explained by several different mechanisms. First, inactivation might reflect an obligatory and stochastic conformational change that follows Ca\(^{2+}\)-dependent activation, like some forms of inactivation of voltage-dependent ion channels (32). This “coupled” model predicts that potentiation and inactivation will show a similar sensitivity and specificity for Ca\(^{2+}\) and that conditions which promote potentiation will similarly promote inactivation. Alternately, potentiation and inactivation may be mediated by “independent” Ca\(^{2+}\)-dependent processes. This model predicts that inactivation could be separable from activation. For each of these two mechanisms, external Ca\(^{2+}\) could act either by binding to a site on the outside of the channel or it could enter the cell, through TRPA1 channels, to act internally.

To determine if the processes of Ca\(^{2+}\)-dependent potentiation and Ca\(^{2+}\)-dependent inactivation are independent, we tested if the two could be separated based on their sensitivity to extracellular Ca\(^{2+}\) or other divalent cations. Following Cin activation of TRPA1, we varied the concentration of external Ca\(^{2+}\) that was introduced into the bath. Surprisingly, potentiation of TRPA1 currents could be induced by a Ca\(^{2+}\) concentration as low as 12 μM, and at 50 μM Ca\(^{2+}\) the magnitude of the response was saturated, although the response kinetics were slowed as compared to responses to 2 mM Ca\(^{2+}\) (Fig. 2A, C; 1.26 ± 0.05 fold increase at 12 μM Ca\(^{2+}\), n = 4; 2.22 ± 0.16 fold increase at 50 μM Ca\(^{2+}\), n = 4). In contrast, the rate of inactivation varied with Ca\(^{2+}\) concentration over the entire range that we tested (12 μM to 10 mM) (Fig. 2A, D). Importantly, at low Ca\(^{2+}\) concentrations that still induced maximal potentiation, (e.g. 50 μM), inactivation was substantially slower as compared to control conditions (2 mM Ca\(^{2+}\)), suggesting that inactivation does not derive its Ca\(^{2+}\) dependence from activation.

**Potentiation and inactivation of TRPA1 by other divalent cations**

In a similar series of experiments, we tested whether divalent cations other than Ca\(^{2+}\) could potentiate or inactivate TRPA1 currents. Introduction of Ba\(^{2+}\) (2 mM) produced both robust
potentiation and rapid inactivation of TRPA1 currents (fold increase of 1.83 ± 0.05; T50 of 12.0 ± 1.9 s; n = 4). In contrast, introduction of 2 mM Mg²⁺ caused little or no potentiation, but significant inactivation (fold increase of 1.13 ± 0.09; T50 of 47.5 ± 8.1 s; n = 6) (Fig. 2B,C,D). A higher concentration of Mg²⁺ (20 mM) promoted more rapid inactivation (T50 of 11.6 ± 2.2; fold increase of 1.03 ± 0.01; n=6; Fig 2C,D). Similar results were obtained using a clone of rTRPA1 that was not fused to YFP (see supplemental Fig. 1). We conclude that potentiation and inactivation are mediated by distinct processes that can be separated on the basis of their specificity for divalent cations.

Inactivation of TRPA1 by Mg²⁺ was unexpected given that under physiological conditions this ion is present at millimolar concentrations inside and outside cells. If inactivation is mediated by entry of divalent cations, then it might be predicted that physiologically relevant concentrations of intracellular Mg²⁺ would inactivate the channel. To determine whether this is the case, we measured TRPA1 currents in response to prolonged exposure to Cin in cells perfused with varying concentrations of free Mg²⁺. Inhibition by the TRPA1 specific blocker HC-030031 (15) confirmed that the currents were entirely mediated by TRPA1 channels. Perfusion with 8.8 mM free Mg²⁺ strongly reduced the magnitude of the currents, whereas perfusion with 1.8 mM free Mg²⁺, a physiologically relevant concentration, had no effect when compared with perfusion with a low concentration of free Mg²⁺ (0.35 mM; Fig 2E,F). Therefore, physiological relevant resting levels of free Mg²⁺ are not expected to inactivate TRPA1. Moreover, the steep dependence of the TRPA1 currents on the intracellular Mg²⁺ concentration suggests that the process by which divalent cations speed inactivation of TRPA1 is highly cooperative.

Conventional methods cannot be used to assign a location for Ca²⁺ regulation of TRPA1

Having established that potentiation and inactivation of TRPA1 are mediated by distinct processes, we next examined whether the Ca²⁺ regulation of each process was due to Ca²⁺ binding to the outside of the channel or to entry of Ca²⁺ through the channel and binding of Ca²⁺ to an intracellular target. If Ca²⁺ entry mediates either process, it might be possible to disrupt the effect of extracellular Ca²⁺ with an intracellular Ca²⁺ chelator. The preceding experiments were all performed with 5 mM EGTA, indicating that at this concentration EGTA does not block potentiation or inactivation. Moreover, reducing the buffer to 20 μM EGTA only slightly increased the potentiation of the currents, and had no effect on the rate of inactivation (fold increase of 2.50 ± 0.29, T50 of 5.0 ± 0.5 s; n = 5; Fig 2C, D).

Because Ca²⁺ might act close to the site of entry, where EGTA would be ineffective, we also tested whether potentiation or inactivation of TRPA1 was affected by including the fast Ca²⁺ chelator BAPTA in the pipette. For these experiments, TRPA1 currents were activated by mustard oil, which has a similar mechanism of action as Cin (37). The Ca²⁺-dependent potentiation and inactivation of TRPA1 currents were not different between cells that were dialyzed with 5 mM BAPTA and those dialyzed with 20 μM EGTA (supplemental Fig. 2). In similar experiments, other groups have reported only a partial block of TRPA1 activation by BAPTA (21,24). Therefore, either Ca²⁺ acts on the extracellular side of the channel or the Ca²⁺ buffers at the concentrations we used were not effective in interfering with the binding of Ca²⁺ to its intracellular target. This might be the case if Ca²⁺ acts at a site very close to the permeation pathway, or if the buffer is saturated by the large Ca²⁺ influx through the channels.

