Analysis of phototoxin taste closely correlates nucleophilicity to type 1 phototoxicity

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Edited by Paul Garrity, Brandeis University, and accepted by Editorial Board Member Michael Rosbash May 2, 2019 (received for review April 9, 2019)

Pigments often inflict tissue-damaging and proaging toxicity on light illumination by generating free radicals and reactive oxygen species (ROS). However, the molecular mechanism by which organisms sense phototoxic pigments is unknown. Here, we discover that Transient Receptor Potential Ankyrin 1-A isoform [TRPA1(A)], previously shown to serve as a receptor for free radicals and ROS induced by photochemical reactions, enables *Drosophila melanogaster* to aphotically sense phototoxic pigments for feeding deterrence. Thus, TRPA1(A) detects both cause (phototoxins) and effect (free radicals and ROS) of photochemical reactions. A group of pigment molecules not only activates TRPA1(A) in darkness but also generates free radicals on light illumination. Such aphotic detection of phototoxins harboring the type 1 (radical-generating) photochemical potential requires the nucleophile-sensing ability of TRPA1. In addition, agTRPA1(A) from malaria-transmitting mosquitoes *Anopheles gambiae* heterologously produces larger current responses to phototoxins than *Drosophila* TRPA1(A), similar to their disparate nucleophile responsiveness. Along with TRPA1 (A)-stimulating capabilities, type 1 phototoxins exhibit relatively strong photo-absorbance and low energy gaps between the highest occupied molecular orbital and the lowest unoccupied molecular orbital. However, TRPA1(A) activation is more highly concordant to type 1 phototoxicity than are those photochemical parameters. Collectively, nucleophile sensitivity of TRPA1(A) allows flies to taste potential phototoxins for feeding deterrence, preventing postingestive photo-injury. Conversely, pigments need to bear high nucleophilicity (electron-donating propensity) to act as type 1 phototoxins, which is consistent with the fact that transferring photoexcited electrons from phototoxins to other molecules causes free radicals. Thus, identification of a sensory mechanism in *Drosophila* reveals a property fundamental to type 1 phototoxins.

nucleophile | type 1 phototoxins | chemosensation | photochemistry

Sunlight is actively used by organisms on Earth through photochemical reactions that underlie fundamental biological functions such as photosynthesis, vision, synthesis of vitamin D, and entrainment of circadian rhythm. In contrast, photoexcitation of chemical compounds can result in chemical transformation that generates tissue-damaging and proaging free radicals and singlet oxygens. For this reason, the chemical compounds that yield the toxic photochemical products on light exposure are regarded as phototoxins, categorized into two classes: radical-generating type 1 and singlet oxygen-generating type 2 phototoxins (1). Pigments with alternating single and double bonds absorb light at specific wavelengths through conjugated electron systems (2, 3). Photoabsorbance by pigments is a prerequisite for photochemical reactions, but theoretical prediction of phototoxins among pigment molecules has been challenging (4).

Riboflavin, also known as vitamin B_2, is a precursor of flavin adenine dinucleotide and flavin mononucleotide (FMN), which are cofactors of redox enzymes essential for cellular respiration, probably, in addition, exerting effects of antiaging and antioxidative stress (5). The vitamin can be amply ingested through many nutritional sources, despite being a potent type 1 and type 2 phototoxin excited by UV to blue light (6, 7). Its blood concentration is strictly controlled via excretion to urine (8, 9), which may thus prevent photosensitive damage in the skin. Such a systematic photoprotection mechanism against riboflavin suggests that organisms have evolved to survive phototoxins present in the diet. To our knowledge, little is, however, known about how organisms detect phototoxins and avoid ingestion.

In insects including *Drosophila melanogaster* and *Anopheles gambiae*, two isoforms of TRPA1, TRPA1(A) and TRPA1(B), arise from the *TrpA1* locus by alternatively opting in one of two different exons, each with its own start codon (10). As a result, the two isoforms are distinct in the distal N-terminal region, but sharing >90% of the primary structure that includes pore-forming transmembrane domains. TRPA1(B) is a highly temperature-sensitive nonselective cation channel, widely used as a thermogenetic actuator in *Drosophila* circuitry studies (11, 12). In contrast, TRPA1(A) has been recently characterized as an ion channel receptor for nucleophilic compounds. One implication of its nucleophile sensitivity was found in UV-dependent feeding deterrence, where UV-induced free radicals and reactive oxygen species activate TRPA1(A) in bitter gustatory receptor neurons via nucleophile sensitivity of the channel (13). Here, we find that TRPA1(A), which acts as a receptor for radicals generated after light illumination, aphotically detects polycyclic phototoxins, which are capable of radical and reactive oxygen species generation on light illumination. Our results show that ingestion of potential

Significance

Light-absorbing pigments undergo chemical changes via excitation of electrons. The electron excitation may generate reactive chemicals such as free radicals, and eventually reactive oxygen species, causing tissue damage like sunburn. Such pigments able to exert toxic effects on light illumination are regarded as phototoxins. Despite advances of our chemical knowledge on phototoxicity, the prediction of intrinsic pigment phototoxicity has been limited. Our study shows that the insect chemical receptor called Transient Receptor Potential Ankyrin 1 (TRPA1) is able to discern intrinsic phototoxins among pigments, for which TRPA1 needs the capability of sensing nucleophiles, chemical compounds with strong electron-donating tendency. Accordingly, understanding insect chemical receptor TRPA1 leads to the discovery of nucleophilicity as a chemical signature of intrinsic phototoxicity.

Author contributions: E.J.D., T.J.A., S.-T.K., and K.K. designed research; E.J.D., T.J.A., H.S., H.J., and S.-T.K. performed research; E.J.D., T.J.A., H.S., H.J., H.-W.K., and K.K. analyzed data; and H.-W.K. and K.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. P.G. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1905998116/-/DCSupplemental.

Published online May 28, 2019.

phototoxins is suppressed through TRPA1(A)-dependent gustatory aversion. Thus, nucleophile sensitivity of TRPA1(A) is critical for detection of chemical cause (phototoxins) and effect (free radicals) of photochemical reactions. Conversely, our results suggest that pigment compounds need to bear nucleophilicity to be strong radical-generating phototoxins.

