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Putative interaction site for membrane phospholipids controls activation of TRPA1 channel at physiological membrane potentials

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Abbreviations: ATR-FTIR, attenuated total reflection Fourier transform infrared; CD, circular dichroism; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; P/L, peptide/lipid ratio; PI(4,5)P₂, L- α -phosphatidylinositol-4,5-bisphosphate; POPC, palmitoyl-oleoyl-phosphatidylcholine; POPS, palmitoyl-oleoyl-phosphatidylserine; PWR, plasmon waveguide resonance; SUV, small unilamellar vesicle; TRPA1, transient receptor potential channel subtype 1; TBS, tris-buffered saline; PBS, phosphate-buffered saline; MDS; microfluidic diffusional sizing

ABSTRACT

The transient receptor potential ankyrin 1 (TRPA1) channel is a polymodal sensor of environmental irritant compounds, endogenous proalgesic agents and cold. Upon activation, TRPA1 channels increase cellular calcium levels *via* direct permeation and trigger signaling pathways that hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP₂) in the inner membrane leaflet. Our objective was to determine the extent to which a putative PIP₂-interaction site (Y1006-Q1031) is involved in TRPA1 regulation. The interactions of two specific peptides (L992-N1008 and T1003-P1034) with model lipid membranes were characterized by biophysical approaches to obtain information about affinity, peptide secondary structure and peptide effect in the lipid organization. The results indicate that the two peptides interact with lipid membranes only if PIP₂ is present and their affinities depend on the presence of calcium. Using whole-cell electrophysiology, we demonstrate that mutation at F1020 produced channels with faster activation kinetics and with a rightward shifted voltage-dependent activation curve by altering the allosteric constant that couples voltage sensing to pore opening. We assert that the presence of PIP₂ is essential for the interaction of the two peptide sequences with the lipid membrane. The putative phosphoinositide-interacting domain comprising the highly conserved F1020 contributes to the stabilization of the TRPA1 channel gate.

Introduction

The human transient receptor potential (TRP) subtype A1 (TRPA1) is a chemosensitive ion channel involved in nociception and inflammatory pain [1-4]. It is considered to be one of the key physiological sensors for many pungent and irritant compounds being activated by thiol-reactive electrophiles such as cinnamaldehyde, oxidants, and various non-electrophilic agonists such as carvacrol or menthol [5, 6]. Cytosolic calcium modulates the activity of TRPA1 in a bimodal manner so that Ca^{2+} activates or potentiates the channel at low concentrations and inactivates it at higher concentrations [7]. The proposed molecular mechanism involves Ca^{2+} -sensing calmodulin that pre-associates with the C-terminal region of TRPA1 (L992-N1008) and enables the channel to distinctly respond to diverse Ca^{2+} signals [8]. Further adding to the complexity of TRPA1 regulation are early signaling events linked to Ca^{2+} -dependent phosphoinositide-specific phospholipase C (PLC) enzymes that hydrolyze phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂; PIP₂) in the inner membrane leaflet. The PLC-mediated PIP₂ depletion may represent an important mechanism involved in TRPA1-mediated nociception triggered by pro-inflammatory agents such as bradykinin [9] and protease-activated receptor PAR2 [10]. Up to date, however, reports are inconsistent regarding the effects of membrane phosphoinositides on TRPA1 [reviewed in [11-13]]. The sites at which these may interact with TRPA1 are not resolved, but most likely involve the membrane proximal regions of the N- and C-termini and inter-subunit regions near the inner leaflet of the membrane [14-16]. Indeed, we have previously shown that a 29 residues peptide (I964-L992) from the C-terminus exhibits a high affinity to PIP₂-containing membranes, relative to other lipids, suggesting that important electrostatic interactions with the negatively charged lipid head groups occur [15]. These results fitted well with a later structural study that attributed the predicted region (then, called H1 in our study) to the TRP-like helix that runs parallel to the inner leaflet of the membrane and forms a central nexus of activating signal convergence [17].

From the TRPA1 electron density map, another putative site for interaction with membrane phospholipids has been proposed that is formed by a poorly resolved flexible linker connecting the TRP-like domain with the C-terminal coiled-coil [17]. This part of the channel (Y1006-Q1031) forms an intracellular vestibule between adjacent subunits connected to the bulk solution through a side fenestration that could provide means for hydrophobic

modulators and lipids to affect channel functioning. The flexible linker contains a short α -helix (shown in violet in Fig. 1A, B) that is almost buried in the inner membrane leaflet. The linker is integrated with a putative three-stranded β -sheet formed by two anti-parallel β -strands from the N-terminus and a contacting strand that follows the C-terminal TRP-like helix. The latter, peripherally exposed β -strand binds the carboxy-lobe of calmodulin and even under resting concentrations of Ca^{2+} (~100 nM) may form a tight complex with the channel [8]. Calmodulin binds to TRPA1 within the region L992-N1008 and it may compete for the same or overlapping binding site with PIP_2 or other phosphoinositides.

Here we examined the functional role of both the above regions (L992-N1008 and Y1006-Q1031) with the aim to extend available knowledge about the putative role of membrane PIP_2 in TRPA1 channel functioning. Using electrophysiological and biophysical studies, we tested the hypothesis that the binding specificity of PIP_2 may depend not only on the presence of positively charged residues in the TRP-like domain that likely interact with polar head groups as demonstrated in [15] and [18], but also on specific hydrophobic residues presumably involved in contacting the lipid hydrophobic tails.

Results

Specific peptides corresponding to the flexible linker or calmodulin binding site rely on the presence of PIP_2 to establish superficial contacts with the lipid membrane

Different biophysical approaches were employed to characterize the peptide/lipid interaction in terms of the affinity (Plasmon Waveguide Resonance - PWR and Microfluidic Diffusional Sizing - MDS), effect in lipid membrane organization (PWR and Polarized ATR-FTIR, p ATR-FTIR), membrane integrity (Rhodamine leakage) and peptide secondary structure (Circular Dichroism - CD) (Fig. 2 A-D). To investigate the importance of PIP_2 in these interactions, lipid model systems such as planar membranes, large unilamellar vesicles (LUVs) and polymer-based nanodiscs (styrene maleic acid lipid particles, SMALPs) composed of POPC (main lipid of mammalian cell membranes) and POPC with PIP_2 (5-20%) were employed. To test the binding of the peptides to the lipid model systems, three different approaches were employed: polarized ATR-FTIR [19], PWR [20] and MDS, a new technology developed by Fluidic Analytics that allows to determine the concentration and size of proteins or peptides or any primary amine containing molecule [21, 22]. Since the higher