Mutation of a proposed EF hand does not affect potentiation or inactivation

It has recently been proposed that an EF-hand located within the N terminus of TRPA1 mediates Ca²⁺ activation (20,21). To confirm this result and determine if this site also mediates inactivation of TRPA1, we mutated each of 3 residues proposed to participate in activation of TRPA1 by Ca²⁺ (SD471RA, L475A, D480A; see supplemental Fig. 3). None of the mutations had any effect on Ca²⁺-dependent potentiation or Ca²⁺-dependent inactivation (supplemental Fig. 3). Similar results were obtained with a clone of rTRPA1 that was not fused to YFP (L475A and D480A were tested; supplemental Fig 4). To determine whether the
differences between our results and those of Doerner et al (21) were due to the use of different species variants of TRPA1 or to different activating chemicals, we introduced mutations into 2 sites on hTRPA1 (L474A, and D479A) and measured potentiation following both Cin and MO activation. Mutation of Leu474 in hTRPA1 had no effect on Ca\(^{2+}\)-potentiation following Cin or MO activation, while mutation of Asp479 abrogated channel function, as previously noted (21) (supplemental Figs 5 and 6). Therefore, as assessed under a variety of conditions, residues in a putative EF hand do not play a critical role in potentiation or inactivation of TRPA1, and EF hand mutant channels cannot be used to assign a location for Ca\(^{2+}\) regulation. We noted that mutation of Asp480 in rTRPA1 destabilized opening of the channel by Cin, and this region, which is in close proximity to N terminal cysteine residues that are the targets for pungent chemicals (17,18), may therefore be more generally involved in channel gating.

A pore region mutation disrupts Ca\(^{2+}\)-dependent potentiation and inactivation of TRPA1

A more direct way to assess the role of Ca\(^{2+}\) entry in potentiation and inactivation of TRPA1 is to reduce the Ca\(^{2+}\) permeability of the channel and determine whether it can still be potentiated or inactivated by extracellular Ca\(^{2+}\). In other structurally related ion channels this has been accomplished by mutation of acidic residues in the pore region (34,38). An alignment of TRPA1 with other cation channels places Asp918 in the selectivity filter at the same position as the tyrosine residue in the GYG motif of the potassium channel (39) (Fig. 3C). We therefore mutated Asp918 and characterized the ion selectivity of the Cin-activated currents by rapidly exchanging the external solution with those containing either Na\(^+\), Cs\(^+\), K\(^+\) or Ca\(^{2+}\) as the only permeable cation. The reversal potential of the current under these bionic conditions can be used to calculate permeability relative to Cs\(^+\), which was the only cation present in the pipette solution (Fig. 3A) (34). Consistent with previous reports (3), we found that wild-type TRPA1 channels are highly Ca\(^{2+}\) permeable (P\(_{\text{Ca}}\)/P\(_{\text{Cs}}\) = 3.28 ± 0.58, n=6). Mutation of Asp918 to alanine (D918A) led to a reduction in calcium permeability of nearly 2 orders of magnitude (P\(_{\text{Ca}}\)/P\(_{\text{Cs}}\) = 0.08 ± 0.01, p<0.05; n = 3; Fig. 3A, B). A charge neutralizing substitution that did not change the size of the amino acid side chain, D918N, produced channels with an intermediate Ca\(^{2+}\) permeability (P\(_{\text{Ca}}\)/P\(_{\text{Cs}}\) = 0.26 ± 0.01, p<0.01; n = 6; Fig. 3A,B) and the charge conserving substitution D918E, increased Ca\(^{2+}\) permeability (P\(_{\text{Ca}}\)/P\(_{\text{Cs}}\) = 13.56 ± 2.63, p<0.05; n = 8; Fig. 3A,B). Note that the D to E substitution is found in TRPA1 orthologs from several species, including zebrafish and drosophila (Fig. 3D).

If Ca\(^{2+}\) entry mediates either potentiation or inactivation, we expected that one or both of these processes would be impaired in the D918A mutant, which is poorly permeable to Ca\(^{2+}\). Indeed the D918A mutant showed virtually no potentiation or inactivation in response to addition of 2 mM Ca\(^{2+}\) following activation with Cin (fold increase of 1.06 ± 0.02, p<0.001; T50 of 105.8 ± 19.3 s, p<0.001; n = 5; Fig. 4B, E,F). According to this logic, the D918N mutant, which has an intermediate permeability to Ca\(^{2+}\), should show a milder deficit in potentiation and inactivation. As seen in Fig. 4, D918N currents showed robust potentiation in response to extracellular Ca\(^{2+}\) (fold increase of 1.52 ± 0.15, n = 5), but potentiation was slowed and resembled the response of wild-type channels to a much lower external Ca\(^{2+}\) concentration (Fig. 4C, compare with Fig. 2A). Inactivation of D918N channels was also significantly slowed as compared with wild-type (T50 of 81.4 ± 9.2 s, p<0.001; n = 5; Fig. 4F). In contrast, the response to Ca\(^{2+}\) of the highly Ca\(^{2+}\) permeable D918E mutant was indistinguishable from that of wild-type currents (fold increase of 1.57 ± 0.19, p>0.05; T50 of 4.5 ± 0.3 s, p>0.05; n = 4; Fig. 4D,E,F).

To determine if the decrease in potentiation of D918A currents by Ca\(^{2+}\) could be attributed to saturation of the activation of the channels by Cin, we measured the magnitude of D918A currents following prolonged Cin exposure, which is expected to activate all available channels. Prolonged Cin exposure elicited currents of 4.1 ± 0.9 nA and -1.9 ± 0.7 nA at +80 mV and -80 mV, respectively, (n=6) which was ~ 2 times the size of the currents used for assessing Ca\(^{2+}\)-potentiation (2.1 ± 0.1 nA and -0.54 ± 0.07 nA at +80 mV and -80 mV, respectively, n=5). Note that current magnitudes of all pore mutants were somewhat reduced as compared with the wild-type current,
but compared with each other were not significantly different (2.3 ± 0.1 nA and -0.27 ± 0.04 nA at +80 mV and -80 mV, respectively, n=5 for D918N; 2.4 ± 0.1 nA and -1.4 ± 0.9 at +80 mV and -80 mV, respectively, n=4 for D918E).

