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TRPA1 is activated by direct addition of cysteine residues to the *N*-hydroxysuccinyl esters of acrylic and cinnamic acids

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ABSTRACT

The nociceptor TRPA1 is thought to be activated through covalent modification of specific cysteine residues on the N terminal of the channel. The precise mechanism of covalent modification with unsaturated carbonyl-containing compounds is unclear, therefore by examining a range of compounds which can undergo both conjugate and/or direct addition reactions we sought to further elucidate the mechanism(s) whereby TRPA1 can be activated by covalent modification. Calcium signalling was used to determine the mechanism of activation of TRPA1 expressed in HEK293 cells with a series of related compounds which were capable of either direct and/or conjugate addition processes. These results were confirmed using physiological recordings with isolated vagus nerve preparations. We found negligible channel activation with chemicals which could only react with cysteine residues via conjugate addition such as acrylamide, acrylic acid, and cinnamic acid. Compounds able to react via either conjugate or direct addition, such as acrolein, methyl vinyl ketone, mesityl oxide, acrylic acid NHS ester, cinnamaldehyde and cinnamic acid NHS ester, activated TRPA1 in a concentration dependent manner as did compounds only capable of direct addition, namely propionic acid NHS ester and hydrocinnamic acid NHS ester. These compounds failed to activate TRPV1 expressed in HEK293 cells or mock transfected HEK293 cells. For molecules capable of direct or conjugate additions, the results suggest for the first time that TRPA1 may be activated preferentially by direct addition of the thiol group of TRPA1 cysteines to the agonist carbonyl carbon of α,β-unsaturated carbonyl-containing compounds.

1. Introduction

TRPA1 is now widely believed to be a nociceptor. This cation channel has an important role in the sensation of pain, inflammation and cough in animals and man [1,2]. It is activated by a wide range of environmental irritants including acrolein and toluene diisocyanate [1,3] as well as plant products such as cinnamaldehyde and mustard oil [4]. Two recently published papers suggested that activation of TRPA1 by this diverse range of compounds was via covalent modification of specific cysteine residues on the cytoplasmic N terminus of the protein [5,6]. Hinman et al. noted it is likely that the structure of the agonist is of less importance, as it is not required to fit into a selective ligand binding pocket, rather it is the chemical reactivity of the compound that is important for activation of the channel [5]. The precise mechanism whereby these compounds form covalent bonds and thus activate TRPA1 is unclear. Both groups carried out site directed mutagenesis and identified cysteine residues important for the activation of the channel [5,6]. Hinman et al. suggest that cysteines, C619, C639 and C663 were covalently modified by electrophilic agonists such as acrolein, allyl isothiocyanate and cinnamaldehyde [5]. Macpherson et al. suggested acrolein and cinnamaldehyde (α,β-unsaturated aldehydes) activate TRPA1 by conjugate addition of the nucleophilic thiol group of cysteine residues C415, C422 and C622 [6]. Formaldehyde in aqueous solution also activates TRPA1 and this appears to be an important mechanism of formalin-induced pain [7]. Like acrolein, formaldehyde has a reactive aldehyde functional group and can readily and reversibly add

Abbreviations: AcCysOMe, *N*-acetylcysteine methyl ester; MA, methyl acrylate; MO, mesityl oxide; MVK, methyl vinyl ketone; NHS, *N*-hydroxysuccinyl ester; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1.

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nucleophiles to form hemiacetal and acetal-type adducts. McNamara et al. [7] noted the resemblance of formaldehyde and acrolein, but this is a rather simplistic structural viewpoint and their chemical reactivity prompts a closer examination. In comparison to formaldehyde, acrolein has two reactive electrophilic sites, namely the carbonyl carbon and the β-carbon, and such α,β-unsaturated aldehydes can undergo both direct and conjugate addition reactions. Whilst the absolute rates of addition by either pathway may vary in different substrates it is known that for a given compound the direct addition product is formed at the faster rate even though the conjugate addition leads to the thermodynamically more stable adduct. Conjugate addition products can be formed however when the faster, direct addition process is reversible. Whilst characterising our cloned TRPA1 channel we observed that the TRPA1 agonist, cinnamaldehyde, acted in a manner compatible with reversible, competitive agonism. It was therefore not immediately obvious to us whether the channel activation caused by acrolein and cinnamaldehyde, was triggered by fast reversible addition at the carbonyl carbon, or as a result of slower, covalent irreversible modification at the β-carbon. Thus we sought to further elucidate the mechanism(s) whereby TRPA1 can be activated by covalent modification by examining a range of compounds which can undergo both conjugate and/or direct addition reactions at differing rates.