resolution limit in terms of size is about 30 nm of radius, herein we have chosen polymer based nanodiscs (SMALPs) that have a diameter of about 10-15 nm as our lipid model system. MDS was used to follow peptide binding to SMALPs composed of POPC and POPC/PIP₂ (9/1 mol/mol) by following the changes in size occurring upon the interaction (further details can be found in the Materials and Methods). From the PWR and MDS data was evident that PIP₂ is essential for lipid interaction as no binding was observed in the case of pure POPC membranes (Fig. 2 A, B). For POPC/PIP₂ membranes, binding was observed for both peptides with affinities ranging from about 300 to 700 nM for L992-N1008 and T1003-P1034 peptides, respectively, as determined by MDS (Table 1). The peptide L992-N1008 showed a stronger affinity than T1003-P1034. This could be related to the difference in the net charge (2 for L992-N1008 and 1 for T1003-P1034) as the peptide L992-N1008 could establish stronger electrostatic interactions with the negatively charged lipid. PWR failed to provide accurate affinities, although a hyperbolic saturating curve was obtained for POPC/PIP₂ membranes, as the spectral changes induced by peptide addition are quite small (≤ 5 mdeg, at saturating peptide concentrations whereas the instrument angular resolution ≥ 0.5 mdeg) (Fig. 2A). Increasing the PIP₂ content to 20% relative to POPC did not increase the PWR signal amplitude (data not shown). This suggests that peptide binding to lipids induces weak perturbations in the lipid ordering. Indeed, this hypothesis is further supported by p ATR-FTIR data that shows no significant changes in the lipid fatty acid. Similar position and dichroic ratio for CH₂ symmetric (at 2852 cm⁻¹) and anti-symmetric (at 2923 cm⁻¹) stretching bonds (Fig. 2D) are observed after incubation with both peptides. To observe only the signal from the membrane-bound peptide, following peptide incubation, the cell was rinsed with buffer to remove all non-membrane bound peptide. In the present studies, the amide I (1640 cm⁻¹) and the amide II (1535 cm⁻¹) were observed before peptide rinsing but became extremely small (detection limit) after rinsing the peptide. Therefore ATR-FTIR data deconvolution of the amide I and amide II region with the intention of obtaining the peptide secondary structure and orientation was not performed here. p ATR-FTIR data agrees well with PWR and MDS data in that no peptide interaction was observed in absence of PIP₂. The presence of Ca²⁺ in the buffer decreased the affinity of both peptides to POPC/PIP₂ lipid membranes as shown by MDS (Table 1). Peptide effect in rhodamine leakage from LUVs was tested to determine if peptide interaction affects membrane integrity (*e.g.* pore formation). No membrane leakage was observed even at peptide/lipid ratios of 1/10 (Table 2). Those two findings suggest that peptide binding to lipid occurs if PIP₂ is present and that the binding is

superficial, most probably involving electrostatic interactions between the anionic lipid and positive charges in the peptide. In terms of the peptide secondary structure, CD data indicates that both peptides are highly non-structured (random coil), with a certain degree of antiparallel β -sheet (Fig. 2C and Table 3). The peptide L992-N1008 also presents a certain degree of helical content. The data suggests no significant peptide conformational changes from a buffer system and upon interaction with POPC or POPC/PIP₂ (9.5/0.5 and 8/2 mol/mol) at P/L ratios ranging from 1/50 to 1/10 (Fig. 2C and Table 3).

Glycine mutation of central phenylalanine F1020 produces strongly rectifying channels

Based on the multiple sequence alignment of TRPA1 proteins over the flexible linker region, the most conserved residue within the putative short α -helix is the central phenylalanine 1020 (Fig. 1B). We substituted this residue with glycine to disrupt the predicted helical structure and measured agonist-induced responses from transfected HEK293T cells using whole-cell patch-clamp electrophysiology (Fig. 3). To stimulate the cells, we used a previously established protocol [23-25] in which currents were first induced by the electrophilic agonist in the absence of external Ca²⁺. The agonist was then washed out for 10 s, and 2 mM Ca²⁺ was added to the extracellular solution to assess the allosteric effects of permeating calcium ions. The membrane potential was linearly ramped up each second from -80 mV to +80 mV (1 V/s) (Fig. 3A). Cinnamaldehyde, a partial agonist of TRPA1, was sufficient to activate the mutant channels at a concentration of 100 μ M. Compared to wild-type channels, mutation F1020G produced a strong outward rectification of currents measured in the absence of Ca²⁺ (Fig. 3B) as was visible from the current-voltage relationships obtained after 30s application of agonist (Fig. 3C, D) and from the time course of rectification ratio measured at -80 mV and at +80 mV (Fig. 3E, F). At negative membrane potentials, the average peak inward currents through F1020G channels were almost three times smaller (-1.0 ± 0.1 nA versus -2.9 ± 0.5 nA for wild-type; $P \leq 0.001$; $n = 13$ and 10). The F1020G-mediated currents, on the other hand, were not significantly different when external calcium was added (-4.0 ± 0.5 nA versus -4.4 ± 0.5 nA at -80 mV; $P = 0.554$; and 5.6 ± 0.7 nA versus 6.1 ± 0.8 nA at +80 mV; $P = 0.637$; $n = 13$ and 10). The mutation thus affected primarily the voltage-dependency of the channel under weakly activated conditions.

Mutation F1020G shifts the voltage-dependence of activation

To further assess the changes in the intrinsic voltage dependence of the F1020G mutant channels, we measured responses to a voltage step protocol from -80 to +200 mV, in 20mV increments, applied in extracellular control solution containing either 1 mM Ca²⁺ or 0 mM Ca²⁺. In both cases, the currents through F1020G exhibited apparently much faster activation and deactivation kinetics (Fig. 4A, B). Mutation F1020G produced a large shift in the steady-state voltage-dependent activation toward more positive potentials (V_{50} from 112 mV to 146 mV; Fig. 4C). The steepness of the Boltzmann relationship (reflecting the apparent number of gating charges z) significantly increased from $z = 0.61 \pm 0.03 e_0$ to $0.75 \pm 0.02 e_0$ ($P \leq 0.001$; $n = 13$ and 16), indicating that PIP₂ may interact with potentially voltage sensing residues. When Ca²⁺ was omitted from the extracellular solution, mutation F1020G also resulted in an ~30-mV rightward shift in the V_{50} of the G - V curve, the estimated z was not significantly different from wild-type channels ($P = 0.343$; $n = 10$ and 15), indicating that Ca²⁺ affects the gating of the wild-type and the F1020G mutant channels to a different extent (Fig. 4D).

The activation kinetics of wild type TRPA1-mediated currents elicited by a depolarizing step from -80 mV to +200 mV in extracellular control solution containing 1 mM Ca²⁺ were best described by the sum of two exponential functions in 7 out of 12 cells ($\tau_{\text{slow}} = 47.2 \pm 9.0$ ms, 60.8 ± 6.6 %, $\tau_{\text{fast}} = 9.6 \pm 2.2$ ms; $n = 7$). In 5 cells, the activation process was best fit by one exponential function (41.0 ± 5.6 ms; $n = 5$). The activation kinetics of F1020G were characterized by $\tau_{\text{slow}} = 32.7 \pm 6.9$ ms, 31.7 ± 4.9 %, $\tau_{\text{fast}} = 4.8 \pm 0.5$ ms in 6 of 16 cells. In 10 cells, the activation process was best fit by one exponential function (12.1 ± 1.8 ms). Repolarization to the holding membrane potential of -70 mV produced pronounced inward 'tail currents' that could be fitted by one exponential function in 8 of 12 cells expressing wild-type channels (16.7 ± 1.4 ms). In four cells, the deactivation process was best fit by a sum of two exponential functions ($\tau_{\text{slow}} = 39.2 \pm 6.0$ ms, 44.1 ± 10.8 %, $\tau_{\text{fast}} = 5.6 \pm 0.5$ ms). Detailed analysis of the F1020G-mediated deactivation kinetics revealed a slow (22.9 ± 2.5 ms; 35.9 ± 4.2 %) and a fast time constant (3.9 ± 0.4 ms). Because the relative weights of fast and slow components differed between the cells, the overall statistics was performed with the weighted, average time constants of the fast and slow components (Fig. 4E, F).