Potentiation and inactivation of TRPA1 channels can be attributed to permeation of Ca^{2+} through the channel

It is well recognized that permeant ions can alter the gating of ion channels, presumably because their occupancy in the permeation pathway impedes channel closure (40). It has also been shown that mutations of the pore of ion channels can affect gating (e.g. (41,42)). Indeed, the activation of the TRPA1 pore mutants was somewhat more sluggish than that of wild-type currents (Fig 4A-D); this was confirmed by measurement of the 50% activation time, which showed a 2-fold slowing for D918A currents as compared to wild-type currents (79.3 ± 6.9 s for D918A and 48.9 ± 5.4 s for wild-type, p<0.01 measured from experiments where Cin was applied until the currents reached saturation, as in Fig 1C). It was therefore important to determine whether mutation of the TRPA1 pore disrupted potentiation and inactivation through reduced Ca^{2+} influx, as we hypothesized, or via a direct effect on the gating of the channel. If the mutation principally affected Ca^{2+} influx, we reasoned that potentiation and inactivation might be rescued by reducing the intracellular Ca^{2+} buffering so as to allow accumulation of intracellular Ca^{2+}. As shown in Fig. 5A, Cin-activated D918A currents were not potentiated or inactivated by 2 or 10 mM extracellular Ca^{2+} when intracellular Ca^{2+} was buffered with 5 mM EGTA (Fig. 5A, B) and lowering the concentration of EGTA to 20 μM partially rescued both potentiation and inactivation (Fig. 5A, B).

We confirmed that the small Ca^{2+} flux through D918A channels produced a significant elevation of intracellular Ca^{2+} under conditions of low Ca^{2+} buffering, by repeating the experiment with the ratiometric Ca^{2+} indicator fura-4F in the pipette. Addition of 2 or 10 mM Ca^{2+} to the bath produced a Ca^{2+} elevation of 1-3 μM in D918A expressing cells activated with Cin (Fig. 5 C, D). Moreover, this elevation of intracellular Ca^{2+} was blocked by including 5 mM EGTA in the pipette (Fig. 5 C, D). Interestingly, in the cells expressing wild-type channels, EGTA did not block potentiation or inactivation, and Ca^{2+} imaging revealed a substantial elevation of intracellular Ca^{2+}, despite the presence of the buffer (Fig 5C, D). These data confirm that potentiation and inactivation are mediated by elevation of intracellular Ca^{2+} and that mutation of Asp^{918} disrupts inactivation by reducing Ca^{2+} permeability.

TRPA1 is potentiated but is not inactivated by uncaging Ca^{2+}

The previous experiments indicate that the ability of extracellular Ca^{2+} to potentiate and inactivate TRPA1 requires Ca^{2+} entry. To determine if elevation of intracellular Ca^{2+} is sufficient to potentiate and inactivate TRPA1 channels, we loaded cells with Ca^{2+} bound to the chelator DMNP-EDTA, which changes affinity for Ca^{2+} from 5 nM to 3 mM upon exposure to UV light (43). Fig. 6A shows that UV uncaging of DMNP-EDTA in the absence of extracellular Ca^{2+} caused a sustained potentiation of the Cin-activated TRPA1 current (2.70 ± 0.20 fold increase at +80 mV, n = 5) (Fig. 6C). The effect of elevating intracellular Ca^{2+} partially occluded further potentiation by extracellular Ca^{2+}, consistent with our previous observations. To confirm that the observed responses were due to elevation of intracellular Ca^{2+}, and not to direct effects of the two photolytic products (44), we loaded cells with DMNP-EDTA without Ca^{2+}. Under these conditions, UV exposure caused only a slow, small increase in TRPA1 currents (Fig. 6B, C; 1.14 ± 0.04 fold increase at +80 mV, n = 5), and this increase was significantly less than what we observed when DMNP-EDTA was loaded with Ca^{2+} (p<0.001).

A surprising result of these experiments was that uncaging Ca^{2+} did not inactivate TRPA1 currents. This could be explained if the Ca^{2+} elevation produced by the uncaging protocol was insufficient to promote inactivation. To test this hypothesis, we compared the Ca^{2+} elevation produced by UV uncaging of DMNP-EDTA with that observed upon subsequent entry of Ca^{2+} through TRPA1 channels. Ca^{2+} levels were measured with the indicator Fluo-5F, which can be visualized with wavelengths of light that do not uncase DMNP-EDTA. Under these conditions, we
found that Ca\(^{2+}\) entry produced a significantly
greater global elevation of intracellular Ca\(^{2+}\) than
did Ca\(^{2+}\) uncaging (p<0.001; Fig. 6D,E). It should
be noted that Ca\(^{2+}\) levels at the plasma membrane
upon entry through TRPA1 channels could be
considerably higher than what we have measured.
Together these data indicate that elevation of
intracellular Ca\(^{2+}\) by uncaging DMNP-EDTA can
potentiate TRPA1 currents, but not inactivate
them. Inactivation may require a greater elevation
of intracellular Ca\(^{2+}\) than can be achieved by Ca\(^{2+}\)
uncaging.

**Effect of intracellular Ca\(^{2+}\) on TRPA1 single
channels.**

To study the effects of Ca\(^{2+}\) on TRPA1
channels in more detail, we recorded the gating of
single TRPA1 channels in cell-attached patches. If
intracellular Ca\(^{2+}\) is sufficient to both potentiate
and inactivate TRPA1 channels, addition of
extracellular Ca\(^{2+}\) to a region outside the patch
pipette should potentiate and inactivate TRPA1
channels under the patch pipette. Fig. 7A shows a
cell-attached patch clamp recording with 100 nM
Ca\(^{2+}\) in the patch pipette from a TRPA1 expressing
HEK 293 cell. Constitutive channel activity was
observed, and this activity was increased by
addition of Cin to the bath, indicating that it
represented opening of TRPA1 channels.
Moreover, this activity was never observed in cells
not transfected with TRPA1 (not shown).
Measurement of single channel amplitudes in
recordings of voltage families gave a slope
conductance of 139 ± 2 pS (n = 3; Fig. 7B).
Following Cin activation, addition of Ca\(^{2+}\) to the
intact cell produced dramatic potentiation
followed by a rapid decay in channel activity.
Measurement of single channel currents and fitting
of all points histograms indicated that the single
channel conductance was unchanged in response
to Cin activation or Ca\(^{2+}\) potentiation (141 ± 12
pS, n = 3, before Cin, 148 ± 3 pS, n = 5, after Cin
and 143 ± 4 pS, n = 5, after Ca\(^{2+}\); Fig. 7 C,D,E).
Following inactivation, sparse channel openings
were observed, and these openings were of
variable amplitude, possibly reflecting the sparse
opening of TRPA1 channels as well as opening of
smaller endogenous Ca\(^{2+}\)-activated channels (our
unpublished observations). We conclude that
potentiation and inactivation of TRPA1 can be
induced by intracellular Ca\(^{2+}\) in a manner that is
not membrane delimited and that potentiation
involves a change in channel gating and not in
conductance.