Results and Discussion

To examine whether riboflavin is phototoxic to the genetically tractable model organism *Drosophila melanogaster*, larvae grown in riboflavin-enriched (0.3 or 10 mM) media were illuminated with blue light (*Materials and Methods*). Indicative of riboflavin phototoxicity, survival of the larvae to adults was dramatically reduced by the irradiation, compared with equally illuminated larvae grown in normal food (Fig. 1*B* and *SI Appendix*, Fig. S1 *A–C*). Note that the larvae in riboflavin-rich food showed similar degrees of ingestion amounts when spectrophotometrically assessed (*SI Appendix*, Fig. S1 *E–H*), and irradiated larvae were no longer subject to riboflavin-enriched ingestion, as they were subsequently placed in normal food. When a single choice of food is offered as in our survival assay, the ingestion amount is estimated to be little affected by excessive riboflavin, as riboflavin in food did not lead to adult weight loss (*SI Appendix*, Fig. S1*I*), a marker of larval starvation (14). Next, we investigated whether riboflavin evoked gustatory aversion, using the capillary feeder assay (CAFE) (15). Although feeding avoidance to riboflavin alone at its water-saturating concentration of 0.3 mM was not observed, riboflavin was well avoided in the presence of 1 mM *N*-methyl maleimide (NMM), a concentration also singly subefficacious for robust feeding deterrence (Fig. 1*C*). NMM is a well-established electrophilic agonist activating TRPA1s from mammals to insects through covalent modification of cysteine sulfhydryl residues (10, 13, 16, 17). Such functional interaction between riboflavin and NMM implies that the *dTrpA1* gene may be important for riboflavin gustation. Indeed, the $dTrpA1^{ins}$ loss-of-function mutant (10, 17, 18) was impaired for NMM-aided riboflavin aversion (Fig. 1*C*). The defective feeding deterrence of the mutants was fully restored by *dTrpA1(A)*, but not *dTrpA1(B)* cDNA with *Gr89a-Gal4* (Fig. 1*D*) covering *dTrpA1(A)*-expressing bitter-sensing neurons (10, 19). The feeding deterrence was little affected by a typical indoor lighting condition (white light at ~0.2 mW/cm^2), despite involving the phototoxin (Fig. 1*E*). Riboflavin-evoked action potentials were readily recorded in the *dTrpA1*-positive gustatory receptor neurons (GRNs) of labella i-a bristles (10, 13, 16, 19) in the presence of 1 mM NMM, regardless of lighting condition (Fig. 1 *F* and *H*). The GRN responses also required *dTrpA1* (Fig. 1 *F* and *G*), revealing coherence between the behavioral and cellular riboflavin-tasting mechanisms. Despite its chemical reactivity, NMM does not seem to covalently modify riboflavin and generate new chemical species, as spectral absorbance of riboflavin was unchanged after 30 min incubation with NMM (*SI Appendix*, Fig. S1*J*).

In addition to riboflavin/NMM-dependent potentiation of dTRPA1(A) in vivo, we examined whether the riboflavin is able to sensitize bitter gustation, as *dTrpA1* is expressed in bitter GRNs (10). Caffeine and berberine at suboptimal concentrations of 0.2 and 0.001 mM, respectively, failed to induce appreciable avoidance in the CAFE assays (*SI Appendix*, Fig. S2 *A* and *E*). However, when the low concentrations of bitter compounds were offered with riboflavin, the avoidance was significantly increased. Such synergistic feeding aversion was absent in $dTrpA1^{ins}$ (*SI Appendix*, Fig. S2 *B* and *F*). Consistent with the behavioral outcome, we obtained very similar results in GRN responses (*SI Appendix*, Fig.S2 *C*, *D*, *G*, and *H*). Again, *dTrpA1(A)*, but not *dTrpA1(B)*, was necessary for the riboflavin-dependent GRN and feeding responses to the low concentrations of bitter compounds. Thus, ingestion of riboflavin that is phototoxic to flies can be suppressed by a *dTrpA1*-dependent mechanism, as the