Docking experiments suggest different PIP₂ conformations within the sensor domain

The lower segment of the S1-S4 sensor domain with the C-terminal TRP-like helix below forms an integrated nexus that has been previously proposed to be capable of binding membrane phosphoinositides including PIP₂ [25]. Our ligand docking studies showed that the negatively charged inositol trisphosphate head group of PIP₂ may adopt several different conformations within the inner crevice formed by the S1-S4 helical bundle of the sensor to contact polar residues in the inner cavity of the sensor (H719, N722, K787, K796, R852) and the TRP-like helix (K989). Mutations at these residues resulted in a partial loss of functional response to depolarizing voltage in the absence or presence of electrophilic agonists and similar effects are observed as a consequence of the depletion of membrane PIP₂ [18, 25]. However, the identification of the positively charged residues that might interact with PIP₂ head group is usually not sufficient to support a conclusion that these are the major binding sites. It is possible that mutations of other contacting residues would give similar defects in channel functioning once they are identified [26]. To gain further insights into possible orientations of PIP₂ within the inner sensor cavity, we performed subsequent ligand docking experiments and found that hydrophobic regions near the PIP₂ hydrocarbon chains may also contribute to the stabilization of the TRPA1-PIP₂ complex (Fig. 5 A-C). The density map for the flexible loop T1003-Q1031 at one side extends to the vicinity of the poorly resolved intracellular S2-S3 linker of the S1-S4 sensor domain. At its other side, it contacts the sensor from an adjacent subunit through stacking interactions with the side chains of F716 and H719 from S1 helix and F853 from the S4-S5 linker (Fig. 5B). We have previously shown that the substitution of H719 by a small residue alanine induced serious functional defects manifested as significant changes in rectification and kinetics of the currents that were blocked but not potentiated by external calcium [25]. To further substantiate the functional importance of this putative contact region, we constructed the F853A mutant and tested its chemical sensitivity (Fig. 5D). The electrophilic full agonist allyl isothiocyanate at a concentration of 100 μ M was used because cinnamaldehyde, a partial agonist of TRPA1, was not sufficient to activate the mutant channels. Control cells transfected with wild-type TRPA1 responded robustly to application of agonist in agreement with our previous reports (e.g. [23-25]). In contrast, the HEK293T cells expressing F853A produced only very small currents in response to allyl isothiocyanate even when combined with voltage-ramp stimulus from -80 mV to +80 mV. The currents developed slowly and were further diminished by the addition of external

calcium, thus resembling H719A [25]. These results together support the structurally predicted importance of the stacking interactions within the S1 and S4 helices of the sensor domain and indicate that the channel functioning can be strongly affected through this region.

Putative mechanism of coupling of PIP₂ signal to the gate involves serine 972

The TRPA1 density map indicates another possible mechanism through which the flexible loop T1003-Q1031 might directly affect functional coupling of stimulus sensing and gate opening. The C-terminal end of the flexible linker (I1033) is located only about 4 Å from serine 972 (Fig. 5B). This serine residue is situated at the beginning of the TRP-like helix, a critically important locus of channel regulation that is directly connected to the lower gate (Fig. 5B). To explore the possibility of such interaction, we compared responses from two TRPA1 constructs in which the serine 972 was substituted with either smaller (alanine) or larger (aspartate) residue (Fig. 6A to F). As above (Fig. 5D), the transfected cells were first activated by the electrophilic agonist in the absence of external Ca²⁺. The agonist was then washed out for 10 s, and 2 mM Ca²⁺ was added to the extracellular solution to assess the allosteric effects of permeating calcium ions. The membrane potential was linearly ramped up each second from -80 mV to +80 mV (1 V/s). Compared to wild-type channels, S972A mediated whole-cell currents were not significantly different, producing only a slight relative decrease in the rectification throughout the time course of the experiment (Fig. 6A, C and E). In contrast, S972D produced a strong outward rectification of currents measured in the absence of Ca²⁺ (Fig. 6B) as was visible from the current-voltage relationships obtained after 30s application of agonist (Fig. 6D) and from the time course of rectification ratio measured at -80 mV and at +80 mV (Fig. 6F). This result, analogous to that obtained from F1020G (see Fig. 3), indicates that the two residues may share a common intramolecular pathway for affecting the gating of the channel. PIP₂ could then regulate TRPA1 specifically at negative membrane potentials, whereas it loses its activity upon strong depolarization or in the presence of calcium. When external calcium was added, the maximum S972A-mediated currents were not significantly different from wild-type TRPA1 (-5.5 ± 0.6 nA versus -4.4 ± 0.5 nA at -80 mV; *P* = 0.230; and 5.5 ± 0.7 nA versus 6.1 ± 0.8 nA at +80 mV; *P* = 0.667; *n* = 4 and 10). The maximum currents measured in the presence of Ca²⁺ were significantly increased in S972D at both negative and positive membrane potentials (to -9.9 ± 0.8 nA, *P* = 0.027 at -80 mV and to 9.6 ± 0.8 nA at +80mV; *P* = 0.01; *n* = 7). This latter

result indicates changes in voltage-dependent gating rather than in trafficking or expression. Secondly, S972 is a residue involved in Ca^{2+} -dependence of TRPA1.

Allosteric model indicates changes in voltage-coupling and basal-open equilibrium induced by mutation F1020G

The double exponential fit needed for fitting the activation phase of voltage-induced responses and the decay of the tail currents (Fig. 4) reflects a complex gating process, apparently including multiple closed and open states. Such analysis does not indicate a possible mechanism through which the mutation may affect the channel gating. The faster voltage-induced activation of F1020G compared with the wild-type channels suggests that the mutation may strengthen the allosteric coupling of putative voltage sensor activation to channel opening. On the other hand, a rightward shift in V_{50} could be related to a direct change in gate equilibrium or a decrease in the voltage sensor equilibrium constant. By plotting the G - V curves described in Fig. 4C and D on a semi-logarithmic scale (Fig. 7A, B), it is obvious that the G - V shift in F1020G observed in the presence of external Ca^{2+} could be due to changes in the (basal-open) gate equilibrium [27]. An allosteric model to explain the activation of thermosensitive TRP ion channels by voltage has been previously proposed by Brauchi et al. [28]. This model is based on the presumption that an independent voltage sensor exists that is coupled to channel gating. At a constant temperature and low voltages, the channel is confined to the equilibrium between closed (C_1) and open (O_1) states with a small equilibrium constant L . Voltage drives transitions between resting and activated states of the sensor (C_1 - C_2) with equilibrium constant $J = J_0 \exp(zFV/RT)$, where J_0 is the equilibrium constant for the transition at 0 mV. In turn, the voltage sensor is coupled to channel gating by an allosteric coupling constant D . A simplifying assumption is that the voltage sensors from each subunit act in concert. The open probability is then given by $P_o = 1/[1+(1+J)/(L(1+JD))]$.

We found that this simple allosteric model could reproduce voltage-dependent activation of the wild-type and the mutant F1020G channels. By simultaneously fitting the normalized steady-state G - V data and the time course of the activation phase of normalized conductances induced by depolarization to +200 mV, we obtained parameters D , L and J_0 for representative cells (Fig. 7C-F and Table 4). For wild-type TRPA1 measured in the presence of external Ca^{2+} , a good fit yielded the equilibrium constant L of 0.0200 ± 0.0027 and the allosteric factor D of $29,562 \pm 3,571$ ($n = 10$). Mutation F1020G did not affect the

equilibrium constant L (0.0172 ± 0.0031 ; $P = 0.490$; $n = 9$) but significantly decreased the allosteric factor D ($P \leq 0.001$; Fig. 7G). The estimated value of the voltage sensor equilibrium J_0 was significantly increased ($P = 0.020$) and this effect was particularly pronounced ($P \leq 0.001$; $n = 11$) when Ca^{2+} was not present in the extracellular solution (see Fig. 7G and Table 4). This result suggests that the F1020G mutation could abolish the allosteric coupling of the putative voltage sensor to channel gating and increase the equilibrium constant for the transitions between resting and activated states of the sensor. External Ca^{2+} affected the basal-open gate equilibrium (L) in F1020G but not in wild-type channels, indicating Ca^{2+} -dependence of conformational changes caused by mutation.

