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Contact sensitizer 2,4-dinitrochlorobenzene is a highly potent human TRPA1 agonist

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Abstract

2,4-dinitrochlorobenzene (DNCB) is widely used in human clinical studies and in experimental animal studies to evoke allergic contact dermatitis. DNCB is a potent immunogen capable of inducing contact sensitization in all humans exposed. However, the mechanism by which DNCB evokes such symptoms is presently unknown. TRPA1 is a nonselective cation channel that is expressed in peptidergic sensory neurons and fibroblasts. TRPA1 activation was recently implicated in the pathophysiology of atopic dermatitis especially in transducing cutaneous itch signals. Here we test the hypothesis that DNCB acts as a TRPA1 agonist and thereby evokes allergic symptoms. We found that DNCB activates human TRPA1 dose-dependently in FLIPR experiments with an EC₅₀ of 167 nM, an effect that was fully blocked by selective TRPA1 antagonists Chembridge-5861528 and A-967079. Similarly, DNCB activated nonselective TRPA1 current in patch clamp studies. Neutralization of 3 critical cysteines in TRPA1 resulted in a loss of DNCB agonism.

Introduction

2,4-dinitrochlorobenzene (DNCB) is a well known allergic sensitizer which is widely used to evoke contact hypersensitivity symptoms in both human clinical studies and experimental animal studies (1-3). It is still unknown how the skin sensitization/allergic symptoms are brought about. TRPA1 is a nonselective cation channel that is expressed in peptidergic sensory neurons (4), fibroblasts and keratinocytes (5). TRPA1 is activated by pungent and reactive compounds through cysteine and lysine reactivity (6). There is extensive literature support for TRPA1 activation in neurogenic inflammation and pain/itch (7). Interestingly, recent studies suggest that activation of the TRPA1 ion channel in peptidergic sensory neurons plays a key role in animal models of atopic dermatitis and contact hypersensitivity (8-11). Here we test the hypothesis that DNCB may directly activate the TRPA1 ion channel and thereby evoke allergic symptoms through activation of TRPA1-expressing keratinocytes, fibroblasts and peptidergic sensory neurons.

Materials and methods

See (12) for a thorough description of automated FLIPR fluorescence imaging and manual patch clamp methods and conditions used to study human TRPA1 and rat TRPA1 stably expressed in HEK cells. The human -3C TRPA1 CMV plasmid was identical to wt hTRPA1 apart from C619S, C639S and C663T mutations (Cell Culture Service GMBH, Hamburg, Germany). Human wt TRPV1 plasmid was obtained from Origene, USA. Both plasmids were transfected at 0.13 µg to 30 000 HEK cells/well by using Lipofectamine (Invitrogen). STC-1 cells were cultured in DMEM supplemented with 15% horse serum and 2.5% fetal calf serum. Measurements were typically performed 24 h post-transfection. 2,4-dinitrochlorobenzene was obtained from Sigma.

Results

DNCB evoked a dose-dependent and biphasic activation of hTRPA1 with an EC_{50} value of 167 ± 12 nM ($n=8$ wells) for the sustained phase, the more relevant phase in view of long term effects (Fig 1A). At concentrations higher than 1 μ M the biphasic activation became a sustained monophasic activation. In order to show that the DNCB-induced sustained calcium influx is TRPA1 mediated, selective TRPA1 antagonists Chembridge-5861528 and A-967079 were used (7). Both compounds inhibited the DNCB-evoked sustained TRPA1 response with IC_{50} values of 6.3 ± 0.2 μ M and 128 ± 8 nM ($n=8$) respectively (Fig 1B). In patch clamp experiments with hTRPA1, DNCB evoked inward current at the holding potential and an outwardly rectifying current that reversed close to 0 mV with voltage ramps (Fig 2). This current was rapidly and reversibly blocked by Chembridge-5861528. To investigate the possible mechanism by which DNCB activates hTRPA1, 3 critical cysteines were neutralized (13). The DNCB-evoked agonism was robustly attenuated by neutralization of these 3 cysteines, since the first phase was entirely absent and the sustained activation phase was significantly inhibited (Fig 1C). As a positive control 300 μ M icilin, a non-reactive agonist, elicited TRPA1 activation in -3C TRPA1 transfected cells, while in mock-transfected cells icilin was without effect.