Finally, we were interested in knowing
whether Ca\(^{2+}\)-potentiation and Ca\(^{2+}\)-inactivation of
TRPA1 was retained in excised patches. A
technical difficulty with these experiments is that
TRPA1 activity induced by mustard oil or
cinnamaldehyde is not preserved upon patch
excision (45). Recently it was reported that
polyphosphates can restore TRPA1 activity after
patch excision (45), making it now possible to
study TRPA1 channels in excised patches. As
shown in Fig. 8A, following activation by Cin, and
in the presence of polyP3, TRPA1 channel activity
is a rapidly and reversibly potentiated by
intracellular Ca\(^{2+}\). The dose dependence of
activation can be fit with the Hill equation with
K\(_{1/2}\) of 225 nM and slope of 1.8 (n = 6; Fig. 8B).
We also tested whether the channels undergo
inactivation under these conditions. TRPA1
currents showed only modest decay in response to
12 μM intracellular Ca\(^{2+}\) (Fig. 8C). At higher Ca\(^{2+}\)
concentrations, more substantial decay could be
observed (Fig. 8D,E). Thus in cell-free excised
patches pre-activated with Cin, TRPA1 current
can be potentiated and inactivated by cytoplasmic
Ca\(^{2+}\), and potentiation occurs at lower Ca\(^{2+}\)
concentrations.

**DISCUSSION**

Inactivation of TRPA1 can rapidly terminate
signaling and therefore may be one of the key
mechanisms that limit sensory activation by
painful or irritating substances. Previous work has
demonstrated that extracellular Ca\(^{2+}\) both
potentiates and inactivates TRPA1 (4,24). In this
study we show that inactivation of TRPA1 is not
coupled to potentiation and that both processes are
mediated by elevation of intracellular Ca\(^{2+}\). In
addition to these findings, we have discovered a
key residue in TRPA1 that controls Ca\(^{2+}\)
permeability of the channel, providing
experimental evidence that the S5-S6 linker of this
channel forms part of the pore region. Together
these results give insights into the mechanisms of
regulation of TRPA1 and provide tools for future
research.

**Identification of the ion selectivity filter in TRPA1.**
An important outcome of our work is the identification of an amino acid residue that determines Ca\(^{2+}\) selectivity of TRPA1. Ca\(^{2+}\) influx through TRPA1 undoubtedly plays an important role in the physiology and pathophysiology of sensory neurons, possibly mediating local release of inflammatory mediators, as well as generating cross-desensitization of co-expressed TRPV1 channels (46).

The architectural similarity between TRP channels and voltage-gated ion channels suggests that the pore (P) region in TRP channels will be formed from the S5-S6 linker as it is in K\(^+\) channels (39,47). Within the P region, a “selectivity filter” is formed in the K\(^+\) channel by a short sequence of highly conserved amino acids, with the motif TVGYG, the backbone carbonyls of which form a series of binding sites for ions within the permeation pathway (47). Recent studies suggest that the pore of Ca\(^{2+}\) and Na\(^+\) selective ion channels is structurally similar to that of K\(^+\) channels (48,49). The reported crystal structure of NaKbc, a nonselective cation channel from bacteria that shares homology with cyclic nucleotide-gated (CNG) ion channels, shows that the carboxyl groups of conserved acidic residues do not line the permeation pathway, as previously assumed, and are instead tangential to it, acting through electrostatic interactions to stabilize permeating divalent cations (48,50).

An alignment of TRPA1 with NaKbc and KcsA shows that Asp\(^{918}\), which we have identified as a key determinant of the Ca\(^{2+}\) permeability of TRPA1, aligns with the acidic residue in NaKbc that stabilizes permeating Ca\(^{2+}\) and with the tyrosine (Y) in the K\(^+\) channel that is essential for K\(^+\) selectivity (Fig. 2C). It also aligns with a methionine in TRPV4 and an aspartate in TRPV5 which are key determinants of ion selectivity in these channels (51,52), and it is near an aspartate in drosophila TRP that has been shown to determine ion selectivity in vivo (38). Mutation of Asp\(^{918}\) to Ala nearly eliminated Ca\(^{2+}\) permeability of the channel while substitution with the size conserving uncharged Asn residue caused a more moderate decrease in Ca\(^{2+}\) permeability, and substitution with the charge conserving larger Glu residue, led to an increase in Ca\(^{2+}\) permeability. These results are inconsistent with a direct coordination of Ca\(^{2+}\) by Asp\(^{918}\) within the constrained environment of the ion permeation pathway, and instead indicate that Asp\(^{918}\) stabilizes the permeating Ca\(^{2+}\) ions at a distance by electrostatics, like the stabilization of Ca\(^{2+}\) by acidic residues in the pore of the NaKbc channel (48,50).

**Potentiation of TRPA1 by intracellular Ca\(^{2+}\)**

Our data support and extend previous observations that TRPA1 channels can be activated by elevation of intracellular Ca\(^{2+}\) (20,21,23). Gating of TRPA1 by intracellular Ca\(^{2+}\) may be the mechanism by which TRPA1 is activated downstream of bradykinin and other inflammatory mediators that bind to G protein-coupled receptors, although a role for other signaling molecules has also been proposed (8,9,53). Activation of TRPA1 has been observed in response to intracellular dialysis of Ca\(^{2+}\), with half activation concentrations of 900 nM and 6 \(\mu\)M measured by two different groups (20,21). Activation of TRPA1 by intracellular Ca\(^{2+}\) has also been observed in excised patches (20,21), but recent data indicate that a co-factor lost upon patch excision is required to maintain these responses (23,45). In the presence of polyP3, which restores TRPA1 activity (45), we found that TRPA1 channels could be activated at concentrations of Ca\(^{2+}\) just slightly higher than the resting level  (K\(_{1/2}\) ~225 nM); this is in contrast to two other Ca\(^{2+}\)-activated TRP channels, TRPM4 and TRPM5, which in excised patches require micromolar concentrations of Ca\(^{2+}\) to be activated (27,30,54-57).