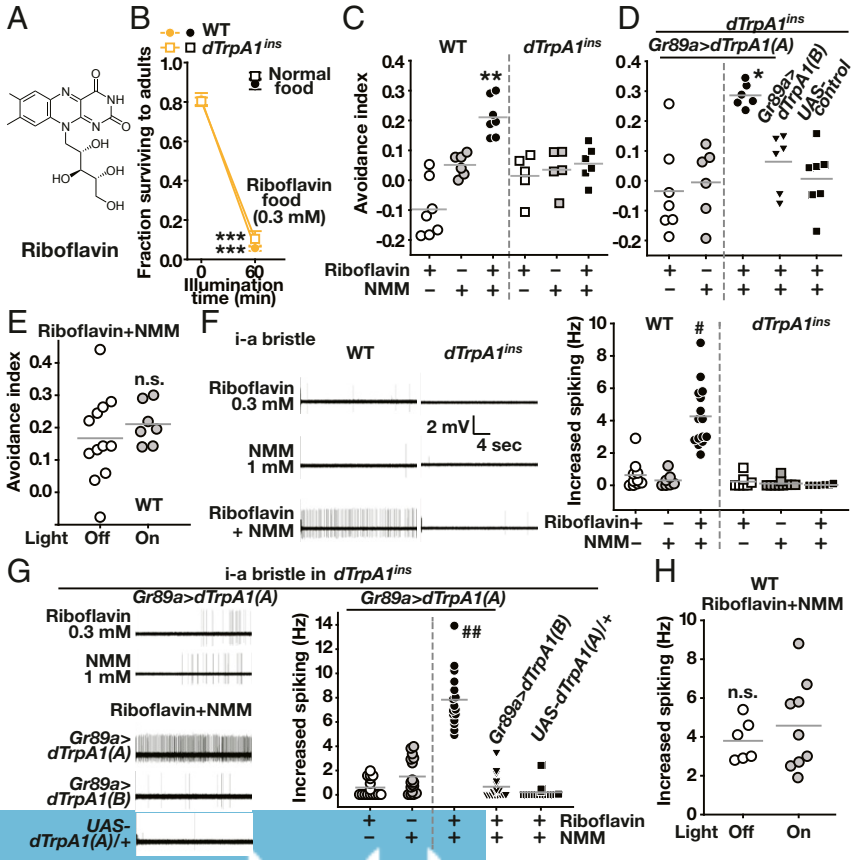

Fig. 1. Riboflavin, phototoxic to *Drosophila*, causes gustatory aversion through *dTrpA1(A)*. (*A*) Riboflavin structure. (*B*) Phototoxicity of riboflavin to *Drosophila* (n = 4–11). ***P < 0.001, unpaired t test. (*C*) Riboflavin gustatory aversion assessed by NMM-aided CAFE assays with wild-type (WT, Canton S) and $dTrpA1^{ins}$ animals (n = 5–7). (*D*) Rescue of feeding aversion in $dTrpA1^{ins}$ by *dTrpA1(A)*, but not by *dTrpA1(B)* (n = 6–7). (*E*) Riboflavin CAFE with or without ambient light of ~0.2 mW/cm^2 (n = 7–12). (*F*) Riboflavin responses in *dTrpA1*-expressing bitter taste neurons in i-a bristle sensilla of indicated genotypes (n = 7–15). (*G*) Rescue of impaired spiking in $dTrpA1^{ins}$ by *dTrpA1(A)* (n = 16–17). (*H*) GRN responses in indicated lighting conditions (n = 6–9). On: ~0.25 mW/cm^2. n.s., not significant, paired t test. *P < 0.05; **P < 0.01, ANOVA Tukey or Welch's ANOVA Games-Howell test; #P < 0.05; ##P < 0.01, ANOVA Dunn's rank test.

dTrpA1(A)-dependent riboflavin gustation can enhance the responses to diverse chemicals stimulating bitter GRNs. Dramatic riboflavin accumulation was reported in yeast mitochondria up to a millimolar range when moderate concentrations of riboflavin were provided from the environment (20). Moreover, there are flavogenic yeasts that can produce riboflavin several-hundred-fold in certain conditions (21), together implying that there may be natural situations in which high doses of riboflavin are available for feeding, and that flies may encounter the high riboflavin concentration used in our experiments.

Heterologously expressed in *Xenopus* oocytes, dTRPA1(A), but not dTRPA1(B), showed dose-dependent current responses (Fig. 2*A* and *SI Appendix*, Fig. S3*A*), as the riboflavin current was normalized with respect to the current acquired by 0.1 mM NMM. This result indicates that dTRPA1(A) serves as a direct receptor for riboflavin in an isoform-dependent manner. Moreover, agTRPA1(A) from malaria-transmitting mosquitoes, *Anopheles gambiae*, yielded much larger current responses to riboflavin than dTRPA1(A), whereas isoform dependence persisted (Fig. 2*A* and *SI Appendix*, Fig. S3*C*). The current acquired with 0.1 mM NMM is close to the maximum for both dTRPA1(A) and agTRPA1(A), as the apparent conductance at −50 mV after NMM perfusion is saturating to the maximum similarly for both channels (*SI Appendix*, Fig. S3*F*). Therefore, data normalization with 0.1 mM NMM semiquantitatively compares highly divergent current responses to riboflavin between species, regardless of possibly varying NMM sensitivities of TRPA1(A)s. In contrast to the conservation of riboflavin-sensing function in the two insect TRPA1s, human TRPA1 (hTRPA1), deficient for nucleophile sensitivity (13), failed to respond to riboflavin (Fig. 2*B* and *SI Appendix*, Fig. S3*E*). Ambient light (0.5 mW/cm^2) is dispensable for riboflavin-induced TRPA1 current responses despite riboflavin phototoxicity, as the response of heterologous agTRPA1(A) insignificantly varies in darkness and light (Fig. 2 *C* and *D*). Together with observation on hTRPA1 lacking riboflavin responses, the isoform and species dependences are reminiscent of previously described nucleophile sensing by TRPA1(A) (13), suggesting that the nucleophile detection mechanism may pertain to riboflavin sensing by TRPA1(A). In the previous study, nucleophile sensitivity of dTRPA1(A) was drastically reduced by substitutions of the conserved residues (C105A and R113A/R116A) in the amino-terminal isoform-specific region, whereas electrophile-dependent activation was preserved. We here show that riboflavin responses of dTRPA1(A) were also severely decreased by the mutations (Fig. 2*E* and *SI Appendix*, Fig. S3*B*). To extend implication of this result to agTRPA1(A), C85A and R93A/R96A substitutions corresponding to the dTRPA1(A) mutations above were introduced. The mutant channels agTRPA1(A)C85A and agTRPA1(A)R93A/R96A in oocytes showed severe impairment in the capability of detecting nucleophilic reductant DTT, which potently activated agTRPA1(A)WT (Fig. 2*F*). Similarly, riboflavin responses of these mutant agTRPA1(A)s were dramatically reduced, reflecting intimate relationship between the mechanisms sensing riboflavin and nucleophilic DTT (*SI Appendix*, Fig. S3*D*). The abrogation of riboflavin responsiveness by the mutation was also recapitulated in in vivo GRNs and feeding behavior (*SI Appendix*, Fig. S3*G*); expression of *dTrpA1(A)R113A/R116A* cDNA dominant-negatively suppressed GRN and feeding responses to riboflavin/NMM mixture. Unlike a previous report of successful expression of hTRPA1 in *Drosophila* (22), we failed to observe consistent functional expression of hTRPA1 in fly GRNs (*SI Appendix*, Fig. S3*H*), hindering us from examining its riboflavin responsiveness in flies. To corroborate that a common activation pathway exists for riboflavin and nucleophiles, heterologous dTRPA1(A) fully activated by 0.3 mM riboflavin was further stimulated by applying 10 mM DTT premixed with 0.3 mM riboflavin. The current induced by 0.3 mM riboflavin was insignificantly increased by an additional 10 mM DTT, although dTRPA1(A) was able to show larger currents in response to electrophilic NMM at 0.1 mM (Fig. 2 *G* and *H*). Thus, we present four observations as evidence of intimate functional correlation between riboflavin and nucleophile sensitivities: both similar isoform and species dependences, covarying responses of loss-of-function dTRPA1(A) and agTRPA1(A) mutants, and saturation of nucleophile-induced activation by riboflavin. These results strongly advocate that riboflavin detection is a physiological function of TRPA1(A)-mediated nucleophile sensing. In addition, riboflavin analogs, lumiflavin, lumichrome, and FMN were also sensed by TRPA1(A) in vitro and in vivo, and induced gustatory aversion in manners parallel with the riboflavin response (*SI Appendix*, Figs. S4 and S5), implying the importance of the core structure that accommodates the light-absorbing conjugation system consisting of delocalized electrons in connected π orbitals. This is consistent with the Grutthuss-Draper law that photoabsorption is requisite for photochemical reactions.

