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Analysis of *Drosophila* TRPA1 reveals an ancient origin for human chemical nociception

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Abstract

Chemical nociception, the detection of tissue-damaging chemicals, is important for animal survival and causes human pain and inflammation, but its evolutionary origins are largely unknown. Reactive electrophiles are a class of noxious compounds humans find pungent and irritating, like allyl isothiocyanate (in wasabi) and acrolein (in cigarette smoke)^{1–3}. Insects to humans find reactive electrophiles aversive^{1–3}, but whether this reflects conservation of an ancient sensory modality has been unclear. Here we identify the molecular basis of reactive electrophile detection in flies. We demonstrate that dTRPA1, the *Drosophila melanogaster* ortholog of the human irritant sensor, acts in gustatory chemosensors to inhibit reactive electrophile ingestion. We show that fly and mosquito TRPA1 orthologs are molecular sensors of electrophiles, using a mechanism conserved with vertebrate TRPA1s. Phylogenetic analyses indicate invertebrate and vertebrate TRPA1s share a common ancestor that possessed critical characteristics required for electrophile detection. These findings support emergence of TRPA1-based electrophile detection in a common bilaterian ancestor, with widespread conservation throughout vertebrate and invertebrate evolution. Such conservation contrasts with the evolutionary divergence of canonical olfactory and gustatory receptors and may relate to electrophile toxicity. We propose human pain perception relies on an ancient chemical sensor conserved across ~500 million years of animal evolution.

Keywords

pungency; electrophile; irritation; pain; TRP channel

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Reactive electrophiles are tissue-damaging agents that modify nucleic acids, proteins and other biomolecules. Reactive electrophiles are aversive to both vertebrates and invertebrates^{1–3}; plants and animals use them as deterrents³. Despite their importance as natural repellents, the cellular and molecular mechanisms by which reactive electrophiles deter insects have not been established. We examined *Drosophila* responses to reactive electrophiles using feeding. When a droplet of food (350 mM sucrose) contacts the legs of a hungry fly, the fly extends its proboscis to drink. This proboscis extension response (PER) is robust and sustained; >90% of the second through fifth offerings of food elicited PER (Fig. 1b). Adding the reactive electrophile allyl isothiocyanate (AITC, Fig. 1a) to the food dramatically inhibited this response (Fig. 1b). This effect was generalized to other reactive electrophiles using N-methyl maleimide (NMM) and cinnamaldehyde (CA) (Fig. 1a). Both NMM and CA robustly inhibited feeding (Fig. 1b). This inhibitory effect appeared gustatory, not olfactory, because NMM is non-volatile (m.p. 93°C) and avoidance required ingestion. When only leg contact with food was permitted, reactive electrophiles did not affect PER (Fig. 1c), suggesting that chemosensors along the path of food intake rather than the legs mediate their inhibitory effects. The bitter compound caffeine, for which there are tarsal receptors^{4,5}, robustly inhibited PER even when ingestion was not permitted (Fig. 1c).

In vertebrates, the cation channel TRPA1 is a molecular receptor for reactive electrophiles, forming covalent adducts with these chemicals and activating sensory neurons to mediate irritation and pain^{6–11}. Previous *in vitro* physiological analyses suggested that *Drosophila* TRPA1 relatives dTRPA1 and Painless were not activated by electrophiles^{6,12}, raising the possibility that flies might use different mechanisms to detect these chemicals. We reexamined the possible involvement of dTRPA1 and Painless *in vivo*, assessing the gustatory response to reactive electrophiles. In contrast to wild type, *dTrpA1* loss-of-function mutants showed no reduction in PER when offered food containing AITC, NMM, or CA (Fig. 1b). Similar defects were observed using two loss-of-function *dTrpA1* alleles (*dTrpA1^{ins}* and *dTrpA1^{fs}*)¹³ and dTrpA1 cDNA expression rescued this defect (Fig. 1d). Thus this response to reactive electrophiles is entirely TRPA1-dependent. *dTrpA1* mutants responded to other deterrents, as caffeine inhibited PER (Fig. 1b). In contrast, *painless* mutants remained responsive to reactive electrophiles (Supp Fig. 1), although responses were less robust than controls, suggesting a possible auxiliary function consistent with previous report¹⁴.

dTRPA1 protein expression was detected in the mouthparts (Fig. 2a–c), but not legs or labellum. Within the mouthparts, dTRPA1 was expressed in neurons innervating sensilla #8 and #9 of the labral sense organ (LSO) (Fig. 2b,e). LSO sensilla contain pores that open onto the esophagus lumen, providing access to chemicals in ingested food. Thus, dTRPA1 is expressed in an appropriate place to mediate ingestion-dependent responses.