By studying the D918A TRPA1 channel, in which Ca\(^{2+}\) permeability is reduced, we provide evidence that potentiation of TRPA1 by extracellular Ca\(^{2+}\) is mediated by Ca\(^{2+}\) entry. Importantly, Ca\(^{2+}\)-potentiation of D918A currents was blocked by including 5 mM EGTA in the pipette, and could be restored by lowering the concentration of EGTA. In addition to demonstrating that intracellular Ca\(^{2+}\) potentiates TRPA1, these data suggest that, under some conditions, TRPA1 channels can respond to a global change in the intracellular Ca\(^{2+}\) concentration (58). This was further confirmed by our observation that TRPA1 channels in cell-attached patches could be potentiated by addition of Ca\(^{2+}\) to a region outside of the patch. Similar results were recently reported for activation of TRPA1 in the absence of a pungent chemical (23).
We noted that potentiation was accompanied by an increase in channel activity, without a change in channel conductance. A previous report showing a decrease in channel conductance during potentiation may be attributed to a concomitant block of the channel by extracellular Ca\(^{2+}\) (24).

Our report is also the first to show that TRPA1 can be potentiated by elevation of intracellular Ca\(^{2+}\) through UV uncaging of the Ca\(^{2+}\)-cage DMNP-EDTA. This method has been extensively used in assessing the role of Ca\(^{2+}\) signaling in synaptic transmission and vesicle release, as well as for studying Ca\(^{2+}\) regulation of ion channels, including TRP channels (33,43-45,57,59). In studying TRPA1, which can be modified by a number of reactive compounds, it was important to establish that the effects of uncaging could not be attributed to effects of the two iminodiacetic acids generated by the photolysis of DMNP-EDTA (43,44). Our observation that UV uncaging of DMNP-EDTA in the absence of intracellular Ca\(^{2+}\) caused only a small, slow increase of the TRPA1 currents, indicates that the potentiation of TRPA1 currents upon UV uncaging of DMNP-EDTA could be largely attributed to Ca\(^{2+}\) elevation. Therefore, with the proper controls, flash photolysis of DMNP-EDTA is a useful method for studying Ca\(^{2+}\)-activation of TRPA1. Interestingly, with this method we observed a long lasting potentiation of TRPA1, which was not accompanied by inactivation. In similar experiments with other Ca\(^{2+}\)-activated TRP channels, a transient activation of the currents was observed, consistent with the observed transient elevation of Ca\(^{2+}\) (33,57). The absence of inactivation could be explained if UV uncaging of DMNP-EDTA did not produce sufficiently high intracellular Ca\(^{2+}\) levels. This interpretation is supported by our observation that the Ca\(^{2+}\) levels attained by UV uncaging of DMNP-EDTA were significantly lower than those attained through entry of Ca\(^{2+}\) through TRPA1 channels.

**Intracellular Ca\(^{2+}\) elevation mediates inactivation of TRPA1**

Inactivation of heterologously expressed TRPA1 by extracellular Ca\(^{2+}\) has been well documented (3,4,8,19,20,24) and is also observed in neurons (e.g. (12)). Our results show that like potentiation, inactivation by extracellular Ca\(^{2+}\) requires Ca\(^{2+}\) entry. Thus D918A channels, which are poorly permeable to Ca\(^{2+}\), inactivate only slowly in response to extracellular Ca\(^{2+}\), and inactivation can be largely restored by lowering intracellular Ca\(^{2+}\) buffering. Moreover, TRPA1 channels in excised and cell-attached patches could be inactivated through elevation of intracellular Ca\(^{2+}\), in the absence of extracellular Ca\(^{2+}\). It is perhaps not surprising that elevation of intracellular Ca\(^{2+}\) mediates inactivation of TRPA1, as it induces inactivation/desensitization of many ion channels and receptors, including both Ca\(^{2+}\)-permeable and Ca\(^{2+}\)-impermeable TRP channels (25-31,60).

Given that intracellular Ca\(^{2+}\) potentiates and inactivates TRPA1, is there a mechanism that insures that potentiation precedes inactivation? In voltage-gated Na\(^{+}\) and K\(^{+}\) channels, inactivation is coupled to voltage-dependent activation, such that the channels first open and then inactivate (61). A similar coupling between Ca\(^{2+}\)-dependent activation/potentiation and inactivation of TRPA1 appears unlikely for several reasons. First, in response to moderate elevation of intracellular Ca\(^{2+}\) by UV uncaging of DMNP-EDTA, potentiation can be observed in the absence of inactivation, indicating the two processes are not strictly coupled. Moreover, inactivation can be induced by extracellular Mg\(^{2+}\), in the absence of potentiation, suggesting that the two processes are mediated by intracellular signaling events with different ionic specificities. Excised patch recording suggests that inactivation follows potentiation because inactivation is relatively slow at the low Ca\(^{2+}\) concentrations that maximally activate the channel. Moreover, these data predict that a modest Ca\(^{2+}\) elevation in response to signaling by G protein-coupled receptors or receptor tyrosine kinases might activate, without inactivating TRPA1. The subsequent entry of Ca\(^{2+}\) through TRPA1 channels may then terminate the signal.