Nucleophiles act as electron donors in chemical reactions. Similarly, type 1 photochemical reactions transfer photoexcited electrons from phototoxins to other molecules, thereby generating free radicals and exerting phototoxicity (1). In addition to

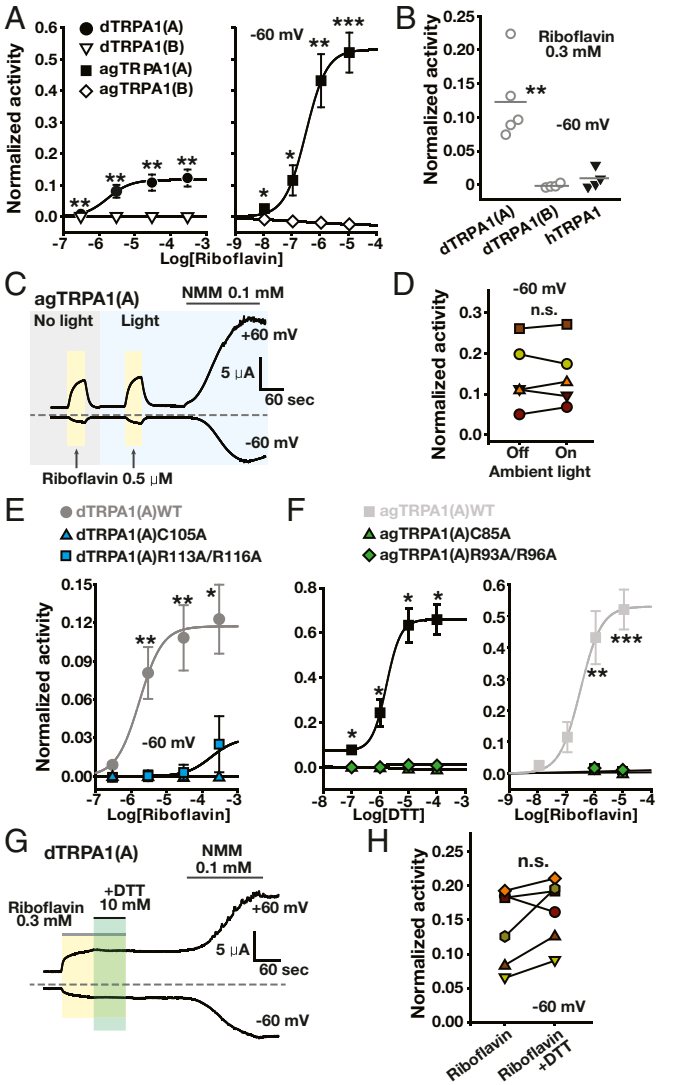

Fig. 2. Riboflavin stimulates TRPA1(A)s through the nucleophile-dependent activation pathway. (*A*) Riboflavin dose dependence of TRPA1 isoforms from *Drosophila* and *Anopheles* expressed in *Xenopus* oocytes (n = 4–5). (*B*) Lack of riboflavin responses of hTRPA1 (n = 4–5). (*C* and *D*) Riboflavin-induced currents from agTRPA1(A) with or without ambient light (~0.5 mW/cm^2; n = 5). (*E* and *F*) Riboflavin and DTT responses of nucleophile-insensitive TRPA1(A) mutants (n = 4–5). (*G* and *H*) Saturation of nucleophilic DTT-evoked activation of dTRPA1(A) by riboflavin (n = 6). NMM, electrophilic agonist, *N*-methylmaleimide; n.s., not significant, paired t test. *P < 0.05; **P < 0.01; ***P < 0.001, Tukey or unpaired t test.

the intimately linked riboflavin and nucleophile sensitivities of TRPA1(A), the conceptual agreement between nucleophilicity and type 1 phototoxicity prompted us to investigate whether the nucleophile receptor TRPA1(A) indeed selectively detects type 1 photochemical potential of pigment molecules. To this end, we first set out to identify more pigments activating TRPA1(A) in darkness. Of 11 color-expressing dyes and four cyclic chemicals with the π conjugation system (*SI Appendix*, Fig. S7 and Table S1), five pigments, such as rose bengal, eosin Y, porphine (meso-tetraphenylporphine-4,4′,4″,4‴-tetrasulfonic acid), methylene blue, and merocyanine 540, were found to stimulate agTRPA1(A) in addition to riboflavin and its analogs (Fig. 3 and *SI Appendix*, Figs. S6 and S7). Isoform and species dependences were observed as with riboflavin. Moreover, agTRPA1(A)C85A and agTRPA1(A) R93A/R96A mutants, defective for both nucleophile and riboflavin sensing, showed severely reduced responses to the dyes activating wild-type agTRPA1(A). Compared with other pigment agonists of TRPA1(A), rose bengal provoked relatively large currents from dTRPA1(A) and agTRPA1(A). This is probably a result of dual stimulation of TRPA1(A)s via both electrophile- and nucleophile-dependent activation pathways, given that dTRPA1(B) and agTRPA1(B), which sense electrophilic but not nucleophilic reactivity (13), were moderately stimulated by rose bengal. TRPA1(A) can be activated by either nucleophiles or electrophiles, and responds more robustly to amphiphilic chemicals, as other polymodal TRP channels are synergistically stimulated by two different activation modalities (23, 24). For example, TRPA1(A) and TRPA1(B) yielded rose bengal-like activation profiles in response to hydrogen peroxide (H_2O_2) bearing both electrophilicity and nucleophilicity (13). Porphine shows inhibition of agTRPA1(A) at a high concentration (0.2 mM; *SI Appendix*, Fig. S6). As porphine is a structural backbone of heme in oxygen-delivering red blood cells, hematophagous mosquitoes may have evolved to accept rather than feel averse to porphine by repressing TRPA1(A) activity at the high concentrations of porphine. Detection of these pigments by agTRPA1(A) is independent of ambient light, as observed with riboflavin (*SI Appendix*, Fig. S8).

