The Anoctamin Family Channel Subdued Mediates Thermal Nociception in Drosophila*

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Calcium-permeable and thermosensitive transient receptor potential (TRP) channels mediate the nociceptive transduction of noxious temperature in Drosophila nociceptors. However, the underlying molecular mechanisms are not completely understood. Here we find that Subdued, a calcium-activated chloride channel of the Anoctamin family, functions in conjunction with the thermo-TRPs in thermal nociception. Genetic analysis with deletion and the RNAi-mediated reduction of subdued show that subdued is required for thermal nociception in nociceptors. Further genetic analysis of subdued mutant and thermo-TRP mutants show that they interact functionally in thermal nociception. We find that Subdued expressed in heterologous cells mediates a strong chloride conductance in the presence of both heat and calcium ions. Therefore, our analysis suggests that Subdued channels may amplify the nociceptive neuronal firing that is initiated by thermo-TRP channels in response to thermal stimuli.

Sensory nociceptive cells (or nociceptors) detect temperatures in the noxious range and initiate thermal nociception (perception of noxious temperature). Noxious temperatures activate ion channels, which then transduce noxious thermal stimuli into neuronal activation. In mammals, nociceptors express the TRPV1 channel, a member of the transient receptor potential (TRP) family, that is activated by capsaicin and temperature. Subdued mutants exhibited defects in food transport to the gut and defects in immunity to consumed virulent bacteria (13). However, its role in thermal nociception has not yet been determined. Here we find that the Subdued channel is required for thermal nociception in collaboration with thermo-TRPs in Drosophila nociceptive behaviors to heat.

Experimental Procedures

Drosophila Strains—The flies were reared on standard yeast/cornmeal agar medium at 25 °C. The c240 enhancer trap-Gal4 line was from a collection described previously (14). The md-Gal4 line (md(109(2)-80), UAS-eGFP) (15) was provided by

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The abbreviations used are: TRP, transient receptor potential; md, multidendritic; FLP, flippase; TNT, tetanus toxin; ANOVA, analysis of variance; FRT, flippase recognition target.
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FIGURE 1. Subdued expression in the class IV md neuron. A, the Subdued (CG16718) locus. The c240-Gal4 enhancer trap P-element is inserted 3’ from the translational start site (labeled as +1). A transcription start site (angled arrow) is 307 bp upstream from the translation start. PiggyBac P-elements (CG16718b22777 and CG16718d1958) inserted into the introns were used to yield a 5265-bp deletion via FLP-FRT-mediated recombination (indicated as Deleted, subdued). The UTR sequence, coding sequence, and introns are denoted in gray, black, and white, respectively. The deficiency Df(3R)Exel6184 overlaps CG16718, as indicated. B, long-range PCR confirms a subdued deletion mutant. A primer set used for genomic PCR is indicated by arrows. Genomic DNAs from subdued homozygotes yielded an expected size (~7.5 kb) of PCR products. Genomic DNAs from control (w1118) flies yielded an expected size (5.8 kb) of PCR products. C, RT-PCR of subdued in wild-type flies (w1118, subdued), homozygotes, and subdued transheterozygotes (subdued/Df(3R)Exel6184, subdued/Df(3R)Exel6184). The primers used for PCR located in exons 12 and 13 are indicated by short vertical red lines in A. The ribosome protein rp49 was used as a control. The absence of bands suggests that subdued homozygotes and subdued transheterozygotes (subdued/Df(3R)Exel6184) are null or severely hypomorphic in a subdued expression. D, c240-Gal4 expression in the class IV md neurons. Confocal images of the skin of a (UAS-mCD8::GFP+/c240-Gal4/ppk-CD4-tdTOM) third instar larva. C240-Gal4 (green), ppk-CD4-tdTOM (red), and merged images are shown. ppk-CD4-tdTOM directs CD4-ttdTOM expression under the promoter of ppk, which is expressed in the class IV md neurons.

FIGURE 2. c240-Gal4 neurons mediate nociception to a heated probe in Drosophila larvae. Shown are larval nociceptive responses to a heated probe. Rolling within 10 s of being touched with a heated probe is considered to be a nociceptive response. Error bars indicate mean ± S.D. of more than three independent experiments (n > 50; one-way ANOVA with Tukey post test; *** p < 0.001; n.s., not significant). A, nociceptive response of c240-Gal4->UAS-shits, ppk-Gal4->UAS-shits, and dTrpA1-C/D->UAS-shits larvae to a heated probe (42 °C) without (permissive) or with (restricted) heat shock (37 °C, 15 min). Gr33a-Gal4->UAS-shits larvae were used as a control. Temp., temperature. B, nociceptive response of c240-Gal4->UAS-shits larvae to a heated probe (46 °C) without (permissive) or with (restricted) heat shock (37 °C, 15 min). Rolling was considered to be a nociceptive response. Response < X s indicates a rolling response within X s minus rolling within X = 1 s. C, nociceptive response of control (md-Gal4/+, md-Gal4->UAS-IMP-TNT (inactive tetanus toxin light chain), c240-Gal4/+, c240-Gal4->UAS-IMP-TNT, md-Gal4->UAS-IMP-TNT) larvae to a heated probe (42 °C). Y. N. Jan. Ppk-Gal4, dTRPA11 and painless (catalog nos. 32079, 26504, and 27895) were from the Bloomington Drosophila Stock Center. The CG16718 (subdued) mutant lines (e02779 and e01958), CG16718 RNAi lines (catalog nos. 37472 and 108953), and Df(3R)Exel6184 (catalog no. 7663) were obtained from the Bloomington Drosophila Stock Center, the Vienna Drosophila RNAi Line Center, and the Exelixis Drosophila Stock Collection at Harvard Medical School, respectively. w1118 from the Bloomington Stock Center was used as a control.