**Where is the site for Ca\(^{2+}\) potentiation and inactivation of TRPA1?**

An attractive hypothesis is that Ca\(^{2+}\) activates TRPA1 by binding directly to a putative EF hand on the N terminus of TRPA1, as proposed recently by two groups (20,21). Within the stretch of 12 amino acids that comprise the Ca\(^{2+}\) binding site on a canonical EF hand, 6 residues coordinate Ca\(^{2+}\)
(62). Doerner et al (21) found that of these sites, mutations of only one, (Leu 474), significantly reduced Ca\(^{2+}\) activation of the channels. This is surprising in that Leu\(^{474}\) occupies a position that contributes a backbone carbonyl to the Ca\(^{2+}\) binding site of a canonical EF hand and Leu is not commonly observed at this position (62). Zurborg et al. (20) found, in contrast, that mutant channels with charge neutralizing substitutions of the acidic residues that flank the EF hand were no longer sensitive to Ca\(^{2+}\), as assayed by intracellular Ca\(^{2+}\) dialysis and other methods. Our experiments show that neither Leu\(^{474}\) nor Asp\(^{479}\) is necessary for Ca\(^{2+}\)-potentiation of TRPA1. We observed robust potentiation of hTRPA1 L474A and of rTRPA1 L475A (analogous to L474A in hTRPA1), using both a fusion protein with YFP and the unfused channel. The absence of potentiation of hTRPA1 L474A currents in the experiments by Doerner et al (21) could be due to a saturation of the response to the agonist (MO), which can occlude activation by Ca\(^{2+}\) (supplemental Fig. 6). In agreement with Doerner et al (21), and contrary to the report by Zurborg et al (20), we found that the D479A mutation of hTRPA1 abrogated channel activity. The equivalent mutation in rTRPA1, D480A did not interfere with Ca\(^{2+}\) potentiati, but appeared to destabilize channel activation. Together these results make it unlikely that an N-terminal region proposed to form an EF hand contributes to Ca\(^{2+}\) potentiation of TRPA1, although it may otherwise regulate gating of TRPA1.

Other mechanisms by which Ca\(^{2+}\) could potentiate, or inactivate TRPA1, include direct binding to other acidic residues on the intracellular side of TRPA1, of which there are more than one hundred, or by binding to other proteins that regulate TRPA1 either directly or indirectly. A prime suspect for the calcium sensor is calmodulin (CaM), which mediates the Ca\(^{2+}\)-dependent facilitation and inactivation of voltage-gated Ca\(^{2+}\) channels (63-68), desensitization of Ca\(^{2+}\)-permeable cyclic-nucleotide gated ion channels (69,70), and which binds to several TRP channels (71-76). However, expression of TRPA1 with a dominant negative form of CaM did not abrogate Ca\(^{2+}\) potentiation or inactivation (20) (Y.Y. Wang and E.R. Liman, unpublished observations), suggesting that CaM is unlikely to be the sensor for Ca\(^{2+}\) regulation of TRPA1. Alternatively, elevation of intracellular Ca\(^{2+}\) could activate enzymatic processes that modulate channel activity. Other TRP channels are regulated by PI(4,5)P\(_2\), which can be depleted by elevation of intracellular Ca\(^{2+}\) (77). Depletion of PI(4,5)P\(_2\) underlies desensitization of TRPV1, TRPM4, TRPM5, TRPM7 and TRPM8 (27-31,78-80). PI(4,5)P\(_2\) depletion has also been proposed to underlie sensitization of TRPV1 in response to the pro-algesic agents bradykinin and NGF that activate receptors coupled to PLC (81,82). A role for PI(4,5)P\(_2\) depletion in the desensitization of TRPA1 is supported by recent experiments showing that cross desensitization of mustard oil evoked currents in sensory neurons by capsaicin, acting on TRPV1, can be blocked by infusion of PI(4,5)P\(_2\) through the pipette (19) and by evidence that PI(4,5)P\(_2\) can restore channel activation following partial, but not complete inactivation in excised patches (83). But other data suggests that PI(4,5)P\(_2\) depletion contributes to activation or sensitization of TRPA1 (9,53). Using newly developed methods for manipulating PI(4,5)P\(_2\) levels (79,84), we found that PI(4,5)P\(_2\) depletion neither activates nor inactivates TRPA1 (see supplemental Fig. 7), although we cannot rule out the possibility that changes in PI(4,5)P\(_2\) levels might have more subtle effects on channel function. Yet another mechanism by which Ca\(^{2+}\) regulates TRP channels is through Ca\(^{2+}\)-activated phosphatase calcineurin (85), although a recent report does not support a role for calcineurin in desensitization of TRPA1 (19).

**Ca\(^{2+}\) regulation of TRPA1 in sensory neurons**

In sensory neurons, intracellular Ca\(^{2+}\) can be elevated through several distinct mechanisms, each of which could affect the gating of TRPA1. For example inflammatory mediators, such as bradykinin can act on cognate GPCRs to release intracellular Ca\(^{2+}\), thermal and chemical stimuli can cause entry of Ca\(^{2+}\) through TRPV1 or TRPA1 channels, and depolarization can activate voltage-gated Ca\(^{2+}\) channels (86,87). How elevation of Ca\(^{2+}\) contributes to activation and inactivation of TRPA1 in sensory neurons is not well understood, and may require a better understanding of the complex spatial and temporal dynamics of Ca\(^{2+}\) signaling in these cells. An example of this complexity is the surprising observation that targeted deletion of either TRPA1 or TRPV1 eliminates bradykinin evoked Ca\(^{2+}\) entry in...
This suggests that the Ca\(^{2+}\) release that is evoked by bradykinin is insufficient to activate TRPA1 and that concurrent entry of Ca\(^{2+}\) through TRPV1 may be necessary. Adding to this complexity is the possibility that changes in other signaling molecules, such as depletion of PI(4,5)P\(_2\), also contributes to activation of TRPA1 in sensory neurons (9, 22). A similar complexity applies to inactivation (or desensitization) of TRPA1 in sensory neurons. In behavioral studies, responses to mustard oil show both homologous desensitization as well as cross-desensitization by TRPV1 activation (88, 89). Recent data indicates that the cross-desensitization of MO responses is Ca\(^{2+}\) dependent (46), and therefore may be mediated by the mechanism we and others observe in heterologous cells. However, homologous desensitization of MO responses in sensory neurons is reported to be Ca\(^{2+}\) independent (46), and may be mediated instead by TRPV1-dependent internalization (19). The absence of Ca\(^{2+}\)-sensitive homologous desensitization may reflect the lower current density of TRPA1 in sensory neurons (e.g. (4)), or differences between native and expressed channels in subunit composition or post translational modification. Clearly additional studies are needed to understand the complex regulation of TRPA1 in native cells.