To test the significance of peripheral dTRPA1 expression, tissue-specific RNAi was performed using three promoters whose expression overlaps dTRPA1-positive LSO neurons: *Dll-Gal4*, expressed broadly within peripheral tissue, *MJ94-Gal4*, expressed in chemoreceptors and the brain¹⁶, and *Gr66a-Gal4*, expressed in chemoreceptors implicated in aversive responses^{4,5} (Fig. 2 f–i). dTRPA1 knockdown using each promoter robustly reduced NMM's effect on PER, consistent with a requirement for dTRPA1 in peripheral

chemoreceptors (Fig. 2j). In contrast, dTRPA1 knockdown in the AC thermosensory neurons of the head using *dTrpA1^{SH}-Gal415* had no effect (Fig. 2j). These data cleanly distinguish the sites of action for dTRPA1 in thermotaxis and gustation, with the former involving AC neurons¹⁵ and the latter peripheral sensory neurons.

dTRPA1 expression in peripheral chemosensors also sufficed to induce reactive electrophile-dependent PER inhibition. dTRPA1 cDNA expression with *Dll-Gal4*, *MJ94-Gal4*, or *Gr66a-Gal4* rescued the mutant phenotype (Fig. 2k). In addition, ectopic expression of dTRPA1 in leg chemoreceptors (using *Gr66a-Gal4*) allowed flies to respond to electrophiles via leg contact (Fig. 2l). Thus, dTRPA1 expression in peripheral chemosensory neurons is both necessary and sufficient for reactive electrophile-induced feeding inhibition.

dTRPA1 has been considered unresponsive to electrophiles^{6,17}; however, we recently found that the original dTRPA1 cDNA contained a partially inactivating mutation¹⁵. Using wild-type dTRPA1, we found dTRPA1 was activated by multiple reactive electrophiles when expressed in *Xenopus* oocytes (Fig. 3a–d; Supp. Figs. 2 and 3). dTRPA1 orthologs from two other *Drosophila* species, *D. mojavensis* and *D. virilis*, and the malaria mosquito *Anopheles gambiae* also responded to these chemicals (Fig. 3e; Supp. Fig. 2). Combined with the sensitivity of mosquito TRPA1 to AITC in HEK cells¹⁷, these findings demonstrate multiple insect TRPA1s respond to electrophiles. Notably, electrophile-activated currents persisted after chemical withdrawal (Fig. 3a–f), contrasting with the transient activation of dTRPA1 by warmth¹⁵. Persistent activation by electrophiles has been observed for mammalian TRPA1s, and it is thought to reflect covalent association between agonists and channel^{10,11}. This similarity suggested reactive electrophiles might activate insect and mammalian TRPA1s via similar mechanisms. Finally, we demonstrated that ectopic expression of dTRPA1 in fly neurons can confer physiological sensitivity to electrophiles. In contrast to controls or motoneurons expressing Painless, dTRPA1-expressing motoneurons were cinnamaldehyde-responsive (Fig. 3f; Supp. Fig. 4). Thus, dTRPA1 acts as an electrophile sensor in multiple contexts.

Reactive electrophiles activate mammalian TRPA1s by forming covalent bonds with cysteine and lysine residues in the channel; six residues (five cysteines and one lysine) are implicated in electrophile detection and mutations in these residues decrease electrophile sensitivity^{10,11}. Insect TRPA1s conserve five of these six residues (Fig. 4a). Mutating dTRPA1 cysteines 650 and 670 to serines (dTRPA1-2C) significantly decreased AITC sensitivity (Fig. 4b–c); this dTRPA1-2C mutant remained robustly warmth-activated (Supp. Fig. 5). The shared requirement for these residues further supports a common mechanism for reactive electrophile sensing by fly and vertebrate TRPA1s. TRPA1s also exhibit some species-specific differences in chemical sensitivity; 2-aminoethoxydiphenyl borate (2-APB) and nicotine, conserved cysteine-independent agonists of mammalian TRPA1s^{10,18}, did not activate dTRPA1 (Supp. Fig. 6).

