Transient Receptor Potential Channel A1 Is Directly Gated by Calcium Ions*

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Members of the superfamily of transient receptor potential (TRP) channels are proposed to play important roles in sensory physiology. As an excitatory ion channel TRPA1 is robustly activated by pungent irritants in mustard and garlic and is suggested to mediate the inflammatory actions of environmental irritants and proalgesic agents. Here, we demonstrate that, in addition to pungent natural compounds, Ca\(^{2+}\) and proalgesic agents. Here, we demonstrate that, in addition to

mediated by TRPA1-mediated activation of sensory neurons (3). The pungent ingredients in mustard (allyl isothiocyanate, AITC) and garlic (allicin) robustly activate TRPA1 currents (4–6). In addition, TRPA1 appears to be regulated by phospholipase C (PLC)-coupled receptors, suggesting that channel opening can be mediated by second messengers (4, 7, 8).

TRPA1 expression was first described in a subset of sensory neurons of dorsal root and trigeminal ganglia that contribute to nociception and co-express calcitonin gene related peptide, substance P and TRPV1 (2, 4). Recent studies on TRPA1 deficient mice support a role of the channel in inflammatory pain and sensation of noxious cold (8, 9). A model suggests TRPA1 activation by bradykinin, a potent allogeneic substance released due to tissue injury and inflammation, in two possible ways: through PLC-mediated increases in intracellular Ca\(^{2+}\) or other metabolites (e.g. diacylglycerol) and via Ca\(^{2+}\) influx through TRPV1 (9). Whether an increase in intracellular Ca\(^{2+}\) is sufficient to activate TRPA1 is still debated (4, 7), but several findigs indicate a role of Ca\(^{2+}\) on TRPA1 function. It was shown that extracellular Ca\(^{2+}\) enhances the current rate and magnitude of AITC-induced currents (4, 10). Furthermore, Ca\(^{2+}\) is thought to be responsible for fast channel closure (10). In addition, single channel recordings of heterologously expressed TRPA1 revealed an AITC-induced conductance, which is reduced in the presence of Ca\(^{2+}\) (10). Together these reports emphasize the importance of Ca\(^{2+}\) for TRPA1 function.

Very recently the existence of a putative EF-hand calcium-binding domain (EF-hand CBD) at the N terminus of TRPA1 was reported (11). The EF-hand CBD is the most common motif among Ca\(^{2+}\)-binding sites of a large number of Ca\(^{2+}\)-interacting proteins (12). The classical EF-hand is a helix-loop-helix motif that coordinates the Ca\(^{2+}\) ion in a pentagonal pyramidal configuration. The domain consists usually of 12 residues, whereas six residues in positions 1, 3, 5, 7, 9, and 12 are postulated to be involved in Ca\(^{2+}\)-binding (12). In this study we set out to clarify the role of Ca\(^{2+}\) on TRPA1 channel activation by studying human TRPA1 transiently expressed in HEK293 cells. Consistent with previous studies on AITC, our results show that Ca\(^{2+}\) potentiates the cinnamaldehyde- and carvacrol-induced responses. In addition, we determine Ca\(^{2+}\) as essential co-agonist for icilin activity on TRPA1. Interestingly, Ca\(^{2+}\) also directly activates TRPA1 (EC\(_{50}\) of 905 nM) in whole-cell and excised inside-out patch recordings. The mechanism underlying Ca\(^{2+}\)-dependent activation is analyzed using pharmacological approaches and mutagenesis studies, thereby identifying the EF-hand CBD as potential Ca\(^{2+}\)-binding site.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney 293 and 293T cells (HEK293) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM l-glutamine, 100 μg/ml penicillin/streptomycin (Invitrogen, Karlsruhe, Germany) at 37 °C in a humidity controlled incubator with 5% CO\(_2\).

Transient Expression of Human TRPA1 and Mutagenesis—For transient expression of human TRPA1 (hTRPA1) we used a recombinant expression plasmid (pcDNA5-FRT) carrying the entire protein coding region for hTRPA1. The plasmid was kindly provided by H.-J. Behrendt. Semiconfluent HEK293 cells were transiently transfected (2 μg of hTRPA1 cDNA per dish) in 35-mm dishes (Flacon, BD Bioscience, Heidelberg, Germany) using the CaP-precipitation method as described previously (13). Co-transfected pIRE-S-EGFP (0.2 μg per dish) served

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2 The abbreviations used are: TRPA1, transient receptor potential channel A1; AITC, allyl isothiocyanate; PLC, phospholipase C; EF-hand CBD, EF-hand calcium-binding domain; HEK293, human embryonic kidney 293 cells; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid; RR, ruthenium red; CMZ, calmidazolium.
as transfection marker. All recordings were performed at room temperature ~24–48 h after transfection. Untransfected cells were used for control recordings.