Next, to test our hypothesis that TRPA1(A) selectively detects radical-generating phototoxins among pigments, we examined whether TRPA1(A)-activating pigments were type 1 phototoxins by adopting polymerization of acrylamide as a reporter of free radical production. Beyond its biochemical utility in gel preparation for polyacrylamide electrophoresis (PAGE), radical-initiated polymerization is a well-characterized phenomenon in the field of polymer science. Indeed, riboflavin and FMN have been used to initiate PAGE gel polymerization in combination with light illumination (25). This was reproduced in our hands with not only riboflavin/FMN but also most of TRPA1(A)-activating pigments at 5 μM (Fig. 4*A*). Typical acrylamide polymerization for PAGE by ammonium persulfate, which chemically, not photochemically, generates free radicals, requires tetramethyl-ethylenediamine (6.67 mM). Consistently, tetramethyl-ethylenediamine was necessary for light-induced pigment-dependent acrylamide gelation. Whereas the polymerization was monitored for 60 min, the gel solution was continuously irradiated with white light at ∼90 mW/cm^2 from a xenon lamp, simulating natural sunlight in wavelength composition and irradiance on the ground (13, 26). Eight of nine TRPA1(A)-activating pigments completed gelation within 30 min (mostly <10 min except porphine), whereas 3.4 mM ammonium persulfate completed gelation within ∼14 min without light irradiation (Fig. 4*A*). Similar results were obtained with 0.1 mM of each pigment that failed to cause the polymerization at 5 μM (*SI Appendix*, Fig. S9*A*; 0.01 mM and 0.05 mM for hypericin and curcumin, respectively, because of solubility). Nevertheless, our photochemical gelation assays may not be sufficiently sensitive, leaving the possibility of false-negative results. Therefore, the phototoxic potential was reappraised with the fluorescent superoxide indicator, dihydroethidium, which is converted to 2-OH-ethidium by reacting with superoxide radicals (27) (*Methods*). Consistent with photochemical gelation, 1-min-long illumination of the white light to the nonpolymerizing pigments/chemicals led

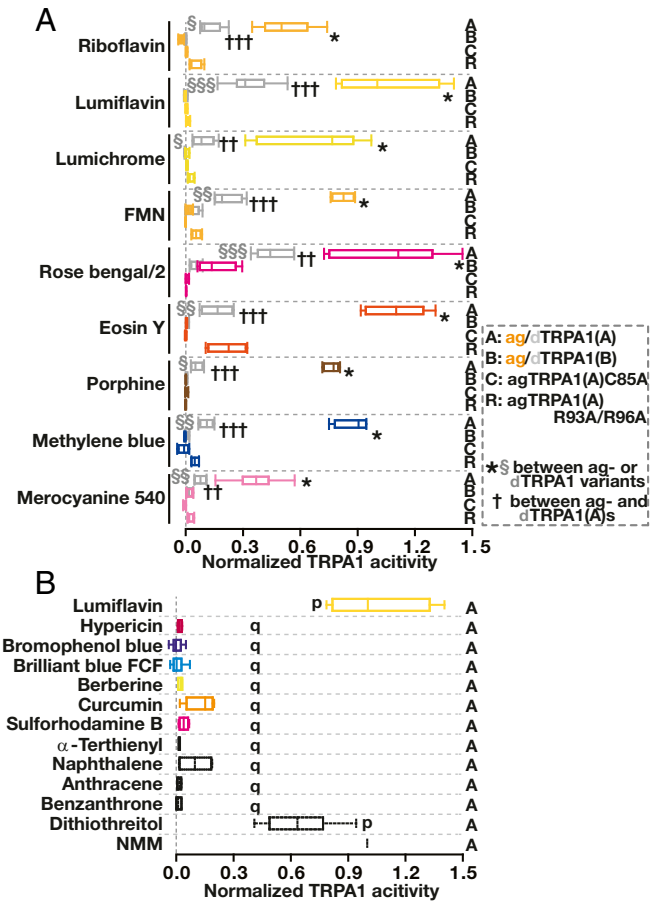

Fig. 3. Identification of pigments activating TRPA1(A) with isoform and species dependences as observed with nucleophiles. (*A* and *B*) Maximum current amplitudes by pigments and chemicals of indicated TRPA1 variants. If current increases are not saturated in dose dependence experiments, the maximally soluble concentrations in Barth's solution were used. Colors of box plots simulate those of pigments. Gray boxes for *Drosophila* TRPA1 isoforms (*A*), and black boxes for chemicals without color expression (*B*). $^*P <$ 0.05, Games-Howell test between four agTRPA1 variants; $^{\S}P < 0.05$, $^{\S\S}P <$ 0.01, and $^{\S\S\S}P < 0.001$, unpaired *t* test between dTRPA1 isoforms; $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$, unpaired *t* test between TRPA1(A)s. $n = 4$–6 (*A*) and 3–10 (*B*). Lowercase letters indicate statistically distinct groups ($P < 0.05$, Tukey).

to little fluorescence increases of 2-OH-ethidium, whereas lumichrome, rose bengal, porphine, and methylene blue showed fluorescence increases (Fig. 4*B* and *SI Appendix*, Fig. S9*B*). Type 2 photochemical reactions excite triplet oxygen to singlet oxygen ($^1O_2$), which is also toxic because of its reactivity. To examine whether TRPA1(A)-activating pigments are associated with the type 2 reaction, we turn to anthracenediyl-bis(methylene) dimalonic acid (ABDA), which is bleached by $^1O_2$ (28, 29). Pigment solutions containing ABDA were illuminated with the xenon light for 0–2 min. These experiments show that type 2 reactions do not clearly correlate with TRPA1(A) activation compared with type 1 reactions (Fig. 4*C* and *SI Appendix*, Fig. S9*C*). One-minute illumination to type 1-photoreactive lumiflavin and lumichrome bleached ABDA to degrees indistinguishable from ABDA-only control, although riboflavin, FMN, rose bengal, eosin Y, porphine, and methylene blue significantly increased ABDA bleaching. Moreover, $^1O_2$ generation can be indirect, as $^1O_2$ is often produced in the reaction of the type 1 reaction product superoxide with peroxides (30). Hypericin and α-terthienyl, previously reported to be natural phototoxins (31), were insignificantly distinct from controls, possibly because they