We examined a series of compounds related to acrolein using calcium signalling with cloned TRPA1 expressed in HEK293 cells and physiological recordings with isolated vagus nerve preparations. In this series of α,β-unsaturated carbonyl compounds the rate of direct addition (at the carbonyl carbon) of a nucleophile diminishes across the series acrolein > methyl vinyl ketone > methyl acrylate ester > acrylic acid > acrylamide (Fig. 1). In this series increasing preference for conjugate addition (at the β-carbon) over direct addition follows the reverse trend, with acrylamide reacting essentially via a conjugate addition process only [8,9]. The rate of conjugate addition processes can also be affected by the steric hindrance at the β-carbon. Mesityl oxide (4-methylpent-3-en-2-one) would be expected to react slower than methyl vinyl ketone (but-3-en-2-one). *N*-Hydroxysuccinyl esters (NHS) are compounds well known in the field of bioconjugation. We therefore sought to examine 'active' NHS esters of acrylic and cinnamic acid, in which the direct addition process leads to an essentially irreversible direct addition, unlike the reversible addition expected of the corresponding aldehydes of the same carbon skeleton. The synthesis of cinnamic acid NHS ester and its subsequent direct addition reaction with the sulfur nucleophile, HS-CoA, has been reported [10]. Nevertheless, before treating TRPA1 with α,β-unsaturated NHS esters we wanted to prove to ourselves that the direct addition process only was occurring by reacting the cinnamic NHS ester with *N*-acetyl cysteine methyl ester as a sulfur nucleophile model of TRPA1 channel.

In this study, we use a series of α,β-unsaturated carbonyl compounds which are capable of either direct and/or conjugate addition processes. The data here shows that TRPA1 is activated by direct addition of the thiol group of TRPA1 cysteines to the agonist carbonyl carbon.

2. Materials and methods

2.1. Materials

Fluo-3 acetoxymethyl ester (Fluo-3 AM) and DMEM were purchased from Invitrogen. FCS and PBS were from Lonza. The TRPA1 inhibitor, HC-030031, was purchased from ChemBridge, San Diego, USA. All other chemicals and reagents were purchased from Sigma (Poole) unless otherwise stated.

2.2. Chemical synthesis

Cinnamic acid *N*-hydroxysuccinyl (NHS) ester was made by EDC-mediated coupling of cinnamic acid and *N*-hydoxysuccininide using the method of Shiosaki et al. [11]. Hydrocinnamic acid NHS ester was made in a similar fashion, but with the addition of 0.05 mole equivalents of 4-(*N*,*N*-dimethylamino)pyridine. Propionic acid NHS ester was made from propionyl chloride and *N*-hydoxysuccinimide.

2.3. Proton NMR

The proton NMR experiment to follow the reaction of cinnamic acid NHS ester (28 mg, 0.114 mmol; NHS ester concentration 0.143 M) and *N*-acetyl cysteine (5 mg, 0.028 mmol, 0.25 eq.) was performed in d_6-DMSO at 400 MHz and 25 °C. Initial data acquisition was after 10 min of mixing and subsequent spectra were recorded at 2 min intervals over a period of 20 min, and then at 50 min and 2 h. The conversion in to the product was monitored by relative integration of the isolated ^2J_H alkenic doublet in starting material (δ 7.91) and product (δ 7.54). Identical experiments were undertaken with hydrocinnamic and propionic acid NHS esters over a longer period of time (see Section 4), with the progress of these reactions monitored by the appearance of the methyl singlets for the product ester and acetyl groups. Two similar reactions with acrylamide were conducted using (i) d_6-DMSO and (ii) a 1:1 (v/v) mixture of d_6-DMSO and 0.5 M phosphate buffer (pH 7.4) in D_2O.