While functional similarities between insect and vertebrate TRPA1s could reflect conservation of an ancestral mechanism for electrophile detection, the electrophile insensitivity of invertebrate TRPA1 relatives like Painless¹² and *C. elegans* TRPA1

(ceTRPA1)¹⁹ raised the possibility that some insect and vertebrate TRPA1s recently converged on similar mechanisms. To test these alternatives, a phylogeny of TRPA proteins was constructed using three different approaches, Bayesian inference²⁰, maximum likelihood²¹, and neighbor joining²². Trees were rooted using TRPAs from the unicellular choanoflagellate *M. brevicollis*. All methods indicated with high confidence that the electrophile-activated TRPA1 channels of invertebrates and vertebrates belong to a monophyletic clade, the TRPA1 clade, distinct from other TRPAs (termed basal TRPAs) by both tree topology and branch lengths (Fig. 4d, e; Supp. Fig. 7, 8 and 9). The TRPA1 clade channels derive from a common ancestral TRPA1 present in the common ancestor of vertebrates and invertebrates (Fig. 4d,e). Consistent with a common evolutionary origin of electrophile detection, sequence reconstruction²³ suggested this ancestral TRPA1 contained all six critical residues associated with electrophile sensing (Fig. 4f). This mode of electrophile detection appears specific to TRPA1 clade members, as no known basal TRPAs conserve more than one of the five cysteines implicated in electrophile detection (Fig. 4a).

These analyses also suggest revisions to proposed relationships among TRPAs. Painless has been called the fly homolog of mammalian TRPA1, and ceTRPA1 considered the nematode TRPA1 ortholog. However, all analyses indicated that neither Painless nor ceTRPA1 descend from the ancestral TRPA1; both are closer to anemone and choanoflagellate TRPAs (Fig. 4d). Consistent with their electrophile insensitivity^{12,19}, Painless and ceTRPA1 lack most cysteines implicated in electrophile detection (Fig. 4a). During evolution, nematodes appear to have lost their TRPA1 ortholog and vertebrates their basal TRPA(s) (Fig. 4d–e).

Functional conservation of TRPA1 provides a simple molecular foundation for the widespread aversion to reactive electrophiles across the animal kingdom. The conservation of reactive electrophile detection differs from other chemical senses like olfaction and gustation whose origins are molecularly diverse and evolutionarily distinct^{24,25}. For example, many fly olfactory receptors are ion channels rather than the G-protein coupled receptors of vertebrates²⁵. Reactive electrophile detection also contrasts with capsaicin detection; capsaicin activates mammalian nociceptors¹, but elicits no acute response in flies or nematodes. The exceptional conservation of TRPA1-mediated nociception could relate to the toxicity of reactive electrophiles²⁶, which could provide selective pressure for maintaining an effective monitoring system.

dTRPA1's ability to mediate aversive responses to natural deterrents suggests insect TRPA1s as targets for developing new deterrents. Insect TRPA1 agonists could be useful against an array of pests, as disease vectors from mosquitoes to lice and agricultural pests from flour beetles to aphids¹⁵ contain dTRPA1 relatives. While natural electrophilic deterrents also activate mammalian TRPA1s, deterring insects and mammals alike, the differential responsiveness of insect and mammalian TRPA1s to other stimuli like temperature²⁷ and nicotine raises the possibility of identifying insect-specific TRPA1 agonists. Such selective agonists could maximize pest deterrence while minimizing irritation to other animals.

TRPA1 activation by reactive electrophiles is a key component of mammalian nociceptor function, eliciting pungency, irritation, inflammation and pain^{6–9}. Our findings demonstrate that the molecular mechanism that initiates these perceptions is not a recent evolutionary innovation and that it is not specific to vertebrates. Rather, we propose that reactive electrophile detection represents an ancient sensory modality conserved in molecular detail across ~500 million years of animal evolution.

Methods summary

Proboscis extension behavior

The proboscis extension assay was modified from ones previously described^{4,5} as detailed in Methods.

Physiology

Oocyte and larval physiology were performed largely as described^{15,28}, with additional details provided in Methods. Chemical sensitivities of wild type and mutant (dTRPA1-2C) channels were assessed by normalizing all currents to currents observed at 1mM AITC. Chemically unrelated insect repellents like DEET, IR-3535, and deltamethrin failed to activate dTRPA1 (K.K. and P.G., unpublished).