are context-dependent phototoxins, as previously observed with berberine, which requires physical binding with DNA for phototoxicity (32). Correlation analysis between pigment-dependent agTRPA1(A) activation and gelation time reveals a negative monotonic relationship (Spearman $\rho = -0.872$; $P = 0.0000002$; Fig. 4*D*), indicating higher radical generation from the dyes more robustly activating agTRPA1(A). Supporting our in vitro results, feeding type 1 phototoxins severely decreased survival of larvae illuminated by white light at 90 mW/cm^2 (*SI Appendix*, Fig. S10). In contrast, pigments lacking apparent radical generation such as brilliant blue and sulforhodamine B did not aggravate the survival compared with normal food (*SI Appendix*, Fig. S10). Note that rose bengal was aphotically toxic, barring us from testing its in vivo phototoxicity even at low concentrations.

To assess how closely TRPA1(A) activation is associated with type 1 phototoxicity, we calculated phototoxicity concordance, the percentage of chemicals specifically concordant with the phototoxicity for each account of pigment properties such as TRPA1(A)-stimulating capability or photochemical properties; compounds with values out of the data range of the other phototoxicity category (either type 1 or non-type 1 phototoxins) were determined to be concordant to type 1 phototoxicity (Fig. 4 *E*–*G* and *SI Appendix*, Fig. S9*D*, arrows). Notably, type 1 phototoxicity is 89% concordant to amplitudes of agTRPA1(A) responses (Fig. 4*E*). Although light absorbance is requisite for photochemical reactions, molar attenuation coefficient (MAC) of pigments yielded low phototoxicity concordance of 42% among tested molecules (Fig. 4*F* and *SI Appendix*, Table S1). Maximum-conjugated π electron numbers (PENMC) (4), which determine wavelengths of absorbed light, also showed marginal phototoxicity concordance (21%; *SI Appendix*, Fig. S9*D*). The energy gap between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) has been proposed to be a predictor of phototoxicity, albeit with a mediocre level of prediction success (4, 33). Concordance of HOMO–LUMO gaps (HLG) to type 1 phototoxicity reached only 63% in our survey (Fig. 4*G* and *SI Appendix*, Table S1), which is lower than that of TRPA1(A) activation, at 89% ($P = 0.0375$, Berger's exact unconditional test). Merocyanine 540, evoking the lowest current amplitude from agTRPA1(A), is the only pigment that failed to exhibit type 1 phototoxicity among those activating TRPA1(A) possibly because its MAC and HLG are suboptimal for robust photosensitization (Fig. 4 *E*–*G*, arrowheads, and *SI Appendix*, Table S1), suggesting that multiple photochemical descriptors such as HLG, MAC, and nucleophilicity must be concurrently fulfilled to constitute type 1 photoreactivity.

Our study on the molecular mechanism for gustatory aversion to phototoxins shows that TRPA1(A) selectively and aphotically recognizes pigments with intrinsic type 1 photochemical potential. Interestingly, agTRPA1(A) exhibited stronger responses to phototoxins than dTRPA1(A) in terms of current amplitudes and, in some cases, EC50s (*SI Appendix*, Table S1). Along with the recent finding on *Anopheles*' sensitive biting cessation by light (34), which would require agTRPA1(A), given the previous study with *Drosophila* (13), natural variation of phototoxin responses between dTRPA1(A) and agTRPA1(A) suggests that malaria-transmitting *Anopheles* need to avoid light and phototoxins more acutely than *Drosophila*. This may be because of evolutionary pressure, possibly from their high susceptibility to phototoxicity, a potential window to controlling mosquitoes that transmit deadly diseases. Although human TRPA1 does not detect nucleophiles (13), mammalian TRPC5 responds to DTT and thioredoxin via reduction of an extracellular disulfide bond (35), implying that phototoxin sensing by nucleophile receptors may be a general phenomenon arising across a wide range of animal species. However, it awaits further investigation if TRPC5 is a nucleophile receptor sensing phototoxins rather than being narrowly tuned for specific reductants.

As nucleophile sensitivity of TRPA1(A) is necessary for its responsiveness to type 1 phototoxins, we propose that type 1 phototoxicity requires nucleophilicity. Association of nucleophilicity with type 1 phototoxicity is unprecedented to our knowledge, but conceptually befitting, as type 1 photoreaction, during which photoexcited electrons in pigments are transferred