2.4. Calcium signalling

The TRPA1 expressing HEK293 cells and calcium signalling method used here have been described previously [12]. Agonist induced increases in intracellular calcium levels in Fluo-3 loaded cells were measured at 530 nm following excitation at 480 nm using a fluorospectometer (Photon Technology International). Concentration effect curves were constructed by measuring peak responses to agonists and expressing them as a percentage of 6 μM calcium ionophore (A23187, maximum response). To investigate the role of the TRPA1 antagonist on acrylic acid NHS responses, cells were pre-incubated for 10 min with increasing concentrations of HC-030031 before addition of 3 μM acrylic acid NHS.

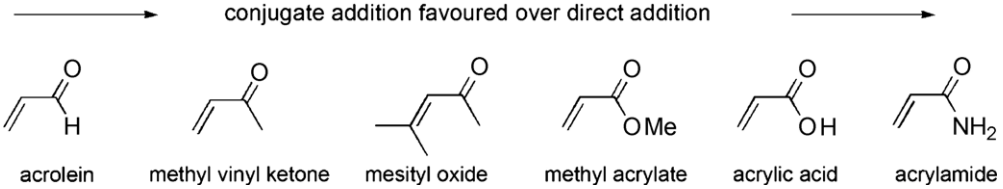

Fig. 1. Analogues of acrolein ordered with regard to their increasing preference for undergoing a conjugate addition reaction (at the β-carbon) over a direct addition reaction (at the carbonyl carbon).