Specific peptides interfere with TRPA1 calcium-dependent potentiation

It has been previously shown that Ca^{2+} /calmodulin complex binds to TRPA1 within the region L992-N1008 and potentiates the channel under resting conditions [8]. This study suggested that basal intracellular Ca^{2+} concentration is sufficient to cause preassociation of calmodulin with TRPA1, but the channels are far from saturated by endogenous calmodulin. On the other hand, our results indicate that the peptide L992-N1008 interacts with lipid membranes if PIP_2 is present and its affinity decreases in the presence of calcium. We reasoned that if PIP_2 binds at the same site under native conditions, then chelating background Ca^{2+} should release the pre-bound Ca^{2+} /calmodulin complex from the channel and enable it to interact with PIP_2 . It has been previously shown that the role of PIP_2 in TRPA1 activation and desensitization is complex and involves the related channel TRPV1 in sensory neurons [29]. In order to explore the possible contribution of the putative PIP_2 -interaction site (Y1006-Q1031) in TRPA1 regulation under close-to-native conditions, we used neuronal F11 cells transfected with human TRPA1. We and others have previously shown that this cell line is a suitable model which maintains broad functional similarities with dorsal root ganglia neurons [30, 31], including phospholipase C pathway and PIP_2 signaling [32, 33]. *TRPA1* and *TRPV1* genes are absent from this cell line [31], which provides an opportunity to observe the human TRPA1 activity in isolation from the TRPV1-dependent pathways.

In our experiments, we aimed: (i) to generalize the previously published observations by Hasan et al. [8] that Ca^{2+} /calmodulin-dependent potentiation of mouse TRPA1 is prevented by including the dominant negative peptide L992-N1008 in the pipette, (ii) to explore,

whether intracellular perfusion with the longer peptide T1003-P1034 has a similar dominant-negative effect as the shorter peptide, and (iii) to examine, whether PIP₂ and Ca²⁺/calmodulin may compete for the same site at TRPA1 and influence the stability/sensitivity of the channel under resting conditions (i.e. at negative membrane potentials and in the absence of calcium). Using the same experimental solutions and application protocol as Hasan et al. (2017), we measured the extent of potentiation of carvacrol (100 μM) responses by external calcium (0.5 mM) under three situations: 1) within 30-50 s after whole-cell formation, 2) after 5-6min perfusion with intracellular solution and 3) after 5-6min perfusion with intracellular solution containing either the L992-N1008 or T1003-P1034 peptide (Fig. 8). Figure 8A shows that initial TRPA1-mediated carvacrol responses recorded in nominal 0 Ca²⁺ 30-50 s after whole cell formation had a much slower onset than responses measured from cells perfused for 5-6 minutes with internal solution with or without the peptide (Fig. 8A). As an apparent consequence, the carvacrol-induced current densities measured at the end of 10s application were significantly smaller in nonperfused cells (median 18.7 pA/pF versus 8.5 pA/pF for the T1003-P1034 peptide; *n* = 12 and 6; *P* = 0.044; Fig. 8C). Intriguingly, Ca²⁺ potentiation was strongly (8.3 ± 1.6 fold) enhanced in the cells recorded within 30-50 s after whole-cell formation (Fig. 8D) and there were no correlation between the peak current amplitudes and the extent of Ca²⁺-induced potentiation (correlation coefficient -0.0979, *P* = 0.749; *n* = 12). Inclusion of the T1003-P1034 peptide in the pipette solution significantly (*P* = 0.028; *n* = 6) prevented Ca²⁺-dependent potentiation similarly as previously seen with L992-N1008 peptide [8]. These results suggest that the T1003-P1034 region is important for Ca²⁺-dependent potentiation and that Ca²⁺/calmodulin complex may compete for the same or overlapping binding site with PIP₂.

Discussion

Although TRPA1 does not necessarily require PIP₂ for function [34], a number of studies have shown that phosphoinositides are involved in TRPA1 regulation under different conditions [10, 29, 35, 36] and a number putative sites of interaction have been proposed [15, 18, 25, 36]. Structural studies [17], together with our results, support the possibility that PIP₂ can regulate TRPA1 either from the inner vestibule of the S1-S4 sensor domain or through a lateral fenestration between two adjacent subunits. Generally, proteins bind phosphoinositides mainly through electrostatic interactions of their positively charged residues and contributing

nearby hydrophobic residues. Previous predictions for mouse TRPA1 protein sequence pointed to several prototypical PIP₂-interacting (pleckstrin homology) domains located in the N-terminus (R46-K56, K443-K448, K592-R605) and the distal C-terminus (K1092-R1097)[36]; based on later structural [17] and mutagenesis [18, 23] studies, the latter region cannot be excluded. Another bioinformatics approach was used by Witschas et al. [15] who mapped the C-terminus of human TRPA1 for prediction of antibacterial activity with AntiBP Server (<http://crdd.osdd.net/raghava/antibp/index.html>). Antibacterial peptides are capable of distinguishing bacterial from mammalian cells based on the differences in plasma membrane composition due to their significant affinity for negatively charged phospholipids [37, 38]. Prediction results identified a high propensity for membrane-active region within the most proximal C-terminal region (later recognized as TRP-like domain) and subsequent biophysical studies indeed confirmed a strong interaction of the corresponding peptide I964-L992 with membranes containing anionic lipids [15]. This assay was complementary to previous electrophysiological studies that identified several positively charged residues within this region (K969, R975, K988 and K989) as critically involved in TRPA1 channel gating [18]. The mapping of antibacterial peptides within the C-terminus predicted also another sequence, with even the highest score: F1017-Q1031. This region has a net charge of zero and contains only one basic residue. Thus, the predicted interaction with phosphoinositides should be predominantly facilitated by hydrophobic/aromatic residues. In this context, it is noteworthy that a missense mutation of a histidine residue two amino acids upstream of F1020 was recently found to be associated with childhood asthma [39]. It could be envisaged that the histidine-to-arginine mutation (rs959976) increases the affinity of PIP₂ to TRPA1 and, thus, influences the gating of the channel at physiological membrane potentials.

We demonstrate that PIP₂ is essential for the interaction of the peptide comprising the above region (T1003-P1034) with membrane lipids. The binding is strong and superficial, inducing only small perturbation in the lipid ordering. By looking at the published structure of TRPA1 [17] and integrating our current knowledge about the interaction capacity of specific TRPA1 peptides with PIP₂, it is tempting to speculate that there might exist at least two sites with separate functions from which PIP₂ can regulate the channel activity. The first, formed by the inner cavity of the sensor and contributed by K989 from the TRP-like domain, and the second, explored within this study, localized between adjacent subunits, capable of directly affecting gating of the channel through the S4-S5 linker and R975 from the TRP-like domain. This scenario also predicts that the effectiveness of PIP₂ in inhibiting or promoting the activity