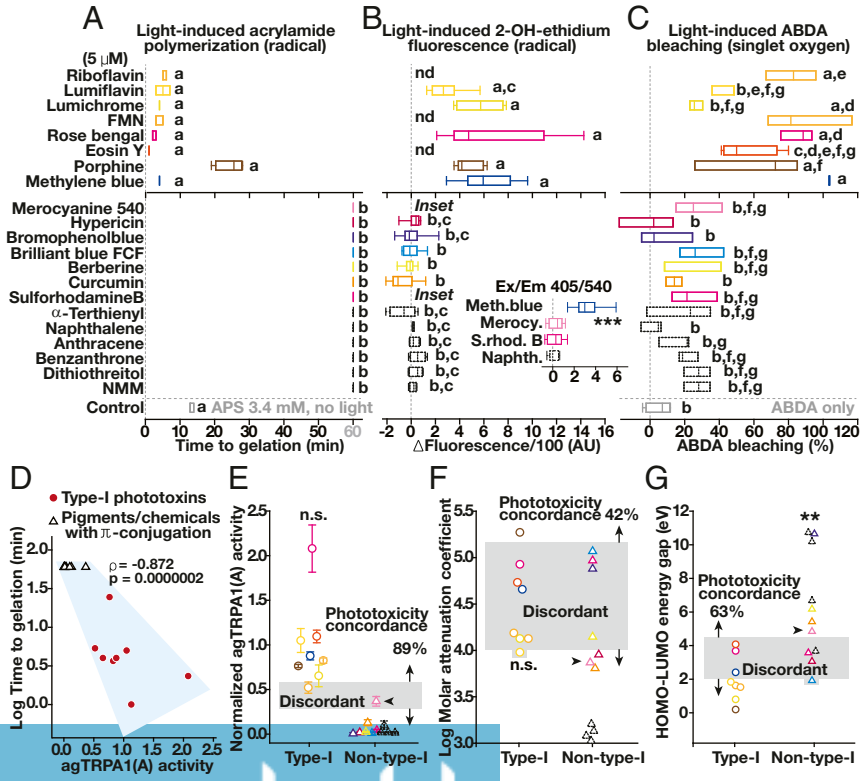

Fig. 4. TRPA1(A) activation by pigments is highly concordant to type 1 phototoxicity. (*A* and *B*) Light-induced radical generation probed by acrylamide polymerization (*A*, n = 3–5) and dihydroethidium transformed to 2-OH-ethidium on reaction with superoxide radicals (*B*, n = 4–12). Excitation/emission: 405/570 (*B*) or 405/540 nm (*Inset*). (*C*) Light-induced singlet oxygen production probed by ABDA bleaching (n = 3–4). Letters indicate statistical differences between groups. $P < 0.05$, Welch's ANOVA Games-Howell tests. (*D*) Spearman correlation analysis showing negative monotonic relationship between agTRPA1(A) activation and acrylamide gelation time. (*E*–*G*) Concordances of agTRPA1(A) activation (*E*), molar photoabsorbance (*F*), and highest–lowest unoccupied molecular orbital energy gaps (*G*) to type 1 phototoxicity. Phototoxicity concordance represents percentage of data nonoverlapping between type 1 phototoxins and nonphototoxins (arrows). n.s., not significant. $^{**}P < 0.01$, unpaired t test. Arrowhead: merocyanine 540.

to other molecules/sites, is a likely manifestation of nucleophilicity that describes electron-donating chemical reactivity. Thus, a sensory function naturally invented through evolution discriminates phototoxins that have been imperfectly predicted by modern chemistry, using phototoxin descriptors such as HLG, MAC, and PENMC. Our findings position TRPA1(A) as the sensory nexus for photoprotection of insects; TRPA1(A) detects not only chemical causes of phototoxicity (phototoxins) but also consequences of photochemical reactions (free radicals and reactive oxygen species) (13, 36, 37), highlighting nucleophilicity as an underlying theme of type 1 phototoxicity.

Materials and Methods

Fly Strains and Chemicals. The *UAS-TrpA1(A)* and *UAS-TrpA1(B)* transgenic lines and *TrpA1^{ins}* were previously described (13). The *Gr89a-Gal4* (20) was a gift from Jae Young Kwon, Sungkyunkwan University, Suwon, Republic of Korea. Wild-type flies are the Canton S strain. Chemical lists and purchase information are provided in *SI Appendix*, Table S1. In addition, dihydroethidium (D1168) was purchased from Thermo Fischer Scientific, and ABDA (75068) was from Sigma Aldrich.

Survival Assay. The second instar larvae grown on pigmented food from the egg stage were illuminated with light indicated in the text. The details are available in *SI Appendix, Supporting Information*.

CAFE Assay. CAFE assays were conducted as previously described (16, 38), with 20 3–4-d-old flies starved for 16–20 h on wet Kimwipes. See *SI Appendix, Supporting Information* for details.

In Vivo Extracellular Single Sensillum Recordings. Action potentials from gustatory receptor neurons were recorded as detailed previously (10, 13, 16). See *SI Appendix, Supporting Information* for details.

Two-Electrode Voltage Clamping on *Xenopus* Oocytes. Two-electrode voltage clamping on oocytes were performed as described (39). *Xenopus laevis* were obtained from the Korean *Xenopus* Resource Center for Research (https://www.aris.re.kr/app/main/mainView.do, Chuncheon, Republic of Korea). See *SI Appendix, Supporting Information* for details.

Acrylamide Solidification Assay. To evaluate type 1 phototoxicity of pigments and chemicals, acrylamide gel solution was mixed as done for standard PAGE preparation, except that SDS was not added. See *SI Appendix, Supporting Information* for details.

Dihydroethidium Assay. Dihydroethidium reacts selectively with superoxides producing 2-OH-ethidium. Dihydroethidium at 10 μM was mixed with each pigment of 5 μM in PBS (140 NaCl, 2.7 KCl, 10 Na_2HPO_4, and 1.8 KH_2PO_4 at pH 7.4, in mM) and illuminated with white light. See *SI Appendix, Supporting Information* for details. Further details are available in *SI Appendix, Supporting Information*.

Statistics. Most statistical calculations were performed with Sigmaplot14.0. See *SI Appendix, Supporting Information* for details.

ACKNOWLEDGMENTS. We thank S. K. Chung, K. H. Kim, J. Y. Kwon, S. Sprecher, S. J. Moon, and E. Chang for discussion and comments on the paper. This work was supported by the National Research Foundation of Korea (NRF-2015R1D1A1A01057288, NRF-2017R1A4A1015534, NRF-2018R1A2B6003321, 2015H-1A2A-1034723, NRF-2014R1A1A2057006, and NRF-2016R1A5A2945889) funded by the Korean Government (Ministry of Education or Ministry of Science, ICP and Future Planning).