For point mutations to the putative TRPA1 EF-hand CBD we followed established protocols. Briefly, overlap extension PCR (14) was used to perform site-directed mutagenesis of the EF-hand domain. The six residues reported to be involved in binding of the Ca\(^{2+}\) ion are in positions 1, 3, 5, 7, 9, and 12 (12). We exchanged the amino acids in these positions to alanine to induce the loss of Ca\(^{2+}\)-binding capability (D468A, S470A, T472A, L474A, N476A, D479A). The primer pairs used for mutations were as follows: external primers, CTGATGATATCGCTCATTCTGGAGG (forward) and GAATCGCTGGAAGGCTGTTATAGA (reverse); pos 1 (D468A), GAGGCTCCTCAAACGCCAAATAGTG (forward) and TCTGATCATCCTATGGGCTTGAGG (reverse); pos 3 (S470A), CTACAAGCATACTGATACGGAG (forward) and AAGGCTCTGATCAGCTATGTCTGTAG (reverse); pos 5 (T472A), ATATAAGGTCGAGGCTTCAATG (forward) and CATGTTGCTTAAGG (reverse); pos 7 (L474A), GATAAGGGGCTGTAAGGTAGTGC (forward) and AGGTCACTCTTCAGAGGCCTGTTATC (reverse); pos 9 (N476A), AGGGCTCTGGCTGAAAGGAGGTCTT (forward) and CATAGAAGGCTCTGCAAGGACCGTCT (reverse); pos 12 (D479A), GAATGGAATGTGCCATCTCAGTGAACTG (forward) and GTCACTTATGAAGGTGACCTTCATT (reverse). The different PCR products carrying the corresponding mutation were cloned into the HpaI/BamHI sites of hTRPA1. The nucleotide sequence of the mutants was verified by sequencing the corresponding cDNA.

**Solutions**—For electrophysiological measurements all solutions were adjusted to pH 7.3. The experimental solutions contained the following (in mM) for whole-cell experiments: standard extracellular solution, 140 NaCl, 5 KCl, 10 HEPES, 2 CaCl\(_2\), 1 MgCl\(_2\); Ca\(^{2+}\)-free solution, 140 NaCl, 5 KCl, 10 HEPES, 1 MgCl\(_2\), 5 EGTA; standard intracellular solution, 140 KCl, 5 KCl, 10 HEPES, 1 MgCl\(_2\), 0.1 CaCl\(_2\), 5 EGTA; standard intracellular solution, 140 KCl, 10 EGTA, 0.1 CaCl\(_2\), 5 EGTA, 10 HEPES. For ramp protocols KCl was replaced by CsCl.

For whole-cell recordings of Ca\(^{2+}\)-activated currents, CaCl\(_2\) was added either to the standard extracellular solution resulting in a concentration of 10 mM CaCl\(_2\) or to the standard intracellular solution resulting in a concentration of 5 mM CaCl\(_2\). The intracellular solution for the Ca\(^{2+}\) dose-response curve contained 140 KCl, 10 HEPES, 1 MgCl\(_2\), 5 EGTA. Ca\(^{2+}\) was added as follows: 4.918 mM CaCl\(_2\) for 100 nM free Ca\(^{2+}\), 7.421 mM CaCl\(_2\) for 300 nM free Ca\(^{2+}\), 9.05 mM CaCl\(_2\) for 1 mM free Ca\(^{2+}\), 9.663 mM CaCl\(_2\) for 3 mM free Ca\(^{2+}\), 9.906 mM CaCl\(_2\) for 10 mM free Ca\(^{2+}\), 9.905 mM CaCl\(_2\) for 30 mM free Ca\(^{2+}\). The free Ca\(^{2+}\) concentration was calculated by the software WCabuf (G. Droogmans, Leuven, Belgium).

The influence of Ca\(^{2+}\) on icilin-induced currents was studied by adding 5 mM BAPTA to the standard intracellular solution or increasing the extracellular Ca\(^{2+}\) concentration to 5 mM CaCl\(_2\). For excised-patch recordings pipette and bath solutions were symmetric (in mM): 140 NaCl, 10 HEPES, 2 EGTA. Solutions were obtained with nano- or micromolar concentrations of free Ca\(^{2+}\) by adding CaCl\(_2\) to this EGTA-based buffer as follows: 1.012 mM CaCl\(_2\) for 100 nM free Ca\(^{2+}\), 1.509 mM CaCl\(_2\) for 300 nM free Ca\(^{2+}\), 1.823 mM CaCl\(_2\) for 1 mM free Ca\(^{2+}\) and 1.940 mM CaCl\(_2\) for 3 mM free Ca\(^{2+}\).

Chemicals were prepared as concentrated stock solutions in either distilled water or Me\(_2\)SO and diluted to the final concentration using standard extracellular solution or standard pipette solution as indicated in the text. AITC, carvacrol, icilin, and BAPTA were obtained from Sigma; ruthenium red (RR), U73122, and calmidazolium (CMZ) were purchased from Calbiochem; CALP2 was obtained from Tocris bioscience; and cinnamaldehyde was from Henkel.

**Electrophysiological Recordings**—All recordings were performed with an EPC7 amplifier (List-Medical Electronic, Darmstadt, Germany). Data were acquired using Pulse software (HEKA, Lambrecht, Germany). Excised patches were sampled at 2 kHz and filtered at 1 kHz. Patch pipettes were pulled from borosilicate glass (GC150TF-10, Harvard Apparatus Ltd.) and fire polished to 3–5 MΩ tip resistance using a horizontal pipette puller (Zeit Instruments, Munich, Germany). Solution exchange was achieved by placing cells in front of a theta-capillary and moving manually from one side of the outlet to the other. Excised patches were placed in front of a micropipette perfusion pipette and solution exchange was achieved by switching from one solution to another under computer control.