of TRPA1 could strictly depend on conformational states of the channel. Our previous mutagenesis study demonstrated that neutralization of either K989 or R975 from the TRP-like domain produced gain of function phenotypes with strong outward rectification upon stimulation by depolarizing pulses. In the presence of a high concentration of agonist (200 μM allyl isothiocyanate), the gating of K989A became voltage-independent whereas the gating of R975A remained strongly voltage-dependent and outwardly rectifying [18]. These experiments were performed in the presence of 1mM concentration of external Ca^{2+} and, thus, we cannot distinguish which of the reported effects may result exclusively from PIP_2 interaction. Given the recently revealed critical and dual role of Ca^{2+} /calmodulin in the regulation of TRPA1 [8], we could only hypothesize that PIP_2 may act in concert with Ca^{2+} and calmodulin to regulate the channel. Notably, the R975A mutant characterized in [18] and the S972D mutant studied here (Fig. 6) could have similar impact on the voltage-sensitivity of the channel due to disrupted contacts with E1032 and I1033 (residues included in the region explored in this study). In any case, our data demonstrate that the peptide corresponding to the calmodulin binding site (L992-N1008) and the peptide overlapping with this site (T1003-P1034) are capable of binding PIP_2 and their affinities are decreased in the presence of Ca^{2+} . Under what physiological circumstances might the PIP_2 -dependent regulation of TRPA1 be relevant? TRPA1 is extensively co-expressed with TRPV1 in sensory neurons and these channels form a complex and functionally interact [29, 40]. Future studies should focus on establishing the physiological relevance of our proposed mechanism (Fig. 8B) in various nociceptive neurons and, particularly, in relation to the TRPV1 expression pattern. Our data demonstrate that lowering the concentration of Ca^{2+} below basal levels increases the apparent affinity of the channel to non-electrophilic agonist carvacrol (Fig. 8A and C). It can thus be imagined that PIP_2 competes with Ca^{2+} /calmodulin for binding at TRPA1 under basal conditions. Because the Ca^{2+} /calmodulin complex primes the channel for activation under weakly activating conditions and inactivates it at higher Ca^{2+} concentrations [8], the effects of PIP_2 should be consequentially complex and Ca^{2+} -dependent. For that matter, a similar degree of complexity of regulation by PIP_2 has been demonstrated (after nearly 15 years of intensive research) for related channel TRPV1 [41, 42].

Materials and Methods

Cell culture, constructs and transfection

Human embryonic kidney 293T (HEK293T, CRL-3216; ATCC, USA) cells were cultured in Opti-MEM I media (Invitrogen, CA, USA) supplemented with 5% fetal bovine serum. The magnet-assisted transfection (IBA GmbH, Germany) technique was used to transiently co-transfect the cells in a 15.6 mm well on a 24-well plate with 200 ng of GFP plasmid (TaKaRa, Japan) and with 300 ng of cDNA plasmid encoding wild-type or mutant human TRPA1 (pCMV6-XL4 vector, OriGene Technologies, Rockville, MD). The cells were used 24–48 h after transfection. At least two independent transfections were used for each experimental group. The wild-type channel was regularly tested in the same batch as the mutant. The mutants were generated by PCR using a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, CA, USA) and confirmed by DNA sequencing (GATC Biotech, Germany). F11 cells (The European Collection of Authenticated Cell Cultures, ECACC 08062601, Porton Down, UK) cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal bovine serum were passaged once a week using trypsin-EDTA (Invitrogen, CA, USA) and grown under 5% CO₂ at 37°C. One to two days before transfection, cells were plated in 24-well plates (2x10⁵ cells per well) in 0.5 ml of medium and became confluent on the day of transfection. The cells were transiently co-transfected with 300 ng of cDNA plasmid encoding human TRPA1 (in the pCMV6-XL4 vector, OriGene Technologies, Rockville, MD) and with 200 ng of GFP plasmid (TaKaRa, Japan) with the use of Lipofectamin 2000 (Invitrogen, CA, USA) and then plated on poly-L-lysine-coated glass coverslips.

Electrophysiology

Whole-cell membrane currents were recorded by employing an Axopatch 200B amplifier and pCLAMP10 software (Molecular Devices). Patch electrodes were pulled from borosilicate glass and heat-polished to a final resistance between 3 and 5 MΩ. Series resistance was compensated by at least 70% in all recordings. The experiments were performed at room temperature (23–25 °C). Only one recording was performed on any one coverslip of cells to ensure that recordings were made from cells not previously exposed to chemical stimuli. A system for rapid superfusion of the cultured cells was used for drug

application [43]. For HEK293T cell recordings, the extracellular bath solutions contained: 150 mM NaCl and 10 mM HEPES, with an added 2 mM HEDTA for the Ca²⁺-free solution, and 2 mM CaCl₂ for the Ca²⁺-containing solutions, adjusted to pH 7.3 with NaOH, 300 mOsm. The current to voltage (*I/V*) relationships were measured in control bath solution containing 160 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 10 mM glucose, adjusted to pH 7.3 and 320 mOsm, or in the Ca²⁺-free solution. The *I/V* relationships were recorded using 100 ms voltage steps from -80 mV to +200 mV (increment +20 mV), holding potential -70 mV, recorded in control extracellular solution ~1 min after whole-cell formation. The internal pipette solution contained 145 mM CsCl, 5 mM EGTA, 3 mM CaCl₂, 10 mM HEPES and 2 mM MgATP, adjusted with CsOH to pH 7.3 and 290 mOsm. The agonist sensitivity was tested with a protocol described previously [25] where the membrane potential was ramped every second from -80 mV to +80 mV (1 V/s). For F11 cell recordings, the extracellular bath solution (nominal 0 Ca²⁺) contained: 140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM glucose, pH 7.4 adjusted with NaOH. Internal pipette solution contained 140 mM KCl, 2 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, pH 7.4 adjusted with KOH. AITC, cinnamaldehyde and carvacrol solutions were prepared prior to use from a 0.1 M stock solution in Me₂SO. To study the effects of peptides on TRPA1-mediated currents in F11 cells, peptides were included in the pipette. All of the chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

Preparation of small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) and polymer-based nanodiscs (SMALPs)

Liposomes were prepared by dissolving the appropriate amount of lipid into chloroform or a mixture of chloroform and methanol 2/1 (v/v), followed by solvent evaporation under nitrogen to deposit the lipid as a film on the wall of a test tube. Final traces of solvent were removed in a vacuum chamber attached to a liquid nitrogen trap during 2-3 h. Dried lipid films were hydrated with TBS buffer, pH 7.6 or with 10 mM phosphate buffer, pH 7.6 (for CD experiments) and thoroughly vortexed at a temperature superior to the phase transition temperature of the lipid to obtain multilamellar vesicles (MLVs). To better mimic biological conditions, the peptide was added to the lipids after vesicle/liposome formation. To form LUVs, the MLV dispersion was subjected to six freeze/thawing cycles and passed 11 times

through a mini-extruder equipped with two stacked 0.1 μm polycarbonate filters (Avanti, Alabaster, AL, USA). For forming SUVs, MLV were tip sonicated on ice, amplitude 40, time 2x 10 min.

SMALPs were prepared by incubating MLVs (1.3 mM) with SMA solution (6.5 mM) both in PBS. To ensure complete solubilisation of the MLVs, a ratio of 0.5 ($[\text{SMA}]/[\text{lipid}]$) was used that is above the reported R_{sol} value (ratio above which the liposome solubilisation is complete) for POPC liposomes [44]. Formation of SMALPs was followed by Dynamic Light Scattering (Vasco, Cordouan) and also by MDS (microfluidic diffusional sizing; in this case we have included 10% POPS in the lipid system in order to have a reactive primary amine for fluorescent labeling). SMALP formation occurred promptly after the mixing and they remained stable for a couple of weeks. SMALPs of about 10-15 nm in size were obtained by this procedure. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The peptides (TIVYPNKPRSGGMLFHIFCFLFCTGEIRQEIP, herein named T1003-P1034 and LWFLRKVDQKSTIVYPN, herein named L992-N1008) were purchased from Biomatik (Cambridge ON, Canada) and used without further purification.