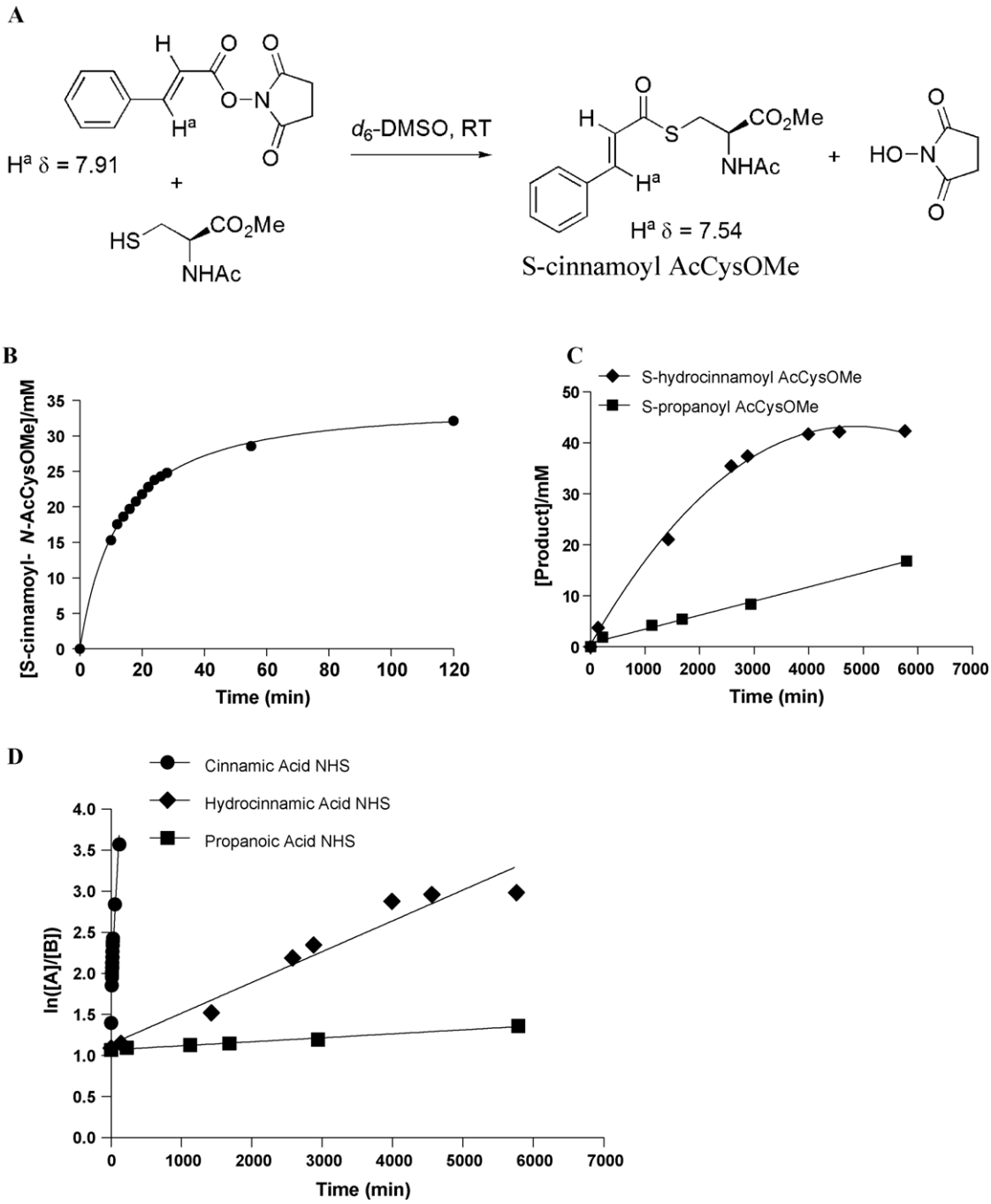

Fig. 2. (A) Conversion of cinnamic acid NHS ester into the corresponding thioester by direct addition of *N*-acetylcysteine methyl ester (AcCysOMe) (at 25 °C in d_6-DMSO). The initial molar ratio of NHS ester to AcCysOMe was 4:1, leading to a final theoretical ratio of NHS ester to thioester of 3:1. (B) Progress of the conversion was monitored by integration of the alkenic ^2J_H doublets Hª in the product and starting material at δ 7.91 and δ 7.54 respectively. (C) Progress of the conversion of hydrocinnamic and propionic acid NHS esters which occurred over a period of two to four days rather than hours. (D) The second order rate plot for *S*-cinnamoyl AcCysOMe, *S*-hydrocinnamoyl AcCysOMe and *S*-propanoyl AcCysOMe production. [A] and [B] indicate the concentrations of NHS ester and AcCysOMe respectively.

2.5. Isolated vagus nerve preparation

Using our fully characterised isolated vagus nerve preparation [13,14], the depolarising effect of the TRPA1 agonists were profiled. Briefly, vagal tissue from male Dunkin Hartley guinea-pigs was collected and mounted in a "grease-gap" chamber to allow the measurement of nerve activity i.e., depolarization. A range of TRPA1 agonists were tested; acrolein (0.03–3 mM), acrylic acid (0.03–3 mM), cinnamic acid (0.03–1 mM), acrylic acid NHS ester (0.03–1 mM), cinnamic acid NHS ester (0.03–0.1 mM), hydrocinnamic NHS ester (0.03–1 mM) and vehicle (0.1% DMSO v/v). Although higher agonist concentrations are required to activate TRPA1 in the isolated nerve preparation the aromatic organic compounds in particular are much less water soluble at these concentrations. Therefore concentrations were chosen based on the solubility of the agonists. Stock solutions were made in DMSO and diluted 1:1000 into Krebs solution. Agonists were applied to the nerve for 2 min, washed with Krebs solution and then the maximum depolarization recorded (mV).

