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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Ligustilide: a novel TRPA1 modulator

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Abstract TRPA1 is activated by electrophilic compounds such as mustard oil (MO). Here, we demonstrate a bimodal sensitivity of TRPA1 to ligustilide (Lig), an electrophilic volatile dihydrophthalide of dietary and medicinal relevance. Lig is a potent TRPA1 activator and is also capable to induce a modest block of MO activated currents. Aromatization to dehydroligustilide (DH-Lig), as occurs during aging of its botanical sources, reversed this profile, enhancing TRPA1 inhibition and reducing activation. Mutation of the reactive cysteines in mouseTRPA1 (C622S, C642S, C666S) dramatically reduced activation by MO and significantly reduced that by Lig, but had an almost negligible effect on the action of DH-Lig, whose activation mechanism of TRPA1 is therefore largely independent from the alkylation of cysteine residues. Taken together, these observations show that the phthalide structural motif is a versatile platform to investigate the modulation of TRPA1 by small molecules, being tunable in terms of activation/inhibition profile and mechanism of interaction. Finally, the action of Lig on TRPA1 may contribute to the gustatory effects of celery, its major dietary source, and to the pharmacological action of important plants from the Chinese and native American traditional medicines.

Keywords TRP channels · TRPA1 · Chemosensation · Electrophiles · Mustard oil · Traditional Chinese medicine

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Introduction

TRPA1 is robustly activated by the pungent constituents of mustard oil (MO), cinnamon and garlic, and mediates the inflammatory actions of environmental irritants and proalgesic agents, paradoxically mediating also their analgesic responses. Ligustilide (Lig) is a major constituent of the aroma of celery (*Apium graveolens* L.) [23] and lovage (*Levisticum officinale* L.) [7], and is contained in high concentrations in popular medicinal plants from the folk medicine of China [dong-quai (*Angelica sinensis* (Oliv.) Dill.) [12], chuanxiong (*Ligusticum chuanxiong* Hort.) [15]] and North America (oshá, *Ligusticum portieri* Coult. & Rose) [15] (Fig. 1). Given the dietary and the medicinal relevance of Lig, its biological activity has been extensively investigated, evidencing a remarkably pleiotropic profile, that involves vasodilatation [4], a decrease of platelet aggregation [27], analgesic effects [6], attenuation of lipopolysaccharide (LPS)-induced proinflammatory response and endotoxic shock [21, 24], neuroprotection [25], and anti-proliferation of smooth muscle cells [13]. Of special interest is the remarkably fast brain bioavailability of Lig by nasal administration, with detectable cerebral concentrations within 20 min after nasal exposure [8, 9], an interesting observation because the biological profile of Lig

has several potential applications in the realm of brain diseases [18, 25]. Despite this burgeoning biomedical literature, very little is known on the molecular target(s) of Lig. Lig is a reactive compound, easily aromatized to dehydroligustilide (DH-Lig) by air oxidation [20], and capable to trap thiol groups in a complex, multiple, and reversible fashion [3]. The unique electrophilic behavior of Lig, its dietary relevance, and its potential clinical relevance for various realms of neurosciences [18, 25], provided a rationale for investigating its action on TRPA1, an ion channel that, due to its expression in the oral and nasal cavity and its sensitivity to electrophiles, might represent a target for this compound [22]. In this work, we demonstrate that Lig is a potent activator of TRPA1 with only a modest inhibitory activity, that this profile of activity is reversed by aromatization to DH-Lig, a potent blocker of MO-activated currents, and that these compounds, despite their structural similarity, have a different mechanism of interaction with TRPA1.

Materials and methods

CHO cell culture

We used a tetracycline-regulated system for inducible expression of mouse TRPA1 in CHO cells. To induce expression of TRPA1, 0.5 μg/ml tetracycline was added to the culture medium, and cells were used 2–3 h after induction [22]. In some experiments, naive CHO cells were transiently transfected with TRPA1 mutant clones in the bicistronic pCAGGS-IRES-GFP vector using TransIT-293 transfection reagent (Mirus). Mutations in *TRPA1* were introduced using the QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene). The nucleotide sequences of the mutants were verified by sequence analysis of the corresponding cDNAs.