Liposome leakage

Sulphorhodamine B-containing LUVs were prepared using the same protocol used to make regular LUVs, except for the hydration step made with 0.5xTBS buffer of the lipid films, which contained 50 mM Sulphorhodamine B. Free rhodamine was separated from rhodamine-containing LUVs using size exclusion column chromatography (Sephadex G-75) with TBS as elution buffer. The concentration of lipids was estimated using Rouser protocol [45]. For the assay, the lipid concentration was set at 1 μM and peptide concentration varied from 1 nM to 500 nM (P/L ratio of 1/100 to 1/10 respectively). All measurements were performed with a BMG Labtech plate reader. Data were collected every 1 s at room temperature using a $\lambda_{\text{exc}} = 565$ nm and $\lambda_{\text{em}} = 586$ nm with an emission and excitation slit of 2.5 nm in a cuvette of 2 mL. The fluorescence from rhodamine at 50 mM concentration was low due to self-quenching, but increased considerably upon dilution. The fluorescence intensity at equilibrium was measured after 60 min. At the end of the assay, complete leakage of LUVs was achieved by adding 100 μL of 10% Triton X-100 solution dissolving the lipid membrane without interfering with the fluorescence signal. The percentage of rhodamine release was calculated according to the following equation:

$$\% \text{ leakage} = (F_t - F_0) / (F_f - F_0) * 100 \quad (1),$$

where the percentage of leakage is the fraction of dye released (normalized membrane leakage), F_t is the measured fluorescence intensity at time t , and F_0 and F_f are the fluorescence intensities at times $t=0$, and after final addition of Triton X-100, respectively. A dilution correction was applied to the fluorescence intensity after injection of the Triton X-100.

Circular dichroism (CD)

CD data was recorded on a Jasco J-815 CD spectrophotometer with a 1 mm path length. Far-UV spectra were recorded from 185 or 195 to 250 nm with a 0.5 nm step resolution and a 1 nm bandwidth at 300 K. The scan speed was 50 nm/min (1 s response time), and the spectra were averaged over 7 scans. CD spectra were collected for peptide samples in a 10 mM phosphate buffer at concentrations varying from 20 to 120 μM for the peptide alone and in presence of the lipid at concentrations of 1220 μM (20% PIP_2 + 80% POPC), 1290 μM (5% PIP_2 + 95% POPC) and 1320 μM (POPC) (P/L ratios investigated ranged from 1/50 to 1/10). For each sample, the background (buffer or liposome/micelle in absence of peptide) was automatically subtracted from the signal. Data were evaluated using BeStSel.org database [46].

Plasmon waveguide resonance (PWR) spectroscopy

PWR experiments were performed on a homemade PWR instrument that had a spectral angular resolution of ≥ 0.5 mdeg. Resonances can be obtained with light whose electric vector is either parallel (s-polarization) or perpendicular (p-polarization) to the plane of the resonator surface. The principles behind the technique and information obtained have been reported elsewhere (see *e.g.* [20, 47, 48]). The sample to be analyzed (a lipid bilayer membrane) was immobilized on the resonator surface and placed in contact with an aqueous medium (TBS buffer, pH 7.6), into which the peptides were then introduced. The solid-supported lipid bilayer was made by incubation of SUVs of lipids with the sensor surface that leads to the spontaneous burst of the vesicles in the silica surface to form a bilayer (further details can be found in [49]). After bilayer formation, a peptide was incrementally added to the cell sample compartment and the spectral changes monitored with both polarizations. Affinities between

the peptide and the lipids were obtained by plotting the PWR spectral changes that occur upon incremental additions of ligand to the cell. Data fitting (GraphPad Prism) through a hyperbolic saturation curve provides the apparent dissociation constants.

Attenuated total reflexion infrared spectroscopy (ATR-FTIR)

ATR-FTIR spectra were recorded on a Nicolet 6700 FT-IR spectrometer (Nicolet Instrument, Madison, WI, USA) equipped with a liquid nitrogen cooled mercury–cadmium–telluride detector (ThermoFisher Scientific, San Jose, CA, USA), with a spectral resolution of 4 cm^{-1} and a one-level zero filling. Lipid bilayers adsorbed on a germanium ATR crystal were obtained by spontaneous fusion of small unilamellar vesicles (SUVs) of pure POPC or a mixture of POPC with PIP₂. The total lipid concentration used was 1.5 mg/mL (ca. 1.96 mM). SUV were prepared by tip sonication for 30 min after direct hydration of lipid films by TBS buffer. TBS was used instead of PBS to avoid phosphate contribution from the buffer. After bilayer formation at the crystal surface, the excess of SUV was removed by a TBS buffer. Since ATR spectroscopy is sensitive to the orientation of the lipid and peptide [50], spectra were recorded with a parallel (p) and perpendicular (s) polarization of the incident light. All the orientation information is then contained in the dichroic ratio $R_{\text{ATR}} = A_p/A_s$, where A_p and A_s represent the absorbance of the considered band for the p or s polarization of the incident light, respectively (for more details see [19]). A peptide at a concentration of 1 mM was incubated for 10 min with the bilayer, the non-interacting peptide was removed by washing three times with TBS buffer.

Microfluidic Diffusional Sizing (MDS)

MDS was performed using the Fluidity One instrument developed by Fluidic Analytics. This newly developed and very sensitive method provides information about sample concentration and size by measuring the rate of diffusion of proteins. Streams of a protein sample and an auxiliary fluid are run in parallel under steady-state laminar flow, with no convective mixing. Consequently, the only way the protein can migrate from the sample into the auxiliary fluid is by diffusion. This is the measurement step in the Fluidity One system, and occurs while the proteins are in their native state, before a label is introduced. Following measurement, the

streams are split and the analytes are labelled via primary amines present in the peptides and lipids (in case of phosphatidylserine or phosphatidylethanolamine lipid headgroups). The hydrodynamic radius of the vesicles is directly related to their diffusion coefficient, D , through the Stokes–Einstein equation

$$D = K_B T / \pi \eta R_h \quad (2),$$

where K_B is the Boltzmann constant, T is the temperature, and η is the viscosity of the buffer used for co-flow and for suspending the sample. Herein we have used this method to follow the interaction of the two peptides with SMALPs composed of POPC and POPC/PIP₂ (9/1). By titrating the peptides (450 $\mu\text{g/mL}$) with increasing concentration of lipids and measuring the size at each point, a binding curve can be obtained based on the size changes occurring upon the peptide/lipid interaction. From the binding curve, the dissociation constant can be obtained by fitting the data with a hyperbolic binding equation (Prism, GraphPad Software, CA, USA). To note that the peptides alone have a size of about 1.5-2 nm and the SMALPs have a size of about 10 nm.

Data analysis, statistics and modeling

All of the electrophysiological data were analyzed using pCLAMP10 (Molecular Devices), and curve fitting and statistical analyses were done in SigmaPlot 10 (Systat Software Inc.) or Prism (GraphPad Software). Conductance-voltage (G - V) relationships were obtained from steady-state whole cell currents measured at the end of voltage steps from -80 to +200 mV in increments of +20 mV. Voltage-dependent gating parameters were estimated by fitting the conductance $G = I/(V - V_{\text{rev}})$ as a function of the test potential V to the Boltzmann equation:

$$G = [(G_{\text{max}} - G_{\text{min}})/(1 + \exp(-zF(V - V_{50})/RT))] + G_{\text{min}} \quad (3),$$

where z is the apparent number of gating charges, V_{50} is the half-activation voltage, G_{min} and G_{max} are the minimum and maximum whole-cell conductance, V_{rev} is the reversal potential, and F , R , and T have their usual thermodynamic meanings. Statistical significance was determined by Student's t -test or the analysis of variance, as appropriate; differences were considered significant at $P < 0.05$ where not stated otherwise. The data are presented as means \pm SEM.