Phylogeny

TRPA sequences were assembled from available genomic and EST data. Multiple sequence alignment was performed using ProbCons²⁹ for region from ~310 amino acids N-terminal of transmembrane regions (containing the residues implicated in chemical sensing) to ~50 amino acids C-terminal of transmembrane regions (Supp. Fig. 5). Bayesian analysis was calculated with the parallel version of MrBayes 3.1.2 using mixed substitution rate matrices and gamma distributed rate variation across sites (8 categories). An exponential prior (mean = 1.0) was assumed for shape parameter of the gamma distribution, an unconstrained exponential prior (mean = 1.0) assumed for branch lengths, and a uniform prior assumed for all labeled topologies. Two independent MCMC analyses were performed (each with one cold and three heated chains), with other parameters set to defaults. Chains were run for 10,000,000 generations, and convergence inferred after cold chain topologies reached a standard deviation of split frequencies of less than 0.005 (~250,000 generations). After convergence, the first half of the chain was discarded as “burnin”. Maximum likelihood analysis was performed with PhyML 3.0, using LG substitution rate matrix, gamma distributed rate variation (8 categories) and was bootstrapped 1000 times. A BioNJ distance-based phylogenetic analysis was performed with PAUP 4b1030 and bootstrapped 1000 times. Ancestral sequence reconstruction was performed with PAML 4.2b23 using the consensus Bayesian phylogenetic tree and mean alpha rate parameter. Branch lengths were fixed.

Methods

Fly strains and immunohistochemistry

dTrpA1^{SH}-Gal4, *UAS-dTRPA1*, and *UAS-dTRPA1^{dsRNA}* transgenic strains¹⁵, as well as *Dll-Gal432*, *MJ94-Gal416,33*, *Gr66a-Gal434*, *UAS-Painless^{AR9}*¹⁴, and *painless35* mutants have been previously described. *UAS-nls:GFP* and *UAS-mCD8:GFP* fly strains were obtained from Bloomington. Anti-dTRPA1 immunohistochemistry was performed as described³¹. Details of the creation of *dTrpA1^{fS}* and *dTrpA1^{ims}* were previously reported^{13,15}. Briefly, *dTrpA1^{fS}* has a 2-bp insertion creating frameshift mutation within the third ankyrin repeat of dTRPA1, prior to the transmembrane regions. *dTrpA1^{ims}* contains two mutated copies of *dTrpA1* that flank vector targeting sequences: one copy lacks the ion pore and sixth transmembrane domain, while the other copy lacks the promoter and upstream sequences, all of exon 1, part of exon 2, and contains the 2bp insertion mutation present in *dTrpA1^{fS}*.

PER behavioral assays—Two to seven day old flies were starved overnight on wet Kim wipes, anaesthetized on ice, and affixed to a glass slide. Flies recovered in a humidified chamber for at least 2 hrs at room temperature prior to testing. During the PER assay, the fly was first satiated with water, then a solution containing tastants was touched to the forelegs as a liquid ball on a pipette tip. If the proboscis was extended and contact with the food was maintained for 2–3 sec, the response was scored as 1. If the proboscis stuttered on the tastant, or contact was brief, a 0.5 was awarded. If the proboscis failed to contact the solution within 5 sec of offering, a 0 was awarded. Each fly was offered tastants five times per experiment, and between offerings water was given to satiation. Because AITC, cinnamaldehyde and NMM were usually accepted on first offering, PER frequency was calculated for the second through fifth offerings (sum of four scores per fly divided by 4). Responses to sucrose resumed within ~10 minutes after pungent chemical exposure, indicating that feeding was not permanently impaired (K.K. and P.G., unpublished). For leg only PER assays, the procedures were as above except flies were not allowed to contact the food with their proboscis. We found that NMM had no effect on ingestion when using a previously published ingestion-independent PER assay for chemical sensitivity¹⁴, suggesting the inhibitory effects of AITC in that assay were not gustatory.