**Data Analysis**—Electrophysiological data were analyzed using the software Pulse (HEKA), IgorPro (WaveMetrics), SigmaPlot (SPSS Science), OriginPro (Origin Lab Corp.), TAC (Bruxton), and Microsoft Excel. Significance was tested using Student’s independent t test (p < 0.05 is marked by an asterisk). The dose–response curve was fitted with a Hill equation of the form y = base + (max–base)/(1 + (x/x_half)\(^n\)). Data are presented as mean ± S.E.

**Western Blotting**—HEK293 cells were transfected with equal amounts of cDNA for wild-type TRPA1 and EF-hand mutants. Whole cell lysates were prepared 24 h after transfection, mixed with Laemmli buffer (30% glycerol, 3% SDS, 125 mM Tris-HCl, pH 6.8) and heated at 95 °C for 5 min. Equal amounts of protein were loaded and resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane (Protran; Schleicher & Schuell). The nitrocellulose membranes were stained with Ponceau S (Sigma) and blocked with TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 2% ECL Advance Blocking Agent (Amersham Biosciences). TRPA1 was detected using two reported antibodies directed either against the C terminus of mouse TRPA1 (1:500) or the N terminus of mouse TRPA1 (1:1000) (10, 15). The primary antibodies were diluted in 2% ECL Advance Blocking Agent in TBST. After washing and incubation with horseradish peroxidase-coupled secondary antibody, detection was performed with ECL Advance (Amersham Biosciences) on Hyperfilm ECL (Amersham Biosciences).

**RESULTS**

**Icilin Requires Ca\(^{2+}\) for Its Agonist Efficacy**—Ca\(^{2+}\) is known to modulate agonist-induced responses of various TRP channels (16–18). Voltage-clamp recordings from TRPA1-expressing *Xenopus* oocytes or HEK293 cells have shown that AITC-induced responses are potentiated by external Ca\(^{2+}\) ions (4, 10). We were interested to examine the effect of external Ca\(^{2+}\) ions...
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**FIGURE 1. Icilin requires Ca\(^{2+}\) for its agonist efficacy.** A–C, 25 μM AITC (A, n = 7), 500 μM cinnamaldehyde (B, n = 10), and 500 μM carvacrol (C, n = 7) activated TRPA1 in the absence of external Ca\(^{2+}\) ions. Addition of Ca\(^{2+}\) to the bath solution potentiated the induced responses. D and E, icilin efficacy was greatly reduced or even absent in Ca\(^{2+}\)-free recording solution. Replenishment of Ca\(^{2+}\) to the extracellular solution induced a recovery of icilin efficacy (100 μM, n = 6; 500 μM n = 2). F, icilin agonist efficacy shows a strong Ca\(^{2+}\) dependence. Icilin (100 μM) displayed little agonist efficacy in the absence of external Ca\(^{2+}\) ions (n = 7). Addition of BAPTA (5 mM) to the pipette significantly reduced the agonist efficacy of icilin (n = 12, p = 0.048) compared with standard conditions (n = 23). Increase of extracellular Ca\(^{2+}\) (5 mM) tends to result in larger current amplitudes (n = 6).

**FIGURE 2. Ca\(^{2+}\) influx is sufficient to activate TRPA1.** A, in the absence of agonist, TRPA1 overexpressing HEK293 cells developed inward currents in standard extracellular solution in 12 out of 15 recorded cells (n = 12/15, V\(_i\) = -60 mV). B, both the slowly developing initial current (inset) and the sharp increase in current were blocked by 1 μM RR (block initial current: n = 7/7, block later current: n = 6/6). C, spontaneous currents did not occur during a period of 8 min at V\(_i\) = +60 mV in standard extracellular solution (n = 4/4). Exposure to AITC (25 μM) evoked outward currents. D, increasing the extracellular Ca\(^{2+}\) concentration to 10 mM CaCl\(_2\) shortened the time to activation at V\(_i\) = -60 mV (n = 10/10). E, the current was absent in untransfected HEK293 cells (n = 4/4). Dashed line indicates zero current level.

on agonist efficacy of various TRPA1 agonists. Therefore, we performed whole-cell patch clamp recordings from HEK293 cells transiently transfected with cDNA of hTRPA1 and applied AITC, cinnamaldehyde, carvacrol, and icilin in the presence and absence of external Ca\(^{2+}\) ions at a holding potential of V\(_h\) = -60 mV (Fig. 1). As expected, AITC (25 μM) induced a slowly developing current in the absence of external Ca\(^{2+}\) ions. Addition of 2 mM CaCl\(_2\) to the bath solution evoked a strong potentiation of the inward current (Fig. 1A). The same was true for cinnamaldehyde (500 μM) and carvacrol (500 μM). The currents developed slowly in the absence of external Ca\(^{2+}\) ions and were boosted when Ca\(^{2+}\) was replenished in the extracellular recording solution (Fig. 1, B and C).