Generation of the Subdued Deletion Line—The CG16718 (subdued) deletion was generated by flippase (FLP)-FRT recombination with two PiggyBac lines, as described previously (16). CG16718e02779 males carrying the PiggyBac [RB] element were mated to females carrying a FLP recombinase transgene. Progeny males carrying both the CG16718e02779 allele and the FLP transgene were then mated to virgin females carrying the CG16718e01958 allele. Two days after the cross, the progeny were heat-shocked for 1 h at 37 °C. The same procedure was repeated after 3 days. Eclosed virgin females were crossed to males carrying balancer chromosomes to generate stocks. The deletion lines were verified by genomic PCR and were outcrossed into the background of the wild-type (w1118) control. LongAmp TaqDNA polymerase (New England Biolabs) was used for long PCR. Primers used for long PCR were as follows: forward, 5’-tgacgactctattgctgcttg-3’; reverse, 5’-agtcttggcctcgagctccttac-3’.

Cloning—The largest CG16718 (subdued) cDNA (catalog no. LD10322, Drosophila Genetic Resource Center) was cloned into a derivative of the pUAST vector to generate transgenic flies (UAS-subdued) and into pcDNA3 (Invitrogen) for electrophysiology recordings in HEK293 cells.
RT-PCR—Flies were collected under CO\textsubscript{2} and rapidly frozen in liquid nitrogen. Total RNA from adult whole flies was extracted using TRIzol reagent (MRC) according to the instructions of the manufacturer. RT was performed by using the AccuPower\textsuperscript{TM} RT Premix (Bioneer K-2041) with 4 μg of total RNA in a 20-μl reaction. PCRs were performed with AccuPower PCR premix (Bioneer K-2016). Primers were as follows: rp49, 5'-agaatcggagcactgct-3' (forward) and 5'-actgagggctggctgct-3' (reverse); subdued, 5'-ggaaatgcgacagtccc-3' (forward) and 5'-cagagttcaaagtcattg-3' (reverse).

Behavioral Assays—The larval thermal nociception assays were performed as described previously (6). Briefly, third instar larvae were placed on 2% agarose medium in 55×12-mm plastic Petri dishes, and their abdominal segments were touched with a soldering iron with a chisel shape 0.6-mm wide. Its temperature was measured with an electronic thermometer. The behavioral responses of the larvae were recorded using a digital camera (Kenox, Samsung) and analyzed.

Adult thermal nociception assays were performed as described previously (10). Briefly, flies aged 5–7 days were placed into behavioral chambers (35×10-mm Petri dish, Nunclon) that were sealed with Parafilm. The flies were rested in the chambers for at least 30 min in darkness. The chambers were then floated in a 46 °C water bath for 4 min. The temperature of the chambers was monitored using an electronic thermometer (Testo 925). Immobilized flies were counted as “incapacitated.” Percentage avoidance was calculated by determining the number of flies avoiding the heated template compared with the total number of flies in the chamber.

Touch sensation was performed as described previously (17). Briefly, the mouth parts of third larvae were gently touched with a fine brush (n = 15, three times) (Kernan score: 0 = no response to touch, 1 = response of pausing mouth-hook movement, 2 = responding by withdrawing the anterior or turning away from the touch, 3 = a single reverse peristaltic wave away from the touch, and 4 = multiple peristaltic waves away from the touch).

Electrophysiological Recordings and Solutions—Current recordings from HEK293 cells expressing subdued were performed in the whole-cell voltage clamp configuration. The patch pipettes were made of borosilicate glass (World Precision Instruments, Inc.) and pulled with a P-87 puller. Patch pipettes filled with the intracellular solution had a resistance of between 3 and 4 MΩ when immersed in the bath solution. Currents were recorded with an Axopatch 200B amplifier controlled by Clampex 8.1 via a Digidata 1200B (Molecular Devices). Data were low pass-filtered at 5 kHz and sampled at 10 kHz. The perfusion solution was heated using a Warner TC-324B single-channel heater controller (Harvard Instruments). The temperature was increased at a rate of 1–2 °C/s. In most experiments, 200-ms voltage steps were applied from a holding potential of 0 mV ranging from −100 to +100 mV. Data were plotted and fitted with the empirical Hill equation using Origin 6.1 (Microcal, Northampton, MA). The standard pipettes and bath solutions contained 140 mM CsCl, 10 mM HEPES (pH 7.0 adjusted with CsOH), 5 mM EGTA, and 2 mM MgCl\textsubscript{2}. Free [Ca\textsuperscript{2+}] was adjusted according to the Maxchelator program to 0, 0.1, and 1.0 μM.

RESULTS

Impairment of the Thermal Nociceptive Response in Subdued Knockdown Animals—To identify cells that were expressing the Subdued channel, we tried both in situ hybridization and immunohistochemistry. Despite many attempts, we failed to obtain a solid Subdued expression pattern when using in situ staining. We also failed to obtain a high titer of Subdued antibodies that allowed us to identify the Subdued protein expression pattern. However, we found an enhancer-trap Gal4 line (c240-Gal4) in which the Gal4 P-element was inserted in proximity to the transcription initiation site of the subdued gene.
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(Fig. 1A). c240-Gal4 is expressed in several non-neural cells, including proventriculus, renal tubule, oviduct, and spermathecal secretory cells (data not shown). In the central nervous system, c240-Gal4 is expressed in some parts of the brain and the ventral ganglion in the larval and adult stages (data not shown). In the peripheral nervous system, c240-Gal4 is expressed in the sensory nociceptors (Fig. 1D), therefore suggesting that c240-neural circuits might mediate nociception.

To examine whether c240-Gal4 neural circuits mediate nociception, we silenced the c240 neural circuits using heat-shocked (37 °C) shibirets1 (shi14), a semi-dominant-negative form of dynamin (Fig. 2, A and B) or tetanus toxin (TNT) (Fig. 2C). IMP-TNT (inactive tetanus toxin light chain) was used as a control for TNT expression. The silencing of c240 neural circuits does not impair motor activity because c240-Gal4>UAS-TNT larvae and adults moved to the same degree as wild-type larvae (data not shown). This result is consistent with c240-Gal4 that is not expressed in muscle cells (data not shown). Notably, silencing of c240 neural circuits resulted in an impaired nociceptive rolling response to noxious thermal stimuli (42 °C or 46 °C) (Fig. 2, A–C). The degree of impairment in this instance was comparable with that induced upon the silencing of nociceptive sensory neurons when using Gal4 lines expressed in nociceptors (Fig. 2, A and C). The silencing of gustatory receptor neurons using Gr33a-Gal4 resulted in no nociceptive response to heat (Fig. 2A).
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To define the Subdued functions in sensory nocceptors, we expressed Subdued RNAi in nociceptive sensory cells. The RNAi-induced reduction of Subdued expression in the c240 line did not impair motor activity because the larvae of c240-Gal4>UAS-subdued RNAi moved in the same degree as wild-type larvae (data not shown). In contrast, the reduction of Subdued expression in the c240 line and in the nociceptive sensory neurons impaired the larval nociceptive heat response (Fig. 3, A and B), indicating that Subdued is required in nociceptive sensory neurons for thermal nociception. Conversely, we examined whether overexpression of Subdued in nociceptive sensory neurons increased the larval nociceptive response to a heated probe. Compared with control larvae harboring one copy of c240-Gal4, larvae expressing subdued (LD10322 from the Drosophila Genetic Resource Center) in c240 neurons (c240-Gal4>UAS-subdued) increased the nociceptive response to a heated probe (Fig. 3C). Larvae, when harboring two copies of UAS-subdued and two copies of c240-Gal4, displayed increased nociceptive thermal responses (Fig. 3C). Therefore, the decrease in the amount of Subdued channel in nociceptive neurons decreased thermal nociceptive behavior. Conversely, an increase in the amount of Subdued channel in nociceptive neurons increased thermal nociceptive behavior. These findings suggest that the Subdued channel is involved in thermal nociceptive transduction.

Impairment of the Thermal Nociceptive Response in Subdued Knockout Animals—To further examine the role of Subdued in nociception, a deletion mutant of Subdued was created by the FLP-FRT-mediated recombination of two PiggyBac elements located in the subdued gene (Fig. 1, A and B). RT-PCR showed that subdued transcripts are not visible in subdued homozygotes and subdued transheterozygotes, indicating that they are null in regard to expression (Fig. 1C). The deletion mutation removed most of the exons and encompassed all of the transmembrane domains of the Subdued. The homozygotes for this mutation were partially lethal at the pupal stage. Eclosed adult flies exhibited partial sterility, but they were morphologically normal and showed normal walking and flying behaviors. The larvae of subdued deletion homozygotes and subdued transheterozygotes impaired the nociceptive heat response to a degree comparable with that found in painless and dTrpA1 mutants (Fig. 4A). This larval rolling behavior exhibited in response to heat was not due to any defect in motor activity, as evidenced by the normal touch response (Fig. 4B). Nocifensive escape behavior to heat was also impaired in adult flies that harbored a subdued deletion (Fig. 4, C and D). These impairments of nocifensive behavior were not due to any defect in locomotion because geotactic movement was normal when compared with control nan flies with locomotor defects (18) (Fig. 4E). Larval heat reduction in subdued mutants was rescued by the expression of subdued in the sensory nocceptors (Fig. 5A). The defect of