Physiology

Two-electrode voltage clamping on *Xenopus laevis* oocytes—Agonist-evoked dTRPA1 currents were recorded as previously described¹⁵, with the following modifications. Agonists of interest were added to the oocyte perfusion buffer (96 mM NaCl, 1 mM MgCl₂, 4 mM KCl, and 5 mM HEPES, pH 7.6). Voltage was initially held at –60 mV, and a 300-ms voltage ramp (–60 mV to 60 mV) per sec was applied to dTRPA1- or AgTRPA1-expressing oocytes during perfusion of agonist-containing buffer. Typical oocyte resting membrane potentials were between –25 and –60 mV. Agonist-elicited currents were specific and TRPA1-dependent; they were absent from uninjected or water-injected oocytes and were significantly reduced by mutation of two cysteine residues within dTRPA1 (Fig. 3a–e, Fig. 4b, data not shown). Furthermore, they were inhibited by ruthenium red, which

partially inhibits warmth-activated dTRPA1 and agTRPA1 currents, and they exhibited the reversal potential and rectification properties previously associated with warmth-activated dTRPA1 and agTRPA1 currents¹⁵. EC₅₀s for wild type dTRPA1 channels were obtained at -60mV, with AITC provided for 60 sec with 30 sec intervals between increasing concentrations. The low sensitivity of dTRPA1-2C to AITC precluded EC₅₀ analysis of the mutant channel.

Larval neuromuscular junction electrophysiology—TRPA channels were expressed in larval motor neurons using OK371-GAL4, a driver specific for glutamatergic neurons, as described²⁸. In all preparations, the ventral ganglion was dissected away, leaving only motor axons and terminals. Larval muscle 6 (m6) was impaled with a sharp electrode (10–20 M Ω) containing 3M KCl. Resting membrane potentials were typically between -40 and -50 mV. Saline was perfused over the preparation, then increasing concentrations of cinnamaldehyde applied using a custom built gravity perfusion system. EJP frequency was measured ~30 sec after application of each concentration using analysis scripts in Spike 2 (Cambridge Electronic Design, Cambridge, UK). Painless was overexpressed using the functional rescue construct *UAS-Painless^{AR}*¹⁴.

Molecular biology

Substitutions of cysteine/lysine residues in dTrpA1 were made by swapping a region of wild type cDNA sequence including codons of cysteine or lysine with mutated cassettes. A pair of mutually complementary oligonucleotide primers with a desired mutation were prepared, and each of them was paired with upstream or downstream primers for the first two PCR reactions. The resulting two PCR fragments overlap in the mutant primer-annealing region that contains the replaced codons, and served as template for the second PCR reaction amplified only with the upstream and down stream primers. The upstream and down stream primers were designed to be just outside of specific restriction endonuclease target sites that were used to clone the second PCR products back in the wild type dTrpA1 cDNA background sequence. The fragments amplified by PCR were confirmed by sequencing after cloning to make sure that only desired mutations were introduced in the final cDNA constructs.

Sequence alignment and phylogeny

Multiple sequence alignments were visualized using JAL2.436. Conservation reflects conservation of physico-chemical properties of residues calculated as described³⁷. Quality is inversely proportional to the cost of mutations in a residue, measure of likelihood of observing mutations³⁶. Consensus reflects percentage of modal residue. The LG substitution matrix was as described³⁸. The input data for the ancestral reconstruction was the consensus Bayesian phylogenetic tree depicted in Figure 4D. We used the “marginal reconstruction” method (RateAncestor = 1) in PAML4²⁵, which determines the posterior probability of each amino acid at each site in the protein alignment for a given node. We fixed the alpha parameter (for gamma distributed rate variation across sites) to the Bayesian expected value as determined by MrBayes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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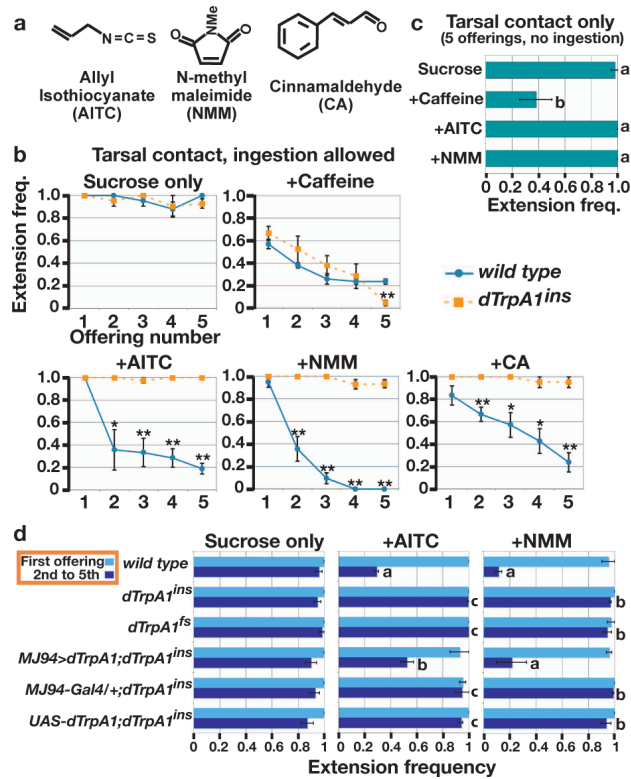