1. L. O. Björn *et al*., *Photobiology-The Science of Light and Life*, L. O. Björn, Ed. (Springer Netherlands, Dordrecht, 2002).
2. A. Gürses, M. Açikyildiz, K. Güneş, M. S. Gürses, *Dyes and Pigments* (Springer, ed. 1, 2016).
3. L. O. Björn, H. Ghiradella, "Spectral tuning in biology" in *Photobiology* (Springer New York, New York, NY, 2008), pp. 155–196.
4. Y. Haranosono, M. Kurata, H. Sakaki, Establishment of an in silico phototoxicity prediction method by combining descriptors related to photo-absorption and photo-reaction. *J. Toxicol. Sci.* **39**, 655–664 (2014).
5. Y.-X. Zou *et al*., Anti-aging effect of riboflavin via endogenous antioxidant in fruit fly Drosophila melanogaster. *J. Nutr. Health Aging* **21**, 314–319 (2017).
6. P. C. Joshi Ultraviolet radiation-induced photodegradation and 1O2, O2-. production by riboflavin, lumichrome and lumiflavin. *Indian J. Biochem. Biophys.* **26**, 186–189 (1989).
7. J.-Y. Liang *et al*., Blue light induced free radicals from riboflavin on E. coli DNA damage. *J. Photochem. Photobiol. B* **119**, 60–64 (2013).
8. A. E. Axelrod, T. D. Spies, C. A. Elvehjem, V. Axelrod, A Study of Urinary Riboflavin Excretion in Man. *J. Clin. Invest.* **20**, 229–232 (1941).
9. Y. Moriyama, Riboflavin transporter is finally identified. *J. Biochem.* **150**, 341–343 (2011).
10. K. Kang *et al*., Modulation of TRPA1 thermal sensitivity enables sensory discrimination in Drosophila. *Nature* **481**, 76–80 (2011).
11. J. G. Bernstein, P. A. Garrity, E. S. Boyden, Optogenetics and thermogenetics: Technologies for controlling the activity of targeted cells within intact neural circuits. *Curr. Opin. Neurobiol.* **22**, 61–71 (2012).
12. F. N. Hamada *et al*., An internal thermal sensor controlling temperature preference in Drosophila. *Nature* **454**, 217–220 (2008).
13. E. J. Du *et al*., Nucleophile sensitivity of Drosophila TRPA1 underlies light-induced feeding deterrence. *eLife* **5**, e18425 (2016).
14. K. Bakker, Feeding period, growth, and pupation in larvae of Drosophila melanogaster. *Entomol. Exp. Appl.* **2**, 171–186 (1959).
15. W. W. Ja *et al*., Prandiology of Drosophila and the CAFE assay. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 8253–8256 (2007).
16. E. J. Du *et al*., The mosquito repellent citronellal directly potentiates Drosophila TRPA1, facilitating feeding suppression. *Mol. Cells* **38**, 911–917 (2015).
17. K. Kang *et al*., Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception. *Nature* **464**, 597–600 (2010).
18. M. Rosenzweig, K. Kang, P. A. Garrity, Distinct TRP channels are required for warm and cool avoidance in Drosophila melanogaster. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 14668–14673 (2008).
19. L. A. Weiss, A. Dahanukar, J. Y. Kwon, D. Banerjee, J. R. Carlson, The molecular and cellular basis of bitter taste in Drosophila. *Neuron* **69**, 258–272 (2011).
20. M. L. Pallotta *et al*., Saccharomyces cerevisiae mitochondria can synthesise FMN and FAD from externally added riboflavin and export them to the extramitochondrial phase. *FEBS Lett.* **428**, 245–249 (1998).
21. K. V. Dmytruk, A. A. Sibirny, Candida famata (Candida flareri). *Yeast* **29**, 453–458 (2012).
22. O. M. Arenas *et al*., Activation of planarian TRPA1 by reactive oxygen species reveals a conserved mechanism for animal nociception. *Nat. Neurosci.* **20**, 1686–1693 (2017).
23. M. J. Caterina *et al*., The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature* **389**, 816–824 (1997).
24. C. L. Stucky *et al*., Roles of transient receptor potential channels in pain. *Brain Res. Brain Res. Rev.* **60**, 2–23 (2009).
25. C. Gelfi *et al*., Kinetics of acrylamide photopolymerization as investigated by capillary zone electrophoresis. *J. Chromatogr. A* **598**, 277–285 (1992).
26. RReDC Reference Solar Spectral Irradiance, ASTM G173-03. https://www.nrel.gov/grid/solar-resource/spectra-am1.5.html. Accessed 30 October 2015.
27. R. R. Nazarewicz, A. Bikineyeva, S. I. Dikalov, Rapid and specific measurements of superoxide using fluorescence spectroscopy. *J. Biomol. Screen* **18**, 498–503 (2013).
28. N. A. Kuznetsova *et al*., New reagents for determination of the quantum efficiency of singlet oxygen generation in aqueous media. *Russ. J. Gen. Chem.* **71**, 36–41 (2001).
29. C. Y. Ang, S. Y. Tan, Y. Zhao, Recent advances in biocompatible nanocarriers for delivery of chemotherapeutic cargoes towards cancer therapy. *Org. Biomol. Chem.* **12**, 4776–4806 (2014).
30. L. A. MacManus-Spencer, K. McNeill, Quantification of singlet oxygen production in the reaction of superoxide with hydrogen peroxide using a selective chemiluminescent probe. *J. Am. Chem. Soc.* **127**, 8954–8955 (2005).
31. R. Ebermann, G. Alth, M. Kreitner, A. Kubin, Natural products derived from plants as potential drugs for the photodynamic destruction of tumor cells. *J. Photochem. Photobiol. B* **36**, 95–97 (1996).
32. K. Hirakawa, T. Hirano, The microenvironment of DNA switches the activity of singlet oxygen generation photosensitized by berberine and palmatine. *Photochem. Photobiol.* **84**, 202–208 (2008).
33. S. Peukert *et al*., A method for estimating the risk of drug-induced phototoxicity and its application to smoothened inhibitors. *MedChemComm* **2**, 973 (2011).
34. A. D. Sheppard *et al*., Light manipulation of mosquito behaviour: Acute and sustained photic suppression of biting activity in the Anopheles gambiae malaria mosquito. *Parasit. Vectors* **10**, 255 (2017).
35. S.-Z. Xu *et al*., TRPC channel activation by extracellular thioredoxin. *Nature* **451**, 69–72 (2008).
36. A. R. Guntur *et al*., Drosophila TRPA1 isoforms detect UV light via photochemical production of H2O2. *Proc. Natl. Acad. Sci. U.S.A.* **112**, E5753–E5761 (2015).
37. E. J. Du *et al*., TrpA1 regulates defecation of food-borne pathogens under the control of the duox pathway. *PLoS Genet.* **12**, e1005773 (2016).
38. M. J. Lee *et al*., Ionotropic receptor 76b is required for gustatory aversion to excessive Na+ in Drosophila. *Mol. Cells* **40**, 787–795 (2017).
39. K. Kang, Exceptionally high thermal sensitivity of rattlesnake TRPA1 correlates with peak current amplitude. *Biochim. Biophys. Acta* **1858**, 318–325 (2016).