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The abbreviations used are: TRPA1, transient receptor potential A1; Cin, cinnamaldehyde; MO, mustard oil; PLC, phospholipase C; PI(4,5)P2, phosphatidyl inositol (4,5) bisphosphate.

FIGURE LEGENDS

FIGURE 1. TRPA1 channels are potentiated and inactivated by extracellular Ca2+. Currents recorded from HEK-293 cells heterologously expressing an N-terminal YFP fusion of rat TRPA1. A, B, In the absence of extracellular Ca2+, brief exposure to TRPA1 agonists, as indicated, elicited a current, which was first potentiated and then decayed to baseline in response to addition of extracellular Ca2+ (2 mM). Inset shows the I-V relationship in response to a ramp depolarization (1 V/s) at the times indicated. Agonists were menthol (30 μM) or Cin (100 μM). Note that following introduction of Ca2+ no additional responses to menthol could be measured, indicating that the channels had entered a long-lasting inactivated state. C, TRPA1 currents in response to prolonged exposure to Cin (100 μM) in the absence of extracellular Ca2+ reached a plateau after ~100s. Addition of 2 mM Ca2+ did not cause further activation, but still promoted rapid inactivation of the currents. Inset shows the I-V relationship in response to a ramp depolarization (1 V/s) at the times indicated. Note the linearization of the I-V curve with strong activation. D, Magnitude of the TRPA1 currents at +80 mV before and after addition of 2 mM Ca2+ from experiments as in (A) and (C). The Cin-evoked currents measured with the brief exposure protocol were ~50% of the maximal Cin-evoked currents, and only these subsaturating responses could be potentiated by Ca2+.

FIGURE 2. Sensitivity and specificity of potentiation and inactivation of TRPA1. A, TRPA1 currents evoked in response to brief exposure to Cin (100 μM) and subsequent addition of varying concentrations of Ca2+ (as indicated). Note that each trace represents a recording from a separate cell and the traces have been aligned by the time at which Ca2+ was introduced. The currents were scaled by the magnitude of the response to Cin, and therefore the Y-axis is represented by an arbitrary unit (a.u.). B, Currents evoked in response to brief exposure to Cin (100 μM) and subsequent addition of Ba2+ or Mg2+ (2 mM). C, Average data from experiments as in (A,B). Potentiation was measured as the fold increase in the current (at +80 mV) following addition of the divalent cation, where “1” represents no change in the current. Note the “threshold” for potentiation of the current by extracellular Ca2+ was ~12 μM. The bar corresponding to the response to “0 Ca2+” was duplicated to allow comparison with responses to Ba2+ and Mg2+. D, Inactivation was measured by the T50 or time, relative to the peak, at which the currents had decayed to 50% of their maximum value (at +80mV). There was a continuous slowing of inactivation as the Ca2+ concentration was lowered. Data represent the mean ± SEM, n = 4-10. Intracellular solution was HB-Cs+ (which contained 5 mM EGTA) for (A, B). E, TRPA1 currents elicited in response to a saturating exposure to Cin (100 μM) with varying concentrations of free Mg2+ in the pipette (as indicated). HC-030031 (10 μM) was introduced at the end of each experiment to confirm that the current was entirely due to the gating of TRPA1 channels. Intracellular solution was HB-Cs+ with 0, 2 or 10 mM MgCl2 added. F, Magnitude of the current (+80 mV) from experiments as in E. Data represent the mean ± SEM.

FIGURE 3. Pore region mutations reduce Ca2+ permeability of TRPA1. A, Whole cell patch clamp recording of wild-type or mutant TRPA1 currents activated by Cin (100 μM). Plots show the current in response to a ramp depolarization (1 V/s) in the presence of the indicated extracellular cation (Na+, NMDG+ or Ca2+). The extracellular solution was rapidly exchanged to minimize changes in the
magnitude of the currents during the recording. Arrow indicates the reversal of the current with 128 mM Ca\(^{2+}\) in the bath. Intracellular solution was HB-Cs\(^{+}\). B, Ion selectivity was calculated based on the reversal potential of the current as measured from experiments as in (A). D918A (n = 3) and D918N (n = 6) showed dramatically reduced Ca\(^{2+}\) permeability, whereas D918E (n = 8) had increased Ca\(^{2+}\) permeability as compared to wild-type (n = 6). C, The S5-S6 region of TRPA1 aligned with that of related cation permeable channels. Conserved acidic residues important for ion permeability are shown in red. Gray shading highlights the conserved GxG sequence in the selectivity filter. Note that Asp\(^{918}\) is at the same position as the Tyr in the GYG motif of the K\(^{+}\) channel. Pore region alignment from multiple channels was based partly on Zagotta (2006). D, Alignment of the putative selectivity filters of TRPA1 from multiple species. Data represent the mean ± SEM. Significance was determined by 2-tailed Student’s t-test and was bonferroni corrected. * p< 0.05, ** p<0.01, *** p<0.001. Ca\(^{2+}\)I and Na\(^{+}\)I refer to the first transmembrane domains of the Ca\(^{2+}\) and Na\(^{+}\) channel respectively. Accession numbers are: POA334 (KcsA), ZP_02523257 (NaKbc), ZP_02523257 (CNGA1), NP_997491 (TRPA1), NP_076460 (TRPV4), NP_446239 (TRPV5), Q13936 (Ca\(^{2+}\)I), NP_062139 (Na\(^{+}\)I), NP_015628 (Human TRPA1), NP_997491 (Rat TRPA1), NP_808449 (Mouse TRPA1), XP_544123 (Dog TRPA1), XP_581588 (Cow TRPA1), NP_001007066 (Fish TRPA1), NP_001097554 (Fly TRPA1).