Figure 1. *dTrpA1* mediates gustatory responses to reactive electrophiles

a, Chemical structures. **b**, Proboscis extension response (PER) frequency at five sequential tastant offerings, ingestion permitted. (* $p < 0.05$, ** $p < 0.01$, unpaired t-test.) **c**, PER, tastant contacts only legs. Five sequential offerings combined ($n = 10$ flies).

d, PER, ingestion permitted: light blue, first offering; dark blue, second to fifth offerings combined. Statistically distinct groups marked by different letters (Tukey HSD, $\alpha = 0.01$). Data are mean \pm SEM. All studies use 12% (350mM) sucrose, alone or with 100 mM caffeine, 2 mM AITC, 10 mM NMM, or 6 mM CA. $n = 3$ groups of 7 flies, unless noted.

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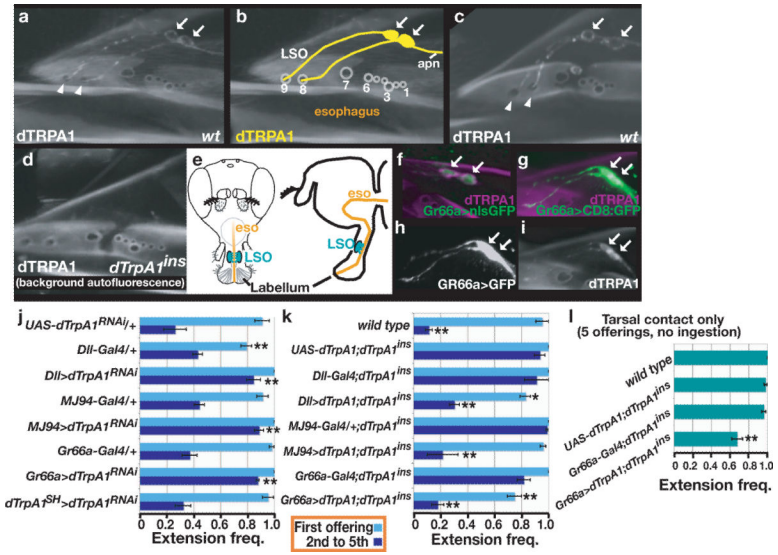


Figure 2. *dTrpA1* functions in chemosensors

a–c, *dTRPA1* expression. Arrows denote cell bodies, arrowheads distal neurites. Cuticle autofluorescence visible. **(b)**, LSO sensilla numbered. apn: accessory pharyngeal nerve. **d**. *dTrpA1* mutants lack *dTRPA1* staining. **e**. eso, esophagus. **f–i**, *Gr66a-Gal4* and *dTRPA1* co-expression in LSO. **(f)** Nuclear and **(g,h)** membrane GFPs expressed using *Gr66a-Gal4*. **j–l**, PER to 350 mM sucrose containing 10mM NMM. **(j, k)** Ingestion permitted. **l**, tarsal contact only. **j**, *dTRPA1* knockdown. **k**, *dTRPA1* rescue. **l**, *dTRPA1* gain-of-function. *: $\alpha=0.05$, **: $\alpha=0.01$, differ from *Gal4* and *UAS* controls, Tukey HSD. **j,k**, $n=3$ groups of 7–8 flies, **l**, $n=10$ flies.