In contrast, icilin (100 μM) displayed very little or no agonist activity in the absence of external Ca\(^{2+}\) ions (40.3 ± 15.5 pA, Fig. 1, D and F). This low icilin efficacy under Ca\(^{2+}\)-free conditions does not seem to reflect a decrease of agonist potency, since increasing icilin concentration from 100 to 500 μM did not produce larger inward currents under Ca\(^{2+}\)-free conditions (Fig. 1E). A recovery of icilin agonist efficacy was observed when Ca\(^{2+}\) was added to the extracellular recording solution (Fig. 1, D and E).

These data let us suggest that Ca\(^{2+}\) serves as co-agonist with icilin by interacting directly with TRPA1, in a manner resembling the effect of Ca\(^{2+}\) on icilin efficacy at TRPM8 (16). Raising the extracellular Ca\(^{2+}\) concentration from 2 to 5 mM did not significantly increase icilin-induced currents (1815 ± 302 pA, Fig. 1F). To test for the hypothesis that intracellular Ca\(^{2+}\) is required for icilin efficacy, we added BAPTA (5 mM) to the pipette solution while recording in standard extracellular solution containing 2 mM CaCl\(_2\). Interestingly, icilin-evoked currents were significantly reduced (863 ± 235 pA, p = 0.048) compared to control conditions with standard intracellular solution (1586 ± 222 pA), indicating a role of intracellular Ca\(^{2+}\) for icilin agonist efficacy (Fig. 1F).

Taken together, our results argue for a distinct mechanism of activation by icilin as compared with other TRPA1 agonists.

\(Ca^{2+}\) Influx Is Sufficient to Activate TRPA1—In light of the clear dependence of agonist activity on Ca\(^{2+}\), we asked whether Ca\(^{2+}\) itself is sufficient to activate TRPA1. Whole-cell voltage-clamp recordings in standard extracellular solution for extended periods of time revealed the activation of an inward current that developed after on average 331 ± 58 s in the absence of any agonist in 12 out of 15 recorded cells (Fig. 2A). The current first showed a slow activation kinetic, which was boosted when the initial current reached on average 26.3 ± 3.3% of the total current. Both the initial current and the sudden sharp increase in current were blocked by 1 μM
investigate the impact of intracellular Ca\textsuperscript{2+}/H\textsubscript{11005}/H\textsubscript{11006}/H\textsubscript{119} oped at within the given time frame of 8 min in which currents developed mV. Indeed, no sudden sharp increase in current developed in current increased faster when extracellular Ca\textsuperscript{2+} is increased to 10 mM (59 \pm 6 s, Fig. 2D). Again, this current was completely blocked by 1 \mu M RR (data not shown) and was absent in untransfected HEK293 cells (Fig. 2E). Taken together, these results indicate that influx of extracellular Ca\textsuperscript{2+} into the cell is sufficient to activate TRPA1.

**Increase in Intracellular Ca\textsuperscript{2+} Elicits TRPA1 Currents**—To investigate the impact of intracellular Ca\textsuperscript{2+} on activation of TRPA1, we directly increased the intracellular Ca\textsuperscript{2+} concentration to 5 mM resulting in a free Ca\textsuperscript{2+} concentration of 23 \mu M. A prominent inward current developed almost instantaneously after establishing the whole-cell configuration in cells recorded either in standard extracellular solution (219 \pm 38 pA) or Ca\textsuperscript{2+}-free solution (214 \pm 68 pA) (Fig. 3A and B, V\textsubscript{m} = -60 mV). This current was blocked by 1–5 \mu M RR (Fig. 3A) and was absent or only exiguous in untransfected HEK293 cells (35 \pm 10 pA, Fig. 3C), supporting the finding that intracellular Ca\textsuperscript{2+} triggers TRPA1 activation.

Next, we quantified the sensitivity of TRPA1 channels to intracellular Ca\textsuperscript{2+} concentrations ranging from 100 nM to 30 \mu M. We chose a holding potential of V\textsubscript{m} = -80 mV to increase the driving force for Na\textsuperscript{+} ions, which should elicit larger inward currents. Ca\textsuperscript{2+} was added to an EGTA-buffered pipette solution to define various Ca\textsuperscript{2+} concentrations. The dose-response relationship was fitted with a Hill equation determining an EC\textsubscript{50} of 905 \pm 249 nM and a Hill-Coefficient of 0.9 \pm 0.2 (Fig. 3D).

**Ca\textsuperscript{2+} Activates TRPA1 in Excised Inside-out Patches**—In whole-cell recordings many receptors or ion channel proteins might be modulated by intracellular processes or factors like enzymatic activity or cytosolic signaling molecules. To reduce a possible influence of cytosolic factors that might control the behavior of channels, we examined the effect of Ca\textsuperscript{2+} on excised patches from transfected HEK293 cells in the inside-out configuration. Interestingly, application of Ca\textsuperscript{2+} in nanomolar concentrations to the intracellular side of the membrane was sufficient to elicit TRPA1 single-channel currents with amplitudes of on average -95 pA at a membrane potential (V\textsubscript{m}) of -80 mV resulting in a single-channel conductance of 119 \pm 6.3 pS (Fig. 4, A and B). Application of higher Ca\textsuperscript{2+} concentrations (1–3 \mu M) led to activation of more channels or even induced macroscopic currents in some patches (Fig. 4A). Furthermore, we observed desensitization of Ca\textsuperscript{2+}-induced currents (see Fig. 4A).