DNCB evoked similar sustained responses with rTRPA1 at concentrations up to 3 μ M, with an EC_{50} value of 826 ± 27 nM ($n=8$). The sustained responses were dose-dependently blocked by Chembridge-5861528 and A-967079. Surprisingly, the first phase responses were absent at concentrations below 1 μ M but above this concentration dose-dependent activation was exhibited eventually leading to inactivation of the sustained response. In mouse STC-1 cells which endogenously express TRPA1, DNCB evoked a sustained dose-dependent intracellular calcium elevation. As with cloned rTRPA1, concentrations of DNCB greater than 3 μ M resulted in a dose-dependent activation of the first phase and subsequent inactivation of the sustained phase. DNCB did not activate hTRPV1 over the concentration range of 30 nM to 30 μ M, whereas positive control agonist capsaicin (1 μ M) elicited marked responses.

Discussion

We show here that the widely used allergic sensitizer DNCB is a potent human TRPA1 agonist. DNCB is almost 40-times more potent than the endogenous TRPA1 agonist 4-hydroxynonenal under identical conditions (12). DNCB appears to selectively target TRPA1, since it was not able to activate TRPV1, another ion channel which has been linked to atopic dermatitis (8,11). Endogenous TRPA1 agonists such as the diabetes-specific methylglyoxal and the oxidative stress-related 4-hydroxynonenal were previously shown to evoke a similar biphasic activation of TRPA1(12). Thus, we suggest that DNCB mimics the natural mode of sustained TRPA1 activation elicited by

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endogenous reactive TRPA1 agonists. In addition, we show that TRPA1 agonism evoked by DNCB was largely abolished by cysteine neutralization (13), which is in agreement with the known cysteine reactivity of DNCB (14). DNCB also activated rodent TRPA1, but with somewhat different kinetics. The most notable difference was the apparent absence of the first phase of activation in rodent TRPA1 at low concentrations. Underlying the species differences is the differential distribution of cysteines along the TRPA1 protein resulting in altered covalent bonding of DNCB with vicinal cysteine thiols. As DNCB was able to activate endogenous mouse TRPA1 in STC-1 cells our results on cloned receptors suggests relevance to *in vivo*.

Topical application of DNCB might target keratinocytes, fibroblasts and epidermal sensory neurons. We suggest that activation of the TRPA1 ion channel in these cell types is a novel mechanism of action for how DNCB elicits allergic dermatitis symptoms. TRPA1 activation in keratinocytes and fibroblasts results in depolarization and intracellular calcium elevation both of which are known to elicit TSLP release. TSLP will in turn directly excite sensory TRPA1-expressing sensory neurons and cause itch (8). Direct stimulation of peptidergic sensory neurons by DNCB further boosts TSLP-induced excitation. In agreement with this, peptidergic sensory neurons were shown to be critically involved in DNCB-evoked contact sensitivity in mice (15). Our finding that DNCB is a TRPA1 agonist supports the emerging literature that TRPA1 activation plays an important role in allergic contact dermatitis and suggests that TRPA1 antagonism offers a novel treatment for allergic dermatitis symptoms.

Figure legends

Figure 1. DNCB induces activation of the TRPA1 ion channel. A) Dose-dependent activation of a biphasic response by DNCB (0.04-30 μ M) from hTRPA1 expressing cells. The initial phase is brought to an end by a decrease fluorescence, after which a sustained phase is observed. B) Dose-dependent inhibition by selective TRPA1 antagonist A-967097 (0.01-1 μ M) of the response elicited by 1 μ M DNCB. C) The response induced by 1 μ M DNCB from hTRPA1 WT and -3C mutant expressing cells. Note that though the 1 μ M DNCB response is qualitatively the same as in B the scale is different due to use of a different FLIPR and settings.

Figure 2. DNCB induces hTRPA1 current. A) Whole-cell patch-clamp recording from a HEK cell stably expressing hTRPA1. The voltage protocol consisted of a ramp (-120 mV to +120mV; 50ms duration) evoked from a holding potential of -70mV every 3s. B) Current-voltage relationship of the CHEM subtracted current. The ramps used are indicated by asterisks in A.

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