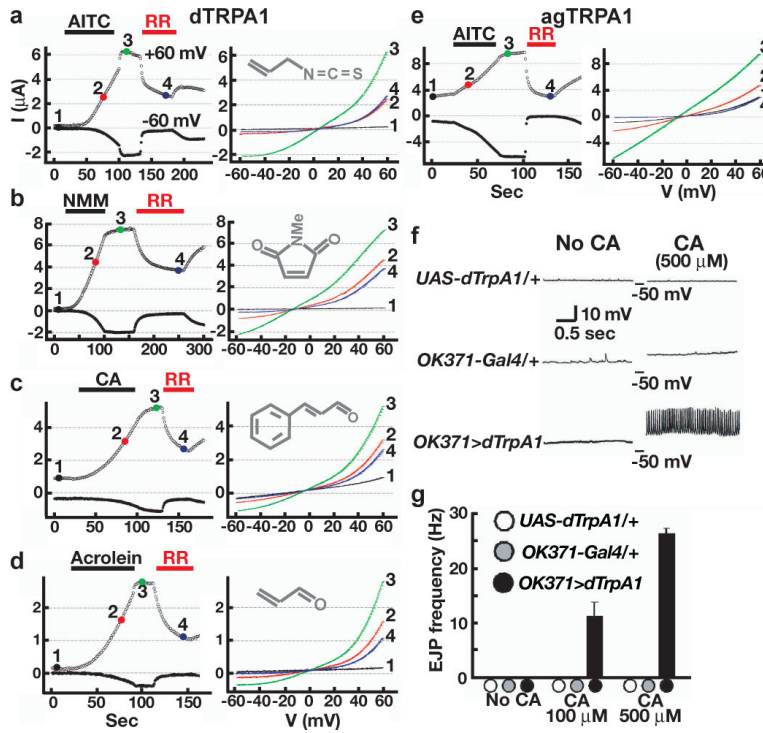


Figure 3. Insect TRPA1s are reactive electrophile sensors
a–e. Representative responses of dTRPA1 (**a–d**) and agTRPA1 (**e**) expressed in oocytes. Left panels, currents at -60 and $+60$ mV. Perfusion buffer containing indicated chemical (**a**, **c**, **d**, $100 \mu\text{M}$; **b**, **e**, $40 \mu\text{M}$) was applied for 60–80 sec. $100 \mu\text{M}$ ruthenium red (RR) applied as noted. Right panels show I–V relationships at points marked on left panels. **f**, **g**, Ectopic dTRPA1 expression confers electrophile sensitivity upon motor neurons. **f**, Motor neuron-driven excitatory junction potentials (EJPs) from third instar larval muscles. **g**, Mean EJP frequencies. In controls, no EJPs were observed.

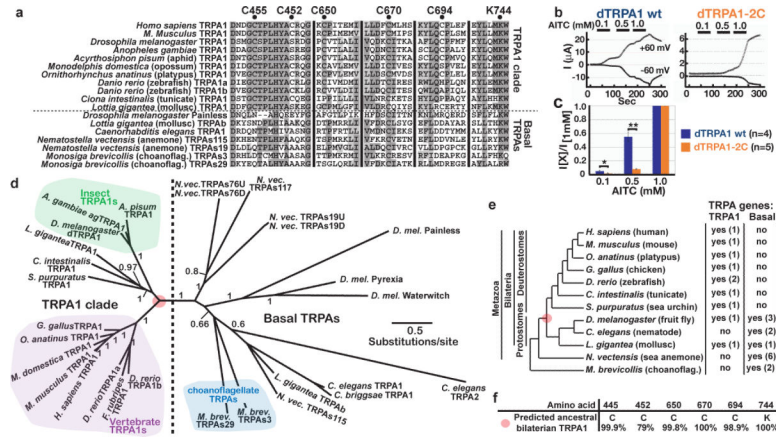


Figure 4. TRPA phylogeny

a, Conservation of residues implicated in electrophile detection. choannoflag, choannoflagellate. **b**, TRPA1-wt (wild type) and dTRPA1-2C channels in *Xenopus* oocytes. 60 sec pulses of AITC (0.1, 0.5, and 1.0 mM) were applied with 25 sec intervals. **c**. +60 mV currents normalized to channel's response to 1.0 mM AITC. **p*<0.05, ***p*<0.001, unpaired *t*-test. **d**, Bayesian consensus phylogeny for TRPAs. Internal branches labeled by posterior probability (<0.5 branches collapsed). Red dot denotes ancestor of TRPA1 clade. **e**, Cladogram showing TRPA complements, including numbers of channels. Red dot denotes bilaterian ancestor. **f**, PAML residue identity estimates for ancestral TRPA1.