**Consistent with the Ca\textsuperscript{2+} dose-response curve for TRPA1 in whole-cell recordings (see Fig. 3D), we also found dose dependence in inside-out patches (Fig. 4C).**

In untransfected control cells we observed an endogenous Ca\textsuperscript{2+}-sensitive ion channel with a single-channel conductance of 40 pS at V\textsubscript{m} = -80 mV, which interferes with the overexpressed TRPA1 in transfected cells (Fig. 4D). Further analysis of single-channel currents in transfected cells during voltage ramp-protocols showed that currents evoked by Ca\textsuperscript{2+} (3 \mu M) or AITC (25 \mu M) have identical reversal potentials and rectification properties arguing that Ca\textsuperscript{2+-}activated currents are due to TRPA1 activation (Fig. 4E).

**Ca\textsuperscript{2+} Activates TRPA1 in a PLC-independent Fashion**—In light of the clear activation of TRPA1 by intracellular Ca\textsuperscript{2+} we asked whether Ca\textsuperscript{2+} directly gates TRPA1 or via a PLC-dependent signaling pathway. Since endogenous PLC activity remains preserved in inside-out patches (19) we stimulated inside-out patches with 3 \mu M Ca\textsuperscript{2+} after 30 s preincubation with the PLC-inhibitor U73122 (10 \mu M). Ca\textsuperscript{2+} was still able to induce TRPA1-mediated currents (Fig. 4F), indicating that Ca\textsuperscript{2+} may directly gate TRPA1 probably by binding to a high affinity Ca\textsuperscript{2+-}binding site at the channels cytosolic side.

**TRPA1 Exhibits a Putative EF-hand CBD**—While screening for Ca\textsuperscript{2+}-binding domains, we identified a putative EF-hand motif at the N terminus of TRPA1 (Asp-468-Leu-480). To test for the hypothesis that Ca\textsuperscript{2+} activates TRPA1 by binding through the EF-hand CBD, we used a 12-mer Ca\textsuperscript{2+} like peptide, CALP2, known to function as antagonist at the EF-hands of calmodulin and troponin C (20, 21). We examined
the effect of CALP2 on Ca\textsuperscript{2+}-induced TRPA1 single-channel currents in excised inside-out patches at $V_m = -80$ mV. Interestingly, Ca\textsuperscript{2+}-induced (1 $\mu$M) activity was strongly reduced in the presence of CALP2 (50 nM). The open probability decreased by ~90%, whereas the single-channel amplitude was unaffected (8.3 ± 0.5 pA before CALP2; 7.7 ± 1.7 pA during CALP2) (Fig. 5A). The inhibitory effect of CALP2 was reversed by removal of the peptide and channel activity reverted back to the prior level (Fig. 5A). To exclude that CALP2 blocks the channel pore, we evaluated the effect of CALP2 on AITC-induced currents (Fig. 5B). Co-application of CALP2 (50 nM) had no influence on AITC-induced (25 $\mu$M) open probability nor on single-channel currents (13.1 ± 4.3 pA before CALP2; 14.5 ± 2.8 pA during CALP2). It should be noted that AITC-induced current amplitudes varied extremely (Fig. 5B) and reached on average larger amplitudes as compared with Ca\textsuperscript{2+}-induced currents (Fig. 5A).

Up to now a role of calmodulin in TRPA1 function has not been reported. However, CALP2 might interact with calmodulin bound to TRPA1. To exclude a role of calmodulin in Ca\textsuperscript{2+}-dependent activation, we preincubated the excised patches with CMZ, a known calmodulin inhibitor. We used a concentration known to be active in other calmodulin-dependent processes (10 nM) (22, 23). After 20 s preincubation we exposed the patches to 1 $\mu$M Ca\textsuperscript{2+} in the presence of CMZ. Ca\textsuperscript{2+} still elicited TRPA1 single-channel currents (8.9 ± 3 pA), arguing that calmodulin is not involved in Ca\textsuperscript{2+}-dependent activation of TRPA1 (Fig. 5C).

A Single Mutation within the EF-hand CBD Impairs Ca\textsuperscript{2+} Sensitivity—Based on the effect of CALP2 on Ca\textsuperscript{2+}-induced single-channel currents, we were interested to see whether sensitivity of TRPA1 to Ca\textsuperscript{2+} could be limited to a single residue within the EF-hand CBD. The EF-hand motif of TRPA1 appears to be highly conserved within different spe-
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468, Ser-470, and Leu-474 were found to retain AITC sensitivity (Fig. 6D). Although slight differences were observed in mean current amplitudes elicited after 10 s application of AITC (Fig. 6E), currents showed identical reversal potentials and rectification properties for mutants as compared with the wild-type channel, arguing for plenary functionality of the mutants D468A, S470A, and L474A (Fig. 6, D and E). No currents could be observed for cells expressing the T472A, N476A, and D479A mutants (Fig. 6D). Even prolonged exposure to AITC (25 \(\mu M\), 60 s) did not induce any inward or outward currents (Fig. 6D). To exclude the possibility that these mutants lost sensitivity to AITC, we applied cinnamaldehyde (500 \(\mu M\), 60 s). Again, we failed to elicit any reliable currents (data not shown). Therefore, we concentrated in further studies on the mutants D468A, S470A, and L474A which showed unaltered AITC responses.