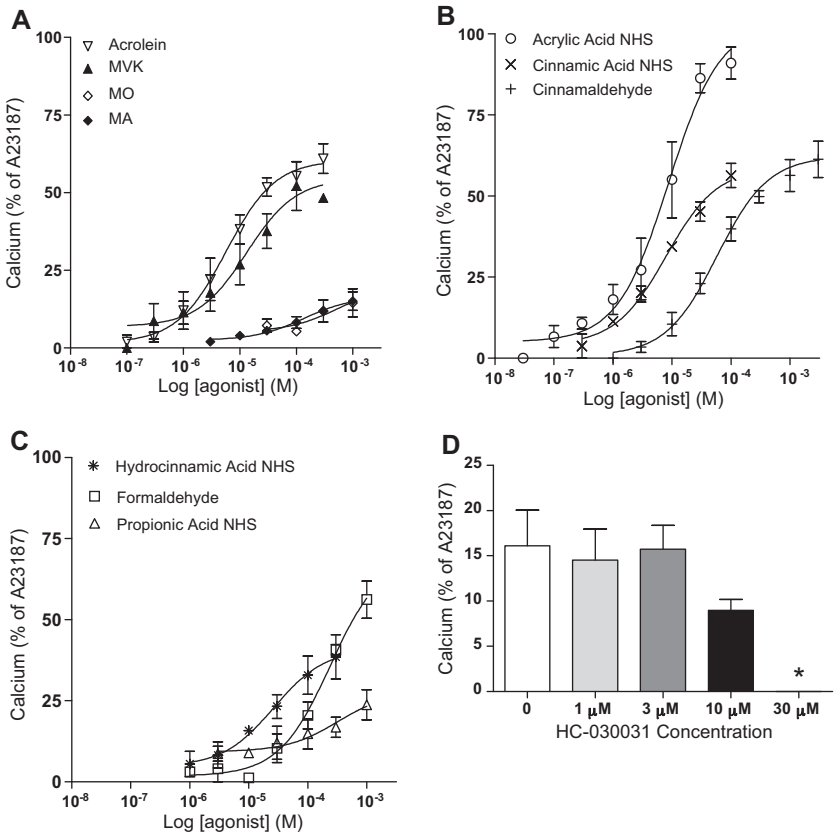

Fig. 3. TRPA1 agonist concentration effect curves. (A) Acrolein, methyl vinyl ketone (MVK), mesityl oxide (MO) and methyl acrylate (MA) TRPA1–HEK concentration effect curves. (B) Acrylic acid NHS, cinnamic acid NHS and cinnamaldehyde TRPA1–HEK concentration effect curves. (C) Hydrocinnamic acid NHS, formaldehyde and propionic acid NHS TRPA1–HEK concentration effect curves. Responses are expressed as the mean percentage of the maximum achievable response (A23187) ± SEM of 3–5 experiments each performed in duplicate. (D) Effect of increasing concentrations of the TRPA1 antagonist, HC-030031, on acrylic acid NHS evoked responses. Results are expressed as the mean ± SEM of 3 experiments each performed in duplicate.

3. Results

3.1. Proton NMR reveals cysteine reacts with NHS esters via direct addition

The model reaction of *N*-acetyl cysteine methyl ester with cinnamic NHS ester was used to confirm that a thiol will react with this substrate by a direct and not a conjugate addition process. Proton NMR was used to monitor the reaction of a four-fold excess of the substrate with the cysteine derivative. Over 120 min a key signal (δ 7.91) from the starting material disappeared and one for the product (δ 7.54) increased almost reaching the theoretical final ratio of remaining cinnamic acid NHS ester to product of 3:1 (Fig. 2A and B). Only direct addition and no conjugate addition was observed in this NMR reaction despite cinnamic acid NHS ester being capable of both. The NMR experiments with hydrocinnamic and propionic acid NHS esters showed that the corresponding direct reactions proceeded over surprisingly much longer periods of time compared to cinnamic acid NHS ester, with reactions occurring over periods of two to four days rather than hours (Fig. 2C). Fig. 2D shows the second order rate plots for direct addition of *N*-acetyl cysteine methyl ester to cinnamic acid NHS ester, hydrocinnamic acid NHS ester and propionic acid NHS ester.

A similarly slow reaction with acrylamide was observed, with evidence of product appearing after 16 h. We were aware that the solvent plays an important role in the conjugate addition reaction, as it proceeds via an intermediate enolate much more basic than a thiolate, and a proton transfer is needed to facilitate the conversion to the product. So the aprotic nature of DMSO was expected to affect significantly the rate of this type of process in particular. Thus the NMR-scale conjugate addition reaction of *N*-acetyl cysteine methyl ester with acrylamide was repeated but in 1:1 mixture of DMSO and 0.5 M phosphate buffer (pH 7.4) in D_2O to better mimic the physiological conditions of the bioassays. In this case the reaction was found to proceed much faster, and over a matter of 4–6 h, i.e. a period not too dissimilar to the cinnamic acid NHS ester reaction. On this scale and under these conditions, evidence of disulfide formation also started to appear in the NMR spectrum.