FIGURE 4. Pore region mutations that reduce Ca\(^{2+}\) permeability disrupt Ca\(^{2+}\) potentiation and inactivation of TRPA1. A-D, Effect of extracellular Ca\(^{2+}\) (2 mM) on wild-type and mutant TRPA1 currents activated by brief exposure to Cin (100 μM). B, Extracellular Ca\(^{2+}\) caused no significant potentiation and only slow inactivation of the mutant D918A, which is poorly permeable to Ca\(^{2+}\). C, Currents carried by the D918N mutant, which has an intermediate Ca\(^{2+}\) permeability, were strongly potentiated by Ca\(^{2+}\), but potentiation was slowed as compared to wild-type. D, Currents carried by the D918E mutant, which has an enhanced Ca\(^{2+}\) permeability, were strongly activated and rapidly inactivated by extracellular Ca\(^{2+}\). E, F, Average data from experiments as in (A-D), quantified as in Fig. 1. Wild-type data were determined by 2-tailed Student’s t-test with bonferroni correction for multiple samples. *** p<0.001. Internal solution was HB-Cs\(^{+}\), which contained 5 mM EGTA.

FIGURE 5. Lowering Ca\(^{2+}\) buffering rescues potentiation and inactivation of D918A currents. A, D918A currents evoked by Cin (100 μM), with 5 mM EGTA in the pipette are not potentiated or inactivated by either 2 mM (left panel) or 10 mM (right panel) extracellular Ca\(^{2+}\) (gray lines). Lowering the Ca\(^{2+}\) buffer in the pipette to 20 μM EGTA, rescued potentiation and inactivation (black lines). Traces are from separate cells. To allow direct comparison of the currents, the responses were normalized to the amplitude of the Cin-evoked current and aligned by the time at which Ca\(^{2+}\) was added to the bath. B, Average data from experiments as in (A). C, Simultaneous recordings of the current magnitude (top) and intracellular Ca\(^{2+}\) level (bottom) in D918A and wild-type (WT) expressing cells loaded through the pipette with the Ca\(^{2+}\) indicator Fura-4F (20 μM) with or without 5 mM EGTA. In the absence of intracellular Ca\(^{2+}\) buffering (no EGTA; left trace), D918A currents were potentiated and inactivated by bath application of 2 mM Ca\(^{2+}\), which produced a large rise in intracellular Ca\(^{2+}\). Including EGTA in the pipette (middle trace) under identical conditions eliminated the rise in intracellular Ca\(^{2+}\), and the currents showed no potentiation or inactivation. Wild-type currents (right trace) supported a large Ca\(^{2+}\) influx that produced a significant elevation of intracellular Ca\(^{2+}\), despite the presence of 5 mM EGTA in the pipette. D, Peak intracellular Ca\(^{2+}\) concentration after addition of extracellular Ca\(^{2+}\) from experiments as shown in (C). In D918A expressing cells loaded with no Ca\(^{2+}\) buffer, Ca\(^{2+}\) levels rose to the micromolar range. This elevation was completely blocked by 5 mM EGTA in the pipette. Data represent the mean ± SEM.

FIGURE 6. TRPA1 currents can be potentiated by elevation of intracellular Ca\(^{2+}\) through UV uncaging of DMNP-EDTA. A, Potentiation of heterologously expressed TRPA1 currents in response to
flash photolysis of caged Ca^{2+} (DMNP-EDTA loaded with Ca^{2+}). TRPA1 currents were activated by brief exposure to Cin (100 μM) and following stabilization of the current magnitude, UV uncaging (100 ms) potentiated the currents. Subsequent bath application of 2 mM Ca^{2+} produced near complete inactivation of the current. B, UV light had little effect on the TRPA1 currents when cells were loaded with DMNP-EDTA in the absence of intracellular Ca^{2+}. C, Average data from experiments as in (A, B). D, Simultaneous recording of current magnitude (top) and intracellular Ca^{2+} (bottom) in HEK-293 cells expressing wild-type TRPA1 channels. The Ca^{2+} indicator Fluo-5F (10 μM) was loaded into the cells through the patch pipette. Cin (100 μM) in the absence of extracellular Ca^{2+} activated TRPA1 currents, and UV uncaging of Ca^{2+} potentiated the currents. Addition of extracellular Ca^{2+} (2 mM) caused a significantly higher Ca^{2+} elevation compared to that achieved by Ca^{2+} uncaging. E, Mean data from experiments as in (D). In the same experiments, the change in fluorescent emission from the Ca^{2+} indicator fluo-5F (ΔF) was measured immediately after UV uncaging and following introduction of 2 mM Ca^{2+} to the bath. Data represent the mean ± SEM, ** p<0.01, *** P<0.001.

FIGURE 7. Potentiation and inactivation of TRPA1 channels by extracellular Ca^{2+} in cell-attached patches. A, TRPA1 channel activity in cell-attached patch clamp recording (V_m = -80 mV) in response to addition of agonists outside the patch. B, Conductance of spontaneously active channels measured from step depolarizations prior to exposure to Cin. The slope conductance was 139 ± 2 pS (n = 3). C, D, E, Single channel activity at an expanded time scale (top panel) before and after bath application of Cin (100 μM), and following subsequent addition of 2 mM Ca^{2+}. All points histograms (bottom panel) fitted with Gaussians (gray line) indicate that the single channel conductance was not altered upon activation by Cin or potentiation by Ca^{2+}. The pipette solution was HB-Cs\textsuperscript{+}, which contained 5 mM EGTA and 100 nM Ca^{2+}.

FIGURE 8. Ca^{2+} potentiates and inactivates TRPA1 channels in excised patches. A, Current activation (V_m = -80 mV) in response to increasing concentrations of Ca^{2+} in inside-out patches from TRPA1-expressing HEK-293 cells. Currents were activated by brief exposure to Cin (100 μM) in cell-attached recording mode and all measurements from excised patches were obtained in the presence of polyP3 (1 mM). B, Average Ca^{2+} dose response curve from experiments as shown in (A) (data were normalized to the peak current in each patch). Fit was with V_max = 1, K_{1/2} = 0.225 μM, n = 1.83. Data are represented by the mean ± SEM, n = 6. C, D, Currents activated by 12 μM Ca^{2+} (C) or 200 μM Ca^{2+} (D) applied to the cytoplasmic side of inside-out patches (V_m = -80 mV). Ca^{2+}. E, Average data for the rate of current decay from experiments as in (C-D). At lower cytoplasmic Ca^{2+} concentrations, the currents decayed more slowly. Data are represented by the mean ± SEM, n = 4–5.
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The nociceptor ion channel TRPA1 is potentiated and inactivated by permeating calcium ions
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