To examine the Ca\(^{2+}\) sensitivity of the EF-hand mutants D468A, S470A, and L474A, we compared mean current amplitudes elicited with a saturating Ca\(^{2+}\) concentration (10 \(\mu M\), see Fig. 3D) in the pipette solution while recording in Ca\(^{2+}\)-free extracellular solution at \(V_h = -80\) mV.

A prominent inward current with an average 999 \(\pm\) 140 pA developed almost instantaneously after establishing the whole-cell configuration in cells expressing wild-type TRPA1.

To identify the site(s) responsible for Ca\(^{2+}\) binding, we performed alanine scanning mutagenesis for the residues proposed to be involved in Ca\(^{2+}\) binding (Fig. 6B) (12). The resulting mutants (D468A, S470A, T472A, L474A, N476A, and D479A) were expressed in HEK293 cells and expression was first confirmed by Western blot analysis (Fig. 6C). Mouse TRPA1 antibodies recognized an expected band of \(\sim 128\) kDa for wild-type TRPA1 and EF-hand mutants, indicating effective expression of all mutants. It should be noted that although the antibodies only recognized one band for the mutant T472A, this was of lower molecular weight than predicted. No band was detected in the whole cell extract from untransfected cells (Fig. 6C).

To analyze for functionality of the mutant receptors, we verified the sensitivity to AITC (25 \(\mu M\)) before examining changes in Ca\(^{2+}\) sensitivity of the mutants. Mutations at positions Asp-468, Ser-470, and Leu-474 were found to retain AITC sensitivity (Fig. 6A). To identify the site(s) responsible for Ca\(^{2+}\) binding, we performed alanine scanning mutagenesis for the residues proposed to be involved in Ca\(^{2+}\) binding (Fig. 6B) (12). The resulting mutants (D468A, S470A, T472A, L474A, N476A, and D479A) were expressed in HEK293 cells and expression was first confirmed by Western blot analysis (Fig. 6C). Mouse TRPA1 antibodies recognized an expected band of \(\sim 128\) kDa for wild-type TRPA1 and EF-hand mutants, indicating effective expression of all mutants. It should be noted that although the antibodies only recognized one band for the mutant T472A, this was of lower molecular weight than predicted. No band was detected in the whole cell extract from untransfected cells (Fig. 6C).

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FIGURE 5. TRPA1 exhibits a putative EF-hand CBD. A, CALP2 (50 \(\mu M\)) strongly reduced Ca\(^{2+}\)-induced (1 \(\mu M\)) TRPA1 channel activity \(n = 12\), \(V_m = -80\) mV). The inhibitory effect was reversed by removal of the peptide. Amplitude histograms from the inside-out patch shown above indicate that single-channel current amplitudes are unaltered in the presence of CALP2 (8.3 \(\pm\) 0.5 pA before CALP2; 7.7 \(\pm\) 1.7 pA during CALP2), whereas the open probability is decreased by \(\sim 90\%\). B, CALP2 (50 \(\mu M\)) did not affect AITC-induced (25 \(\mu M\)) open probability and single-channel amplitudes \(n = 8\). 13.1 \(\pm\) 4.3 pA before CALP2; 14.3 \(\pm\) 2.8 pA during CALP2). The inset shows the same plot with extended y scaling. C, preincubation (20 s) with 10 \(\mu M\) CMZ did not alter Ca\(^{2+}\)-induced (1 \(\mu M\)) activity of TRPA1 \(n = 6\). The amplitude histogram shows that single-channel currents retain unaltered amplitudes (8.9 \(\pm\) 3 pA) as compared with currents elicited in the absence of CMZ. Dashed line indicates zero current level.
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**A** Alignment EF-hand CBD TRPA1

| Homo sapiens | Mus musculus | Rattus norvegicus | Bos taurus | Canis familiaris | Gallus gallus | Danio rerio |
|--------------|--------------|------------------|------------|-----------------|---------------|-------------|
| D I S D T R L L E G D L 480 | D I S D T R L L E G D L 480 | D I S D T R L L E G D L 480 | D I S D T R L L E G D L 480 | D M S D T R L L E G D L 496 | D M K D T R L L E G D K 690 | M V T D T R L L E G D E 478 |

**B** hTRPA1 mutants

| hTRPA1 D468A | hTRPA1 S470A | hTRPA1 T472A | hTRPA1 L474A | hTRPA1 N476A | hTRPA1 D479A |
|--------------|--------------|--------------|--------------|--------------|--------------|
| 468 | 468 | 468 | 468 | 468 | 468 |
| D I S D T R L L E G D L 480 | D I S D T R L L E G D L 480 | D I S D A R L L E G D L 480 | D I S D T R A L L E G D L 480 | D I S D T R L L A E G D L 480 | D I S D T R L L E G A L 480 |

**C**

| 250 kDa | 130 kDa | 95 kDa |
|---------|---------|-------|

**D**

![Graph of voltage vs. current](image)

**E**

![Graph of current vs. voltage](image)

**F**

![Graph of whole-cell current](image)

**G**

![Graph of whole-cell current](image)

**H**

![Graph of whole-cell current](image)