3.2. TRPA1 is activated by compounds capable of direct addition

Using HEK293 cells permanently expressing cloned human TRPA1 (TRPA1–HEK) we performed calcium signalling experiments to determine the structure/activity relationship of TRPA1 activation using a series of related compounds which were capable of either direct and/or conjugate addition processes. Initially we screened chemicals structurally related to the TRPA1 agonists acrolein and cinnamaldehyde, which would react via conjugate addition only. These were cinnamic acid, acrylic acid and acrylamide. These compounds caused little or no TRPA1 activation up to 300 μM (5.2, 1.9 and 0% of A23187 respectively). Acrylamide did cause a very small increase in intracellular calcium in TRPA1–HEK cells but only at 100 mM (4.8% of A23187). Methyl vinyl ketone however is able to react either via conjugate or direct activation, and caused activation of TRPA1 with responses similar to acrolein. Responses were seen from 300 nM reaching a maximum at 100 μM (49% of calcium ionophore). Mesityl oxide and methyl acrylate are also able to react by either conjugate or direct addition however they caused only small increases in intracellular calcium levels (15% of calcium ionophore at the maximum concentration tested, 1 mM,

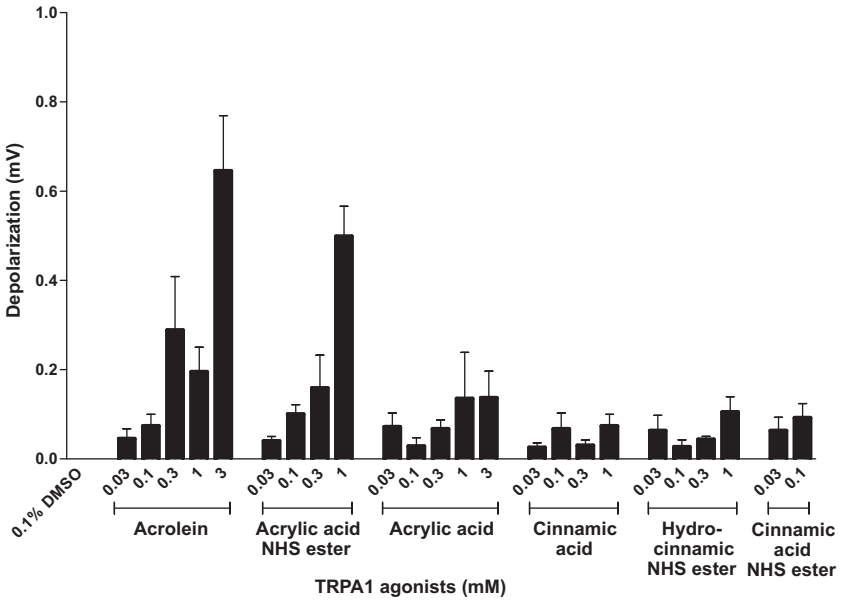

Fig. 4. The effect of TRPA1 agonists on depolarization of the guinea-pig isolated vagus nerves. Responses are expressed as mean ± SEM of 6 experiments. Note differing concentrations used for each agonist, see Section 4.

Fig. 3A). Next we investigated whether the addition of a reactive leaving group (*N*-hydroxy succinimide esters) to the carbonyl carbon of the acrolein and cinnamaldehyde 'skeletons', changed the agonist activity of the compounds. The NHS ester of acrylic acid caused a robust response in TRPA1 (Fig. 3B). Responses were seen as low as 300 nM, reaching a maximum at 100 μM with a maximum responses of 91% of calcium ionophore (A23187, maximum attainable response, Fig. 3B). Cinnamic acid NHS ester (EC_{50} 7.9 μM) caused activation of TRPA1 with responses at concentrations over a half log less than cinnamaldehyde (EC_{50} 58 μM, Fig. 3B). Hydrocinnamic acid NHS ester, propionic acid NHS ester and formaldehyde are only capable of direct addition. Hydrocinnamic acid NHS ester activated TRPA1–HEK cells from 3 μM with an EC_{50} of 30 μM (Fig. 3C). Propionic acid NHS ester also activated TRPA1, but to a lesser extent, reacting from 10 μM and not reaching a maximum by the highest concentration tested, 1 mM (Fig. 3C). Formaldehyde activated TRPA1 from 30 μM and again had not reached a maximum by the highest concentration tested, 1 mM (Fig. 3C). Propionaldehyde only evoked concentration dependent responses from 1 mM to the maximum concentration tested, 100 mM (62.5% of A23187, data not shown). All the TRPA1 agonists mentioned above failed to activate TRPV1–HEK or mock transfected HEK293 cells. Acrylic acid NHS responses (3 μM) could be inhibited in a concentration dependent manner following pre-incubation (10 min) of the cells with the TRPA1 specific antagonist HC-030031, with significant inhibition seen at 30 μM (1 way ANOVA followed by Dunnett's test, $p < 0.01$, Fig. 3D).