Electrophysiology

For electrophysiological recordings in the whole-cell mode of the patch-clamp technique, CHO cells were maintained in an extracellular recording solution containing (in mM) 150 NaCl, 5 $MgCl_2$, 10 Glucose, and 10 *N*-(hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), buffered at pH 7.4 with NaOH. The intracellular standard pipette solution contained (in mM): 100 aspartic acid, 40 KCl, 5 EGTA, 4 ATP, 2 $MgCl_2$, 1.2 $CaCl_2$, and 10 HEPES, buffered at pH 7.2 with KOH. Patch-clamp electrodes were pulled from Vitrex capillary tubes (Modulohm, Herlev, Denmark) on a DMZ-Universal puller (Zeitz Instruments, Augsburg, Germany). When filled with pipette solution, they showed a DC resistance between 3 and 5 MΩ for whole-cell recording. An Ag-AgCl wire was used as reference electrode. Ag-AgCl electrodes of sintered pellets (IVM Systems, Healdsburg, CA, USA) were used to avoid contamination of the bath and pipette solutions. Membrane currents were recorded using an EPC-10 patch-clamp amplifier (List Electronic, Lambrecht/Pfalz, Germany). For control of voltage-clamp protocols and data acquisition, we used the PATCHMASTER software (HEKA Elektronik, Germany) run on an IBM-compatible PC. For whole-cell analysis, data were recorded at 5 kHz and filtered at 2.9 kHz with PATCHMASTER software. The cell capacitance and series resistance were assessed using the analog compensation circuit of the EPC-10 amplifier. Generally, between 50% and 80% of the series resistance was electronically compensated to minimize voltage errors. Experiments were performed at room temperature (20–25°C).

Reagents

Mustard oil (allyl isothiocyanate [AITC], MO) was prepared from ethanol based 100 mM stock solutions that were kept for not longer than 1 month at −20°C. Otherwise, fresh dilutions (1 mM−10 mM in test solutions) were prepared daily from a 10 M MO stock that was kept at 4°C. All products were purchased from Sigma (Bornem, Belgium). Lig and DH-Lig were isolated from the essential oil of *L. chuanxiong* Hort. by gravity column chromatography [8]. All compounds were added from these stock solutions to the extracellular recording solution. The final ethanol concentration in recording solutions was less than 0.1%. In situ trapping experiments with cysteamine were carried out in DMSO-d_6, reacting Lig and DH-Lig with a two-fold excess of cysteamine [2].

Data analysis

Statistical analysis and graphical presentations were performed using the Origin ver. 7.0 software (OriginLab, Northampton, MA, USA). Significance was tested with analysis of variance (ANOVA) and Student's unpaired t-test. All data are presented as mean ± SEM.

Results

Ligustilide and dehydroligustilide activate TRPA1

The dihydrophthalide ligustilide (*Z*-3-butylidene-4,5-dihydroisodenzofuranone, Lig) and its aromatized analogue dehydroligusitilide (DH-Lig) (Fig. 1a, b) were isolated from the essential oil of chuanxiong (*Ligusticum chuanxiong* Hort.), and were extracellularly applied to test their effects on TRPA1 modulation.

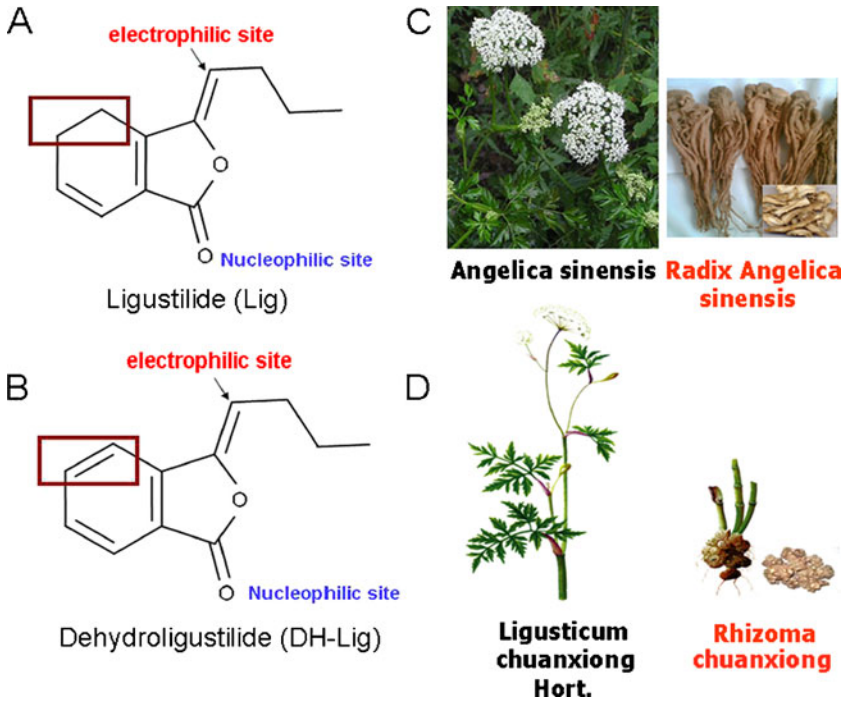

Fig. 1 Chemical structures of ligustilide and dehydroligustilide originated from *Angelica sinensis* and *Ligusticum chuanxiong* Hort. **a** Chemical structure of ligustilide (*Lig*). **b** Chemical structure of dehydroligustilide (DH-Lig), a degradation product of Lig. The differences of Lig and DH-Lig are shown (*box*), the location of the electrophilic and the nucleophilic sites. Note that there is a more extended nature of their reactive moiety as compared to classic TRPA1 activators like acrolein. **c** *Angelica sinensis* and its root, radix *Angelica sinensis*. **d** *Ligusticum chuanxiong* Hort. and its root, *Rhizoma chuanxiong* (plant figures with permission from Shandong University of China and the following free websites: http://www.nature.sdu.edu.cn/artemisia/picright.php?turn=10.1&tblname=Umbelliferae; http://www.nipic.com/show/4/83/abee8e76f3b19b2b.html; http://www.xahjbio.com/showproduct/?7-106.html

We performed whole-cell patch-clamp recordings in CHO cells expressing the mouse isoform of TRPA1. Consistent with previous reports, repetitive application of a voltage ramp from −100 to +100 mV from a holding potential of 0 mV elicited sizable TRPA1 currents, indicative of constitutively open TRPA1 channels [11, 17]. Extracellular application of Lig and DH-Lig induced a fast activation of both outward and inward currents (Fig. 2a, c). Washout of 300 μM Lig did not result in a rebound activation, whereas washout of DH-Lig consistently induced a rapid increase of TRPA1 currents (off-response) followed by a slow recovery (Fig. 2c). Current voltage relationships of the activated currents are shown in Fig. 2b and d. The activated currents show an almost linear *I–V* relationship which is typical for MO activated TRPA1 currents.

Measurements of dose response curves for Lig and DH-Lig resulted EC_{50} values of 44 and 539 μM for Lig and DH-Lig, respectively (n=4, Fig. 2e). If 100 μM MO was applied, additional application of Lig did only induce small changes in the activated current (Fig. 2a), whereas — as expected from the washout off response — application of DH-Lig induced a striking current inhibition (Fig. 2c). This result suggested that lower concentrations of DH-Lig have an agonistic effect on TRPA1, a transient current increase upon washout being observed. Nevertheless, when applied to cells in which TRPA1 is pre-activated by MO application, DH-Lig causes marked inhibitory effects.

Potent block of TRPA1 by dehydroligustilide

To investigate this effect in more detail (Fig. 3), TRPA1 was activated with 100 μM MO. Application of Lig only induced a shallow block of the current at very high concentrations (Fig. 3a), while application of 1 mM induced an average of less than 50% block of TRPA1 in both inward and outward direction. The *I–V* curves clearly show that its effect is not voltage dependent (Fig. 3b). In comparison, application of DH-Lig causes already at 100 μM a nearly complete block of the TRPA1 mediated current, which is again voltage independent (Fig. 3c and d). Inhibition of the MO-induced current was calculated by

$$\mathrm{Inh}(\%) = \frac{I_{\mathrm{pre}} - I_{\mathrm{test}}}{I_{\mathrm{pre}}} \times 100$$

where Inh is the blocking effect in %, I_{pre} is the MO-activated current immediately before Lig/DH-Lig applica-

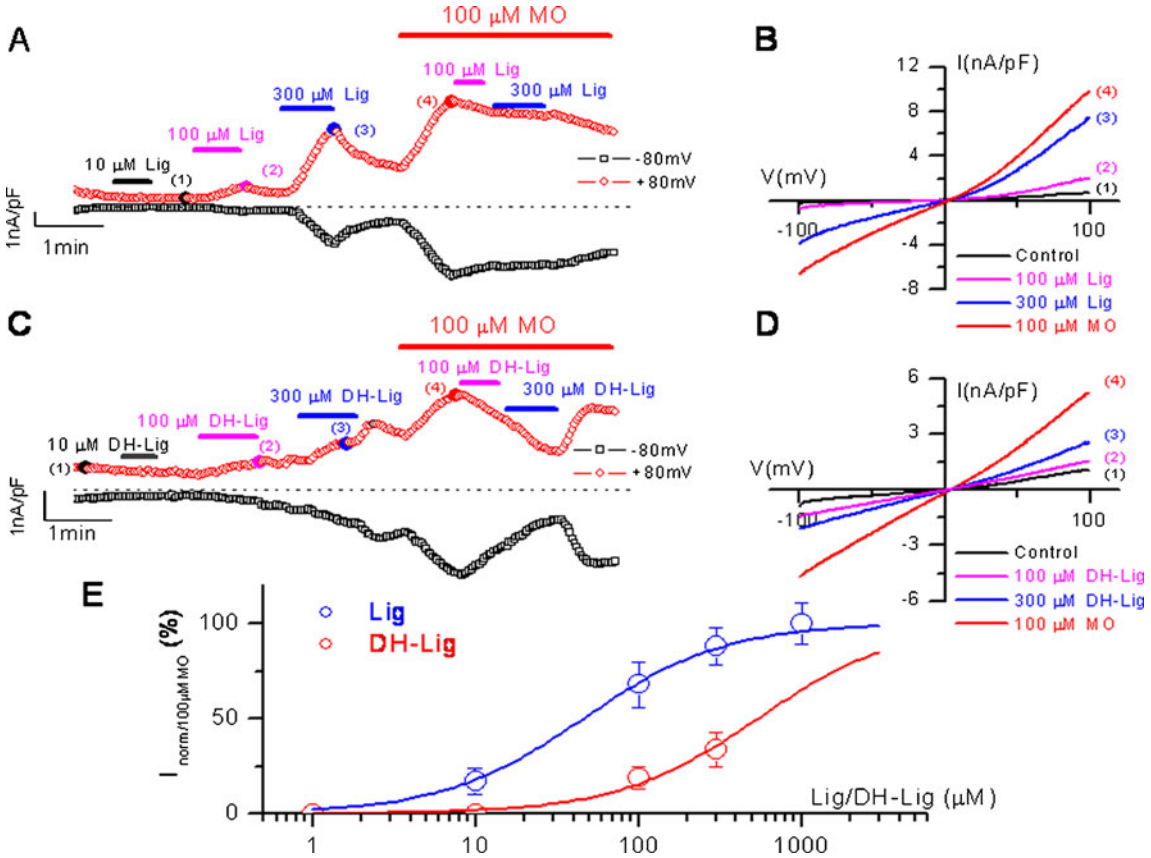

Fig. 2 Effects of ligustilide and dehydroligustilide on whole-cell currents through TRPA1. **a** Time course of whole-cell currents through TRPA1 measured at membrane potential of −80 mV (*black squares*) and +80 mV (*red circles*) during application of Lig and MO at indicated concentrations. Note the fast activation of both outward and inward currents by Lig. **b** Current–voltage (*I–V*) relationships obtained with voltage ramps from −100 to +100 mV at the times indicated in **a**. **c** Current traces of TRPA1-expressing CHO cells at membrane potential of −80 mV (*black squares*) and +80 mV (*red circles*) during application of DH-Lig and MO at indicated concentrations. Note the rapid rebound activation after washout of 300 µM DH-Lig and the striking current inhibition of DH-Lig on TRPA1 pre-activated by 100 µM MO. **d** *I–V* relationships obtained with voltage ramps from −100 to +100 mV at the times indicated in **c**. **e** Dose–response curves of Lig and DH-Lig on the activation of TRPA1. EC_{50} values of Lig and DH-Lig varied greatly, which were 44 and 539 µM, respectively

tion, I_{test} is the minimal current during application of the both compounds. DH-Lig has a much higher blocking efficiency than Lig. The IC_{50} values for Lig and DH-Lig were 1,456 and 23 µM, respectively (n=6, Fig. 3e).

Cross-desensitization between MO and ligustilide/dehydroligustilide

We next tested a possible cross-desensitization to activation of TRPA1 by MO. Application of MO does not allow a second TRPA1 activation, a phenomenon known as desensitization. We tested whether this phenomenon is also present for TRPA1 activation by Lig and DH-Lig. In these experiments, we used solutions with an extracellular Ca^{2+} concentration of 1.5 mM to accelerate TRPA1 inactivation [17]. Figure 4 shows a representative example (n=5). Application of 300 µM Lig resulted in a fast TRPA1 activation with nearly identical inward and outward currents. Washout of Lig caused a current decrease. If 100 µM MO was applied after Lig stimulation, no current was activated (Fig. 4a). Conversely, an application of 100 µM MO resulted in TRPA1 current activation, and no subsequent stimulation by Lig could be observed (Fig. 4b), as expected by a cross-desensitization between MO and Lig. Application of 300 µM DH-Lig resulted in a smaller but transient activation of TRPA1. After washing out of DH-Lig, a dramatic rebound effect could be always observed, strongly suggesting an off-response reaction from TRPA1 inhibition by DH-Lig. Application of MO after this first DH-Lig stimulation failed in any current activation (Fig. 4c). Vice versa, an initial stimulation of TRPA1 by MO prevented a secondary stimulation by DH-Lig. However, DH-Lig inhibited the probably still partially activated TRPA1 current (Fig. 4d). Both compounds therefore caused cross-desensitization with MO.

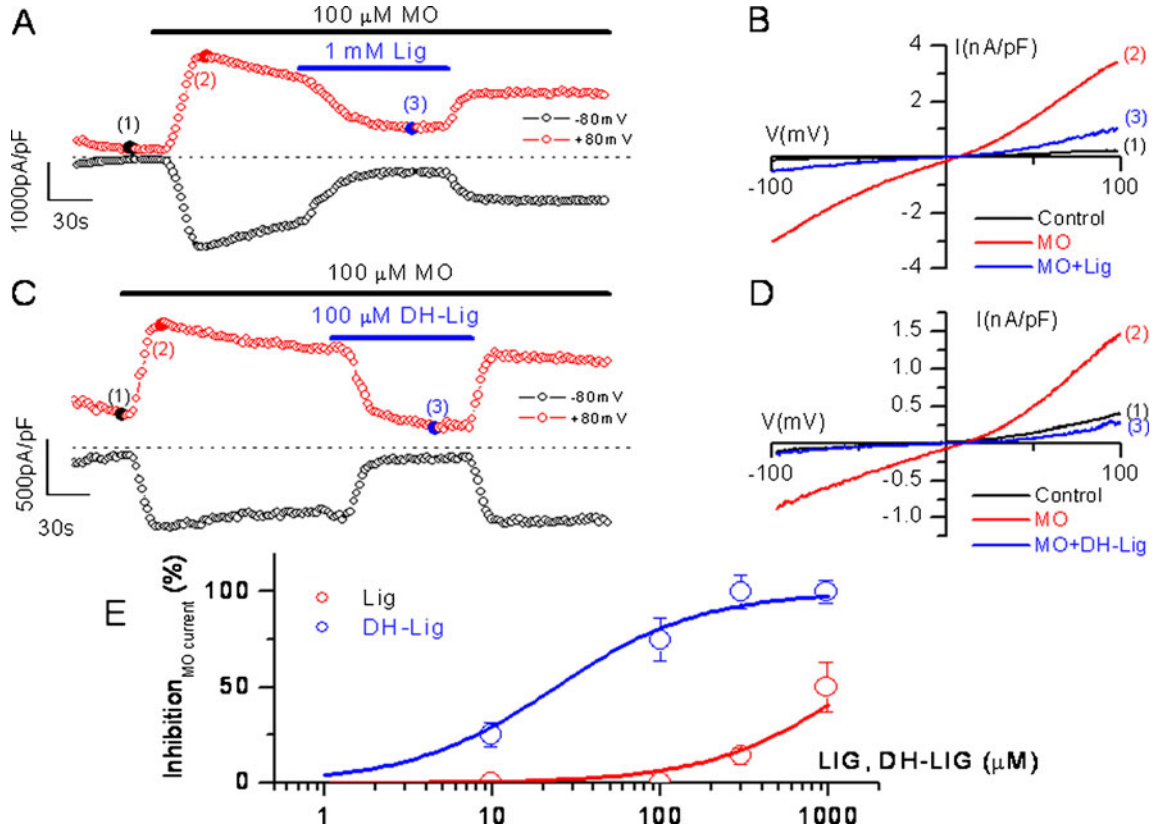

Fig. 3 Differential block effects of ligustilide and dehydroligustilide on whole cell currents through TRPA1. **a** Time course of whole-cell currents through TRPA1 activated with 100 µM MO, followed by a shallow block of 1 mM Lig measured at membrane potential of −80 mV (*black circles*) and +80 mV (*red circles*). **b** *I–V* relationships obtained with voltage ramps from −100 to +100 mV at the times indicated in **a**. **c** Time course of whole-cell currents through TRPA1 activated with 100 µM MO, followed by a nearly complete block of 100 µM DH-Lig measured at membrane potential of −80 mV (*black circles*) and +80 mV (*red circles*). **d** *I–V* relationships with voltage ramps from −100 to +100 mV measured at the times shown in **c**. **e** Dose–response curves for the inhibition on MO-induced currents by Lig and DH-Lig. The IC_{50} values for Lig and DH-Lig were 1,456 and 23 µM, respectively

Differential activation of TRPA1 by ligustilide and dehydroligustilide

To investigate the mechanism of Lig and DH-Lig action on TRPA1 gating in more detail, we used TRPA1 mutations in which three of the most reactive cysteines were exchanged by non-reactive serines [10]. Figure 5a shows that application of 300 µM Lig still activated TRPA1 (see calibration), but to a lesser extent than for the wild-type clone. Stimulation by MO was small and extremely delayed. Application of Lig after MO still resulted in a similar "activation" pattern as for the non-mutant channel. *I–V* curves are again linear in the negative voltage range but show the very variable inactivation at positive potentials [22]. The increase in current after agonist activation is significantly reduced for both Lig and MO in the here shown C622/642S mutant (Fig. 5c, n=5). In contrast, application of DH-Lig to the double mutant resulted in a nearly unchanged activation pattern. After DH-Lig washout, a rebound effect was still measureable, and DH-Lig could also induce the TRPA1 block described above (Fig. 5d–f). In this series (n=5), mutation of the two reactive cysteines resulted in a significant diminution of the MO activated TRPA1 current (Fig. 5d). Obviously, Lig activation of TRPA1 depends much more on the reactive cysteines than on activation by DH-Lig.

We also tested the single (C622S) and triple (C622/642/666S) mutants. The pattern measured with the double mutant (C622/642S) mutation was confirmed. Both, the Lig and the MO response were significantly reduced in the all cysteine mutants (Fig. 6a). However, the DH-Lig response was not significantly affected, and also in the same series, the MO effect was highly significantly inhibited (Fig. 6b; all n=5 and measured at +80 mV). Thus, MO and Lig require at least part of the so far described mouseTRPA1 reactive cysteines, whereas DH-Lig does not require these residues, showing differences in the mode of TRPA1 activation by both compounds.

In addition, we measured effects of Lig and DH-Lig on mouseTRPV1. Both compounds only show a nearly

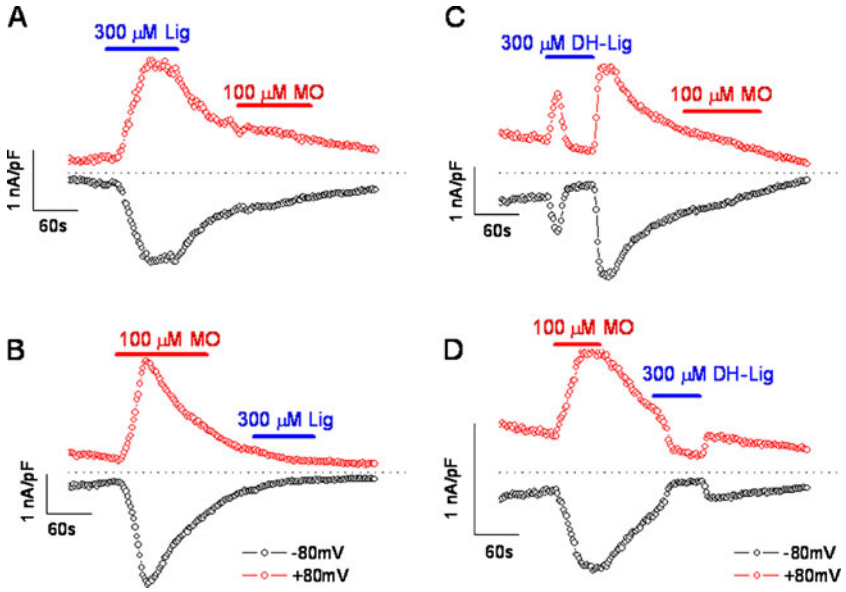

Fig. 4 Cross-desensitization between MO and ligustilide/dehydroligustilide. Note solutions with an extracellular Ca^{2+} concentration of 1.5 mM were used in the experiments to accelerate TRPA1 inactivation. **a** Representative time course of whole-cell currents elicited by 100 µM MO, applied after the washout of 300 µM Lig stimulation on TRPA1. Shown are the currents obtained from voltage ramps at −80 mV (*black circles*) and +80 mV (*red circles*). **b** Time course of whole-cell currents induced by 100 µM MO, without subsequent stimulation by Lig measured at membrane potential of −80 mV (*black circles*) and +80 mV (*red circles*). **c** Representative time course of whole-cell currents elicited by 300 µM DH-lig showed a smaller but transient activation of TRPA1, followed by a dramatic rebound effect after washing out of DH-Lig and no subsequent activation by 100 µM MO. Shown are the currents obtained from voltage ramps at −80 mV (*black circles*) and +80 mV (*red circles*). **d** Time course of whole-cell currents activated by 100 µM MO prevented a secondary stimulation by 300 µM DH-Lig, but followed with an inhibition of DH-Lig. Shown are the currents measured at membrane potential of −80 mV (*black circles*) and +80 mV (*red circles*)

negligible activation of TRPV1 (7.2±2% and 6.8±3% for 300 µM Lig and DH-Lig, respectively, as compared with 0.1 µM capsaicin, +80 mV, n=4; data not shown). We also tested the effects of Lig and DH-Lig on mouseTRPM8. None of the compounds could activated this channel (n=6). However, if TRPM8 was activated by menthol, application of both compounds caused a potent inhibition (100 µM menthol, +80 mV block of 88±6% and 89.8±3% for 300 µM Lig and DH-Lig, respectively; data not shown, n=4).

Discussion

Ligustilide is a plant product of dietary and medicinal relevance, and its biological profile has been extensively investigated in cellular and animal models of disease [4, 6, 13, 21, 24, 25, 27], but very little is known on its biological targets and the molecular mechanism(s) underlying its activity. Despite its structural simplicity, Lig has a complex cross-conjugated di-unsaturated system. It shows Michael-acceptor properties, reacting with thiols to give a mixture of adducts that easily revert to the starting unsaturated compound [20], and has been reported to induce cellular anti-oxidant responses due to by activation of the transcription factor Nrf2 by covalent alkylation [5]. Michael acceptors like α,β-unsaturated carbonyls are believed to activate TRPA1 by exploiting the high reactivity of certain cysteine residues, and two recent reports have indeed provided compelling evidence that activation of TRPA1 by several agonists, including cinnamaldehyde, acrolein and MO, occurs through covalent modification of N-terminal cysteine residues on the channel [14, 22]. The "activate" status for a cysteine residue originates from its location in a molecular environment capable to provide electrophilic activation of the carbonyl oxygen of a Michael acceptor, an increased acidity of the thiol group, or a combination of both [19]. The alkylation reaction requires therefore a precise spatial relationship between the carbonyl activating element and the reactive cysteine thiol group [19]. Compared to other Michael acceptors that activate TRPA1, Lig and DH-Lig, are characterized by a more extended unsaturated system, which spans a double bond in Lig, and a phenyl group in DH-Lig. It was therefore interesting to investigate if they could still act as covalent activators of TRPA1, and, if so, to compare their profile with that of MO, a classic covalent activator.

Surprisingly, Lig and DH-Lig showed a complementary pattern of interaction with TRPA1. Thus, while Lig was a potent activator, with only marginal inhibitory properties on MO-induced activation, DH-Lig could potently inhibit MO-

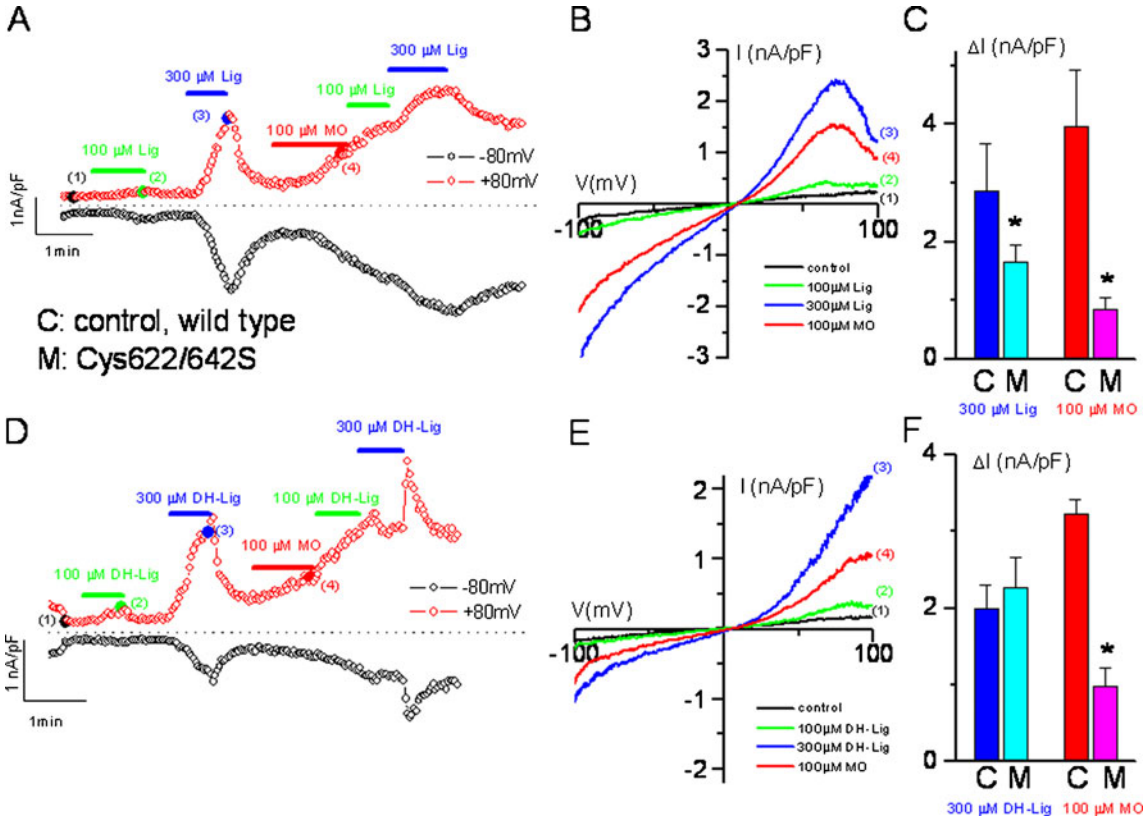

Fig. 5 Differential effects of ligustilide and dehydroligustilide on whole cell currents through C622/642S mutant TRPA1. **a** Representative time course of whole-cell currents elicited by Lig and MO at indicated concentrations through C622/642S mutant TRPA1 measured at membrane potential of −80 mV (*black circles*) and +80 mV (*red circles*). **b** *I–V* relations measured at the times indicated in **a**. **c** Average values for increased currents activated by 300 μM Lig and 100 μM MO through both wild-type and C622/642S mutant TRPA1. **d** Representative time course of whole-cell currents induced by DH-Lig and MO at indicated concentrations through C622/642S mutant TRPA1 measured at membrane potential of −80 mV (*black circles*) and +80 mV (*red circles*). **e** *I–V* relations measured at the times indicated in **d**. **f** Average values for increased currents activated by 300 μM DH-Lig and 100 μM MO through both wild-type and C622/642S mutant TRPA1

induced activation of TRPA1, with only modest activating capacity. The study of TRPA1 mutants characterized by the replacement of the active cysteine residues with serine, evidenced that Lig did not substantially deviate from the activation profile of MO, while, unexpectedly, DH-Lig interacted with TRPA1 in an essentially non-covalent

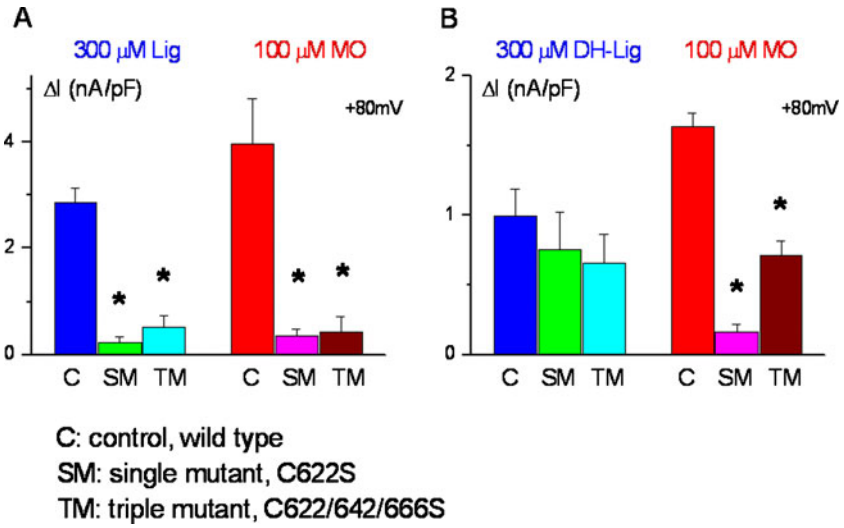

Fig. 6 Differential effects of ligustilide and dehydroligustilide on whole cell currents through C622S and C622/642/666S mutant TRPA1. **a** Average values for increased currents activated by 300 μM Lig and 100 μM MO through wild type, C622S and C622/642/666S mutant TRPA1. **b** Average values for increased currents activated by 300 μM DH-Lig and 100 μM MO through wild type, C622S and C622/642/666S mutant TRPA1

fashion. When subjected to an NMR assay for in situ reaction with cysteamine, an in vitro mimic of the in vivo reaction with an active cysteine residue [2], neither Lig nor DH-Lig showed any reaction. The lack of reaction of DH-Lig is in accordance with observations done for its interaction with TRPA1, which is essentially non-covalent, while the failure of Lig to give a positive assay is in contrast with the results observed with TRPA1 and Nrf2 [5], which show a Michael-type reactivity in cell systems. This discrepancy might be due either to a limitation of the NMR method, that is unable to detect the Michael reactivity of extended electrophilic systems, or to the complexity of the electronic system of Lig, that, after the initial 1,6-addition at the distal carbon of the unsaturated system, can also add a second thiol group in the usual 1,4-fashion [3]. It is therefore not unconceivable to assume that, within the cysteine rich ankyrin loop of TRPA1, Lig is "activated" in situ by a reversible 1,6-addition, generating a more reactive α,β-unsaturated system that is the actual alkylating agent.

Our observation, while qualifying the phthalide element as a privileged structure to modulate the activity of TRPA1, also raises some interesting issues. First, it is not clear to what extent the Lig-sensitive activating site and the DH-Lig-sensitive inhibitory site overlap in TRPA1. The reversal of profile observed for the dehydrogenation of Lig to DH-Lig pair is reminiscent of the one associated to the iodination of RTX or capsaicin, with iodination being capable of reversing the activity at TRPV1 level [1]. Next, as a covalent activator of anti-oxidant response via Nrf2 [5] and of TRPA1-associated sensory responses, Lig seems to basically act as a biological analogue of MO, despite the very different profile of in vivo activities associated to both compounds. On the other hand, the diverse structure of the two compounds and their chemical reactivity might translate into a different pharmacokinetic profile and tissue distribution, diversifying what seems a basically overlapping pharmacodynamic profile [26]. Finally, the broad tuning of TRPA1 and its sensitivity to dietary constituents suggest a certain complementarity to the bitter-sensing system [16], with TRPA1 aimed at sensing electrophilic and oxidant compounds and the bitter receptors at sensing alkaloids, providing us with a sensory shield towards the two most common classes of environmental and dietary toxins.

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