**I**

![Graph of whole-cell current](image)

**J**

![Graph of whole-cell current](image)

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**FIGURE 6. A single mutation in the EF-hand CBD impairs Ca$^{2+}$ sensitivity.** A, sequence alignment of the EF-hand CBD of TRPA1. The motif is highly conserved. The six residues presumably involved in Ca$^{2+}$ binding are indicated by gray shading. B, display of the various EF-hand mutants. Mutated residues are characterized with gray shading. C, expression of wild-type TRPA1 and EF-hand mutants was assessed by Western blotting after transient transfection of each clone into HEK293 cells. Mouse TRPA1 antibodies recognized an expected band of ~128 kDa. The molecular weight of the T472A mutant was lower than predicted. No band was detected in the whole-cell extract of untransfected control cells (untransf.). D, whole-cell recordings in HEK293 cells overexpressing: wild-type (trace a), mutant D468A (trace b), mutant S470A (trace c), mutant T472A (trace d), mutant L474A (trace e), mutant N476A (trace f), and mutant D479A (trace g). Current-voltage relationship of representative cells obtained after 10 s (traces a–c and e) or 60 s (traces d, f, and g) application of AITC (25 μM), respectively. Zero current was subtracted for each trace. E, average inward and outward currents carried by wild-type TRPA1 or EF-hand mutants D468A, S470A, and L474A at -80 and +80 mV (n = 5–7). F, whole-cell recording in Ca$^{2+}$-free solution at V_m = -80 mV from a representative cell overexpressing wild-type TRPA1 perfused with a solution in which Ca$^{2+}$ was buffered to 10 μM. The current was blocked by 1 μM RR. The arrow indicates the time of break-in. G and H, same as in F but showing a representative cell overexpressing the D468A mutant (G) or the S470A mutant (H). I, same as in F but showing a representative cell overexpressing the L474A mutant. 10 μM Ca$^{2+}$ failed to induce a current. For control purposes 25 μM AITC (40 s) was applied. J, average inward currents evoked by 10 μM Ca$^{2+}$ at V_m = -80 mV carried by wild-type TRPA1 (n = 12), mutant D468A (n = 9), mutant S470A (n = 10), and mutant L474A (n = 11). The L474A mutant showed significantly reduced responses to 10 μM Ca$^{2+}$. Dashed line indicates zero current level.

In light of the finding that a single mutation of the residue Leu-474 results in a Ca$^{2+}$-insensitive channel, we next verified the sensitivity of this mutant to AITC in the presence and absence of extracellular Ca$^{2+}$. In Ca$^{2+}$-free solution currents elicited by AITC (25 μM) were about the same for wild-type TRPA1 and the L474A mutant (Fig. 7, A–C, wild-type 1856 ± 345 pA, L474A 1928 ± 479 pA). As expected, addition of 2 mM CaCl$_2$ to the bath solution resulted in a strong potentiation of the wild-type response (Fig. 7, A and C, 3066 ± 430 pA). Interestingly, the L474A mutant showed no potentiation of AITC-induced responses by Ca$^{2+}$ (Fig. 7B). Addition of 2 mM CaCl$_2$ to the bath solution resulted in a reduction of current amplitudes (Fig. 7, B and C, 1423 ± 281 pA), presumably reflecting the reported transition of the channel to a state with lower conductance (10).

Whereas Ca$^{2+}$ was found to potentiate AITC-induced responses, it was shown to be an essential co-agonist for icilin efficacy on TRPA1 (see Fig. 1). Thus, we tested icilin to elicit currents in cells expressing the Ca$^{2+}$-insensitive L474A mutant. Performing whole-cell recordings in standard extracellular solution containing 2 mM CaCl$_2$ showed no substantial currents (9 ± 5 pA), consistent with a loss of Ca$^{2+}$ activation of the L474A mutant (Fig. 7, D and F). In contrast mutations at positions Asp-468 and Ser-470 were found to retain sensitivity for icilin analogue to wild-type TRPA1 (Fig. 4, E and F, wild-type 1217 ± 256 pA, D468A 1159 ± 199 pA, S470A 1310 ± 284 pA).

Taken together, the results indicate that the residue in position Leu-474 participates in Ca$^{2+}$-mediated potentiation of agonist (AITC)-induced responses and also contributes to icilin agonist efficacy on TRPA1.
TRPA1 Is Directly Gated by Ca\(^{2+}\)

In the present study, we investigated the effects of Ca\(^{2+}\) on the gating behavior of the Ca\(^{2+}\)-permeable cation channel TRPA1. We showed that Ca\(^{2+}\) potentiates responses to various TRPA1 agonists and serves as an essential co-agonist for icilin activity. We further demonstrated that Ca\(^{2+}\) activates TRPA1 and shed light on the mechanism in which Ca\(^{2+}\) may directly gate the channel.