Activation time constants (τ_{fast} and τ_{slow}) were measured directly from outward currents by performing a double exponential Chebyshev fit to the rising phase of the activating currents. Fast and slow deactivation was characterized by a single or a double

exponential fit to the current upon repolarization from +200 mV to -70 mV. Initial 1.3ms interval was ignored. Because the relative weights of fast and slow deactivation components differed between the cells tested, we calculated a weighted, average time constant of the fast and slow components of activation/deactivation based on the amplitude of each component.

$$\tau_w = A_{\text{fast}}/(A_{\text{fast}} + A_{\text{slow}}) \times \tau_{\text{fast}} + A_{\text{slow}}/(A_{\text{fast}} + A_{\text{slow}}) \times \tau_{\text{slow}} \quad (4).$$

Fitting to an allosteric model was performed with COPASI 4.22 [51]. Molecular docking of PIP₂ into the TRPA1 sensor domain was performed as described previously [25] with the program AutoDoc Vina integrated in UCSF Chimera 1.12 software using a cubic box of 100×100×100 Å³ with default docking parameters.

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Tables

Table 1. Binding affinity (in nM) for the peptides L992-N1008 and T1003-P1034 for SMALPs composed of POPC and POPC/PIP₂ (9/1 mol/mol) in absence and presence of 2 mM Ca²⁺ obtained by MDS. Data are presented as mean \pm S.D. ($n = 3$).

LIPID/PEPTIDE	L992-N1008	T1003-P1034
POPC	No binding	No binding
POPC/PIP ₂ (9/1 mol/mol) no Ca ²⁺	330 \pm 68	685 \pm 114
POPC/PIP ₂ (9/1 mol/mol) with Ca ²⁺	425 \pm 95	720 \pm 123

Table 2. Rhodamine leakage induced by the peptides L992-N1008 and T1003-P1034 in POPC and POPC/PIP₂ (9.5/0.5 mol/mol) liposomes encapsulating rhodamine. Experiments have been performed 2 times.

Rhodamine leakage				
P/L ratio	T1003-P1034		L992-L1008	
	100% POPC	5% PIP ₂ + 95% POPC	100% POPC	5% PIP ₂ + 95% POPC
1/100	2.5 \pm 0.3	0	2.4 \pm 0.6	0
1/50	1.3 \pm 0.2	0	1.6 \pm 0.4	0
1/25	2.9 \pm 0.3	0	1.9 \pm 0.2	0
1/10	2.5 \pm 0.4	0	2.5 \pm 0.3	0

Table 3. Secondary structure content of the peptides L992-N1008 and T1003-P1034 in buffer and in contact with POPC, POPC/PIP₂ (5% and 20% of PIP₂ were used). Secondary structure content was calculated using BeStSel software as indicated in Materials and Methods.

Secondary structure	L992-N1008				T1003-T1034			
	Buffer	POPC	5% PIP ₂	20% PIP ₂	Buffer	POPC	5% PIP ₂	20% PIP ₂
α -helix	6.4	3.1	6.9	6.9	1.0	0.9	1.1	0.8
Antiparallel β -sheet	30.2	27.5	26.2	30.1	33.9	34.2	33	31.5
Parallel β -sheet	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Turn	15.3	15.4	15.3	15.3	16.5	16.1	16.4	17.0
Random Coil	48.1	54.1	51.7	47.7	48.6	48.9	49.5	50.8

Table 4. Parameters of allosteric model fitted to experimental data obtained in the presence or absence of external Ca^{2+} . The data are presented as means \pm SEM.

Construct/ $[\text{Ca}^{2+}]_o$	D	L	J_0	n
TRPA1/ 1 mM	$29,562 \pm 3,571$	0.0200 ± 0.0027	$9.1 \pm 2.3 \times 10^{-5}$	10
F1020G/ 1 mM	$3,967 \pm 454$	0.0172 ± 0.0031	$2.2 \pm 0.4 \times 10^{-4}$	9
TRPA1/ 0 mM	$41,663 \pm 3,231$	0.0210 ± 0.0029	$9.5 \pm 2.9 \times 10^{-5}$	8
F1020G/ 0 mM	$5,897 \pm 899$	0.0108 ± 0.0023	$8.8 \pm 1.1 \times 10^{-4}$	11

Figure Legends

Fig. 1. Schematic representation of TRPA1 receptor showing regions predicted to interact with membrane lipids. (A) Schematic illustration of human TRPA1 subunit with depicted position of a poorly resolved flexible linker connecting the TRP-like domain with the C-terminal coiled-coil linker region (colored region containing the calmodulin binding domain in green, and an overlapping violet region containing predicted short α -helix). Below, the sequences of the peptides used in this study. (B) Amino acid sequence conservation within the linker of TRPA1 proteins represented as a sequence logo generated from 65 sequences of vertebrate TRPA1 by WebLogo server [52]. The height of the amino acid at each position indicates its probability of occurrence at that position. F1020 in the middle of the short helix (indicated by asterisk) is highly conserved.

Fig. 2. Interaction of the L992-N1008 and T1003-T1034 peptides with model membranes composed of POPC and POPC/PIP₂. (A) PWR resonance position shifts obtained in the p- (blue circles) and s- (green squares) polarization and total internal reflection angle (TIR; black triangles) upon incremental addition of peptide to a planar model membrane composed of POPC and POPC/PIP₂ (95/5 mol/mol). No binding affinities were determined due to the rather small shifts relative to the instrument limits (see text for details). (B) Average radius of the indicated peptides upon titration with increasing concentrations of SMALPs composed of POPC (triangles and squares) and POPC/PIP₂ (9/1 mol/mol) (circles). For experiments performed with POPC/PIP₂, data are presented as mean \pm S.D. ($n = 3$). Binding affinities are presented in Table 1. (C) Secondary peptide structure determined by CD for the two peptides alone in buffer (black) and upon incubation with POPC (blue) and POPC/PIP₂ at 5% (red) and 20% (green) liposomes at P/L ratios of 1/10. Secondary structure content has been calculated and presented in Table 2. (D) Polarized ATR-FTIR spectra obtained with the p- (left panel) and s- (right panel) polarized light of a solid-supported lipid membrane composed of POPC/PIP₂ (8/2 mol/mol) in absence (black) and presence (red) of L992-N1008 peptide.

Fig. 3. Mutation F1020G affects chemical- and voltage-induced gating of TRPA1. (A) Average whole-cell currents induced by 100 μ M cinnamaldehyde (CA) in Ca²⁺-free solution and then exposed to 2 mM Ca²⁺, measured at +80 mV and -80 mV in wild-type TRPA1 (WT).

The membrane potential was linearly ramped up each second from -80 mV to +80 mV (1 V/s) (inset). Grey bars indicate S.E.M. (number of cells; $n = 10$). In some cases, the error bars are smaller than the symbol. (B) Average currents recorded from 13 HEK293T cells expressing the F1020G mutant channels. The average current for the WT is overlaid as a gray dashed line for comparison. (C) and (D) Current-voltage relationships of traces measured at times indicated by arrows in panels A and B for wild-type (black arrow) and indicated mutant (green arrow). (E) and (F) Average rectification of currents shown in A and B, plotted as a function of time. Colored lines with gray bars indicate average \pm S.E.M.. The average rectification of currents through the WT is overlaid as a gray dashed line for comparison.

Fig. 4. Mutation F1020G affects the voltage-dependence of activation. (A) and (B) Representative current traces in response to voltage step protocol indicated above (holding potential -70 mV; 100-ms voltage steps from -80 mV to +200 mV; increment +20 mV) recorded A in extracellular control solution containing 1 mM Ca^{2+} , or B in Ca^{2+} -free extracellular solution. (C) and (D) Average conductances obtained from voltage step protocols as in A and B. Data represent the means \pm S.E.M. (number of cells n indicated in parentheses). The solid lines represent the best fit to a Boltzmann function as described in Materials and Methods. (E) and (F) Activation and deactivation kinetics of TRPA1 and the F1020G mutant. Parameters of the time course of currents elicited by a depolarizing step from -80 mV to +200 mV in extracellular control solution containing 1 mM Ca^{2+} (E left) or in Ca^{2+} -free extracellular solution (F left) was fitted by one or two exponential terms and the weighted time constant (τ_w) was used for statistics. Similarly, the deactivation time constant was obtained from tail currents elicited by stepping from +200 mV to -70 mV (E right and F right). Data are represented as vertical point plots and summary box plots. Boxes encompass the 25th through 75th percentile of the data, the horizontal bar represents the median, and whiskers extend to the 10th and 90th percentile of the data. Number of cells is indicated in panels C and D. The Mann-Whitney Rank Sum Test was used to determine whether there is a statistically significant difference between F1020G and wild-type channels (P values are indicated).

Fig. 5. Docking and functional studies indicate importance of the putative PIP_2 -interacting region. (A) Cryo-EM density with superimposed model of TRPA1 with the completed intracellular loop connecting helices S2 and S3, modeled as described in [25]. The

intracellular side of the sensor cavity has been proposed by Paulsen *et. al.* (2015) to form a putative PIP₂ binding pocket. The negatively charged inositol triphosphate head group of PIP₂ may adopt several different conformations within the sensor domain. The lowest-energy configurations of PIP₂ in the binding pocket obtained by ligand docking experiments are shown. Density map showing the location of a poorly resolved flexible loop between Y1006 and Q1031 with the schematically indicated density map for the α -helix (violet) in the inner membrane leaflet. (B) The loop may contact the sensor domain through stacking interactions with the side chains of F716, F853, and H719. Approximate position of the putative α -helix within the flexible loop is schematically shown (transparent violet). The close proximity of I1033 from the linker and S972 from the TRP-like helix suggests the possibility of a direct impact of the putative PIP₂-interacting region on channel gating. The lower gate of the channel is formed by I957 and V961, indicated in orange. (C) A detailed view from A showing the putative interaction site for PIP₂ centered above the TRP-helix. (D) Time course of average whole-cell currents induced by 100 μ M allyl isothiocyanate (AITC) in Ca²⁺-free solution and then exposed to 2 mM Ca²⁺, obtained from wild-type and indicated mutants according to the voltage-ramp protocol depicted as an inset, measured at +80 mV (open circles with gray bars indicating S.E.M.; *n* indicated in brackets). In some cases, the error bars are smaller than the symbol. The application of AITC and subsequent addition of 2 mM Ca²⁺ are indicated above.

Fig. 6. Mutations at S972 affect chemical- and voltage-induced gating of TRPA1. (A) Average whole-cell currents induced by 100 μ M cinnamaldehyde (CA) in Ca²⁺-free solution and then exposed to 2 mM Ca²⁺, measured at +80 mV and -80 mV in S972A mutant. The membrane potential was linearly ramped up each second from -80 mV to +80 mV (1 V/s) (inset). Grey bars indicate S.E.M. (*n* = 4). In some cases, the error bars are smaller than the symbol. The average current for the WT is overlaid as a gray dashed line for comparison. (B) Average currents (orange squares) and individual traces (light orange lines) recorded from seven HEK293T cells expressing the S972D mutant channels. The average current for the WT is overlaid as a gray dashed line for comparison. Note the desynchronized responses to 2 mM Ca²⁺, most likely due to a low activity at negative potentials that does not permit Ca²⁺ to permeate and modulate the channel from the intracellular side. Number of cells is indicated in brackets for each construct. (C) and (D) Current-voltage relationships of traces measured at times indicated by arrows in panels A and B for S972A (green arrow) and S972D (orange

arrow). (E) and (F) Average rectification of currents shown in panels A and B plotted as a function of time. Colored lines with gray bars indicate average \pm S.E.M.. The average rectification of currents through the WT is overlaid as a gray dashed line for comparison.

Fig. 7. Allosteric model reproduces voltage-dependent activation of TRPA1. (A) and (B) Average normalized conductances obtained as explained in Fig. 4, in the presence or absence of external Ca^{2+} , plotted on a semi-logarithmic scale. Data represent the means \pm S.E.M. (n indicated in Fig. 4C and D). The solid and dashed lines represent the best fit to a Boltzmann function as described in Materials and Methods. (C) The time course of the activation phase of normalized currents measured for a representative cell expressing the wild-type TRPA1, induced by depolarization to +200 mV. (D) The normalized steady-state G - V data simultaneously fitted using a simple allosteric model (shown as inset in C). (E) and (F) The same as in C and D for the mutant F1020G. (G) Bar graph summarizing the results from allosteric model fitted to experimental data obtained for wild-type TRPA1 and F1020G in the presence (white and light green bars; $n = 10$ and 9) or absence (gray and dark green bars; $n = 8$ and 11) of external Ca^{2+} . Asterisk indicates a statistically significant difference from the first bar (Student's t -test; $P < 0.05$). The data are presented as means \pm S.E.M..

Fig. 8. Putative interaction site for membrane phosphoinositides may compete with Ca^{2+} /calmodulin complex. (A) Representative recordings from F11 cells expressing human TRPA1. The cells were exposed to 100 μM carvacrol in absence (10 s) and presence (10 s) of extracellular Ca^{2+} (500 μM) either 30-50 s after whole-cell formation (left), after 5-6min perfusion with internal solution (middle), or after 5-6min perfusion with internal solution containing indicated peptide (right). For the L992-N1008 control peptide, three similar recordings as shown in right panel were measured (not statistically evaluated). (B) Proposed mechanism for regulation of human TRPA1 by Ca^{2+} /calmodulin and PIP_2 . Under basal concentrations of Ca^{2+} , the channels bind Ca^{2+} /calmodulin (left). Chelation of Ca^{2+} upon 5-6min perfusion with internal solution releases calmodulin from TRPA1, enables PIP_2 to bind at the same site and promote the channel activation by carvacrol (middle). When the specific peptide is included in the pipette (5-6min perfusion), binding of PIP_2 increases and Ca^{2+} dependent potentiation is reduced because of the dominant-negative effect of peptide (right). (C) Bar and all point plot summary of time constant (τ_{on}) of TRPA1 activation by 100 μM

carvacrol applied in the absence of Ca^{2+} , obtained by monoexponential fit from experiments similar to those in (A; gray dotted line). Significance (P) tested using Student's paired t -test is indicated above the bars. The number of experiments is given in parentheses. (D) Summary of TRPA1 potentiation induced by $500 \mu\text{M}$ Ca^{2+} in experiments similar to those in (A). The data in panels C and D are presented as means \pm S.E.M..

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Author Contributions

VV and LM conceived the project; VV and IDA designed the experiments; LM, VS, AK, AC and IDA performed experiments; LM, VS, SV, JF, SL, IDA and VV analyzed data; VV and IDA wrote the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.