3.3. Acrylic acid NHS ester activates native TRPA1

The TRPA1 receptor is known to be expressed on airway sensory nerves carried in the vagus nerve [15] and so this system provided an ideal functional assay in whole tissue. A range of TRPA1 agonists were profiled in our isolated vagus nerve system (Fig. 4). Acrylic acid NHS ester caused a much larger depolarization compared to acrylic acid and produced comparable responses at 1 mM to acrolein at 3 mM. The other agonists, cinnamic acid, cinnamic acid NHS ester and hydrocinnamic NHS ester had a similar profile causing a smaller depolarization compared to acrolein and acrylic acid NHS ester. Discrepancies in concentrations were due to solubility problems at higher concentrations.

4. Discussion

TRPA1 can be activated by a number of mechanisms including covalent modification of specific cysteine residues in the N terminus of the channel [5,6]. The data presented here provides evidence for direct covalent modification of cysteine residues at the carbonyl carbon of a family of agonists and further expands the range of noxious chemicals and environmental irritants known to activate the channel.

The diversity of this expanding list of TRPA1 agonists suggests that TRPA1 is not activated by a single mechanism. Menthol for example is different structurally and does not possess a reactive functional moiety such as an unsaturated aldehyde or isocyanate group. Menthol must activate the channel via some other mechanism, which is very likely to be a non-covalent, reversible interaction [16].

Both Hinman and Macpherson [5,6] have shown TRPA1 can be activated by covalent modification of cysteine residues, and inferred that the activation is caused by conjugate addition of a cysteine residue to the β-carbon of acrolein. However, activation of TRPA1 via covalent cysteine modification may also be via multiple mechanisms, dependent on the reactivity of the agonist. A number of recent studies have shown that products of oxidative stress such as hydrogen peroxide and 4-hydroxynonenal (4-HNE) can activate TRPA1 [17–20]. Andersson and colleagues showed using dithiothreitol that 4-HNE and other related naturally occurring alkenyl aldehydes as well as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) activate TRPA1 by conjugate addition where as H_2O_2 caused the formation of disulfide bonds [18]. In addition to the above mechanisms we show for the first time that a group of compounds based on *N*-hydroxysuccinyl esters can activate TRPA1 by covalent cysteine modification via a pathway involving direct addition to the carbonyl carbon of the agonist.

The reaction of *N*-acetyl cysteine methyl ester with cinnamic NHS ester was used as a model system to study the reaction of a thiol with a substrate which may in principle react by either direct or conjugate addition processes. Macpherson and colleagues have showed that NHS, an agent capable of lysine modification, did not activate TRPA1 in their calcium imaging experiments and thus the liberated NHS in our experiments would not be expected to interfere with interpretation of results obtained [6]. A four-fold excess

of the substrate was mixed with the cysteine derivative and then the reaction progress monitored by proton NMR over a period of 120 min (Fig. 2B). The alkenyl signal at δ 7.91, arising from the hydrogen atoms labelled Ha (Fig. 2A), was free from overlap with other signals in the spectrum. If the conjugate addition process was occurring then this signal would have disappeared but been replaced by new alkyl signal at a much lower chemical shift ($<\delta$ 6.0). The disappearance of the signal for Ha (starting material, Fig. 2A) was duly observed, and was replaced by that at δ 7.54 (product). This confirmed the direct addition process was occurring, not conjugate addition and was therefore in agreement with the literature precedence [10]. The final ratio of starting material to product did not reach the theoretical 3:1, undoubtedly due to slow formation of the cysteine disulfide.

We have shown here that these TRPA1 agonists do indeed react with cysteine residues via direct addition and taken together with the results of Hinman et al. [5] and Macpherson et al. [6] which demonstrated that removal of specific cysteine residues ablated agonist responses, the results suggest that these compounds are activating TRPA1 via covalent cysteine modification.

Compounds such as acrylamide, acrylic acid, and cinnamic acid, are effectively only able to react with cysteine residues via conjugate addition. These however failed to activate TRPA1 up to concentrations of 300 μM. Our NMR experiment, backed up by relevant literature [9], showed however that cysteine derivatives react relatively readily with acrylamide in a conjugate fashion when in a protic solvent, and yet the pharmacology does not reveal a correspondingly robust physiological response. In contrast acrolein, methyl vinyl ketone, mesityl oxide, and cinnamaldehyde, which can react via either conjugate or reversible direct addition, caused TRPA1 activation in a concentration dependent manner. These results then may indicate that with reactive aldehydes such as acrolein, it is the reversible direct addition which is responsible for the channel activation in a similar fashion to formaldehyde. This would then explain our observation that TRPA1 agonists such as cinnamaldehyde acted in a manner compatible with reversible, competitive agonism. Acrylic acid and cinnamic acid NHS esters also caused TRPA1 activation in a concentration dependent manner, in which the reaction with the cysteine residues occur via an essentially irreversible direct addition. Pre-incubation of cells with increasing acrylamide concentrations failed to inhibit acrylic acid NHS ester responses confirming that acrylamide does not interact with the TRPA1 cysteines involved in the acrylic acid NHS ester evoked responses (data not shown).

Hydrocinnamic acid NHS ester and propionic acid NHS ester are only capable of direct addition, but both activated TRPA1 expressed in HEK293 cells. The results from these last two cases suggest that TRPA1 can also be activated by acylation of the thiol group of cysteine residues in TRPA1. The NMR reactions of *N*-acetyl cysteine methyl ester with hydrocinnamic acid and propionic acid NHS esters were shown to proceed (Fig. 2D) at much slower rates compared to cinnamic acid NHS ester. It should be noted that whilst these NMR experiments reveal information about the relative reactivity of these compounds, these should not be confused with the absolute rates under the different conditions of the in vitro studies. The relative rates revealed however are consistent with the observed decrease in physiological responses seen with these three agonists.

Sensory nerves innervating the airways express TRPA1 [15]. The isolated vagus nerve preparation, containing sensory nerves, has been previously characterised and is predictive of agents that cause cough in vivo. The guinea pig model used here and human vagus nerve preparations respond similarly to sensory nerve stimulants. This system provides a useful method for pharmacologically characterising compounds on a mixture of fibre types [21]. Acrylic acid and cinnamic acid, only caused small depolarization compared to acrylic acid NHS ester and acrolein. This confirmed the data seen with the TRPA1–HEK cells. Hydrocinnamic acid NHS, the only compound tested in this model which reacts solely by direct addition, failed to produce significant nerve depolarization at the highest concentration tested (1 mM). Poor aqueous solubility of this compound prevented the use of higher concentrations, which are needed for this method, which may have evoked responses. This problem was also observed with cinnamic acid NHS ester.

The activity of formalin, an agent known to induce hyperalgesia, which can only react via direct addition at the carbonyl carbon, confirms the importance of this mechanism of TRPA1 activation and has consequences for our understanding of this process. If agonists work by this mechanism then the activity of acrolein and cinnamaldehyde may be triggered by fast and readily reversible addition of cysteine residues to their carbonyl carbon, and not necessarily by the conjugate addition process that has been inferred by others [5,6]. Addition of a thiol to the carbonyl carbon of a reactive aldehyde is fast, but forms an unstable hemithioacetal and so the reverse process can also occur readily. Conjugate addition however is a relatively slower process, but produces a much more stable β-thioaldehyde. Thus activation of TRPA1 by this newly proposed mechanism is compatible with our initial observation of brief triggering of the cough reflex by acrolein and cinnamaldehyde [2], and may be the principle mechanism of the sensing of these environmental irritants in vivo.

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