Several TRP channels are reported to be regulated or modulated by Ca\(^{2+}\). For example, gating of TRPV4 depends on both extracellular and intracellular Ca\(^{2+}\) (18), and TRPM2 activation requires Ca\(^{2+}\) (24). Here, we show that agonist efficacy of some TRPA1 agonists is modulated by Ca\(^{2+}\). As reported for AITC (4, 10), we found that cinnamaldehyde and carvacrol responses are potentiated by Ca\(^{2+}\), suggesting a common mechanism for these agonists. In contrast, we observed icilin to require Ca\(^{2+}\) for its agonist efficacy, arguing for a distinct mechanism of activation. Whereas concomitant exposure of AITC and Ca\(^{2+}\) displays synergy, the necessity of simultaneous exposure of icilin and Ca\(^{2+}\) points toward a mechanism of co-dependence. Our data obtained by mutagensis support the hypothesis for distinct mechanisms of activation by AITC and icilin. We describe a single residue within the N-terminal EF-hand CBD to participate in Ca\(^{2+}\)-mediated modulation of agonist-induced responses. Ca\(^{2+}\) binding to leucine in position 474 appears to be involved in potentiation of AITC-induced responses and seems to play a major role for icilin agonist efficacy on TRPA1. The ineffective-ness of icilin on the Ca\(^{2+}\)-insensitive L474A mutant argues for a more complex mechanism in icilin-dependent activation of TRPA1 different from that previously reported for TRPM8 (16). Possibly Ca\(^{2+}\) primarily induces activation of TRPA1 and icilin acts modulatory on the Ca\(^{2+}\)-induced responses. Since icilin activates TRPA1 currents with variable delay of onset in the presence of Ca\(^{2+}\) (data not shown) resembling the activation kinetics of Ca\(^{2+}\)-influx induced currents (see Fig. 2), an appropriate scenario is conceivable. However, it remains to be determined whether icilin modulates the response thereby facilitating Ca\(^{2+}\)-mediated activation of the channel rather than directly provoking TRPA1 channel opening.

Activation of TRP channels by Ca\(^{2+}\) ions has been reported previously for the monovalent-selective channels TRPM4 and TRPM5 (25, 26). Although a prior study suggested that TRPA1 can be activated by store depletion (4), the finding is still disputed as a second study failed to reproduce activation of TRPA1 by store depletion (7). Other studies showed that TRPA1 currents can be activated downstream of G protein-coupled receptors (4, 7), arguing for a role of second messengers in TRPA1 activation. Importantly, neither previous study tested whether Ca\(^{2+}\) directly activates TRPA1 channel.

Evidence for Ca\(^{2+}\)-dependent activation of TRPA1 is obtained from our excised-patch and whole-cell recordings. The data show that Ca\(^{2+}\) influx through spontaneously active TRPA1 channels is sufficient to initiate further channel opening. Intracellular Ca\(^{2+}\) activates the channel with an apparent EC\(_{50}\) of 905 nM in whole-cell recordings. The exposure of excised inside-out patches to Ca\(^{2+}\) elicits TRPA1 single-channel currents and the number of Ca\(^{2+}\)-activated channels increased with higher Ca\(^{2+}\) concentrations.

In general, Ca\(^{2+}\) can bind and activate proteins either by itself or by binding through adaptor proteins. A direct interaction of TRPA1 with Ca\(^{2+}\) was analyzed using pharmacological approaches and site-directed mutagenesis. Our data suggest that Ca\(^{2+}\) activates TRPA1 in a PLC- and calmodulin-independent fashion. CALP2 effectively reduces Ca\(^{2+}\)-induced channel activity and a mutation of a single residue within the N-terminal EF-hand CBD induces loss of Ca\(^{2+}\)-dependent activation, favoring the N-terminal EF-hand domain as a putative Ca\(^{2+}\)-binding site.

Within the EF-hand motif six residues are described to be involved in binding of the Ca\(^{2+}\) ion (12). Accordingly, we introduced point mutations at these sites and screened the channel
TRPA1 Is Directly Gated by Ca$^{2+}$

mutants by examining their functionality and Ca$^{2+}$ sensitivity. Of the six potentially involved residues, we observed that a single mutation at position Leu-474 altered Ca$^{2+}$ sensitivity of TRPA1. We find that Ca$^{2+}$-dependent activation is strongly impaired for the L474A mutant, assuming that the leucine contributes to Ca$^{2+}$ sensitivity of TRPA1. The D468A and S470A mutants remain functional and exhibit wild-type responses to a saturating Ca$^{2+}$ concentration, suggesting that mutations at these sites may be accommodated without affecting Ca$^{2+}$ activation. Reports describe a considerable variability in length and amino acid sequence of the N-terminal part of the Ca$^{2+}$-binding loop in EF-hand proteins (27). In natural EF-hand loops the planar position 3 is one of the most variable positions. Depending on the type of mutation the effect can vary over a wide range from nearly no effect up to virtually preventing Ca$^{2+}$ binding (28). The residue in the last coordinating position is reported to be required for the pentagonal bipyramidal coordination geometry and provides commonly two oxygen atoms for liganding (28). The residue in the last coordinating position is reported to be required for the pentagonal bipyramidal coordination geometry and provides commonly two oxygen atoms for liganding (28).

Although our experiments identify the residue Leu-474 within the EF-hand motif as involved in Ca$^{2+}$-dependent activation of TRPA1 and although CALP2 reduces Ca$^{2+}$-induced channel activity, further analysis comprising Ca$^{2+}$-dependent desensitization process (10) that minimizes the Ca$^{2+}$ conductivity of TRPA1 in a self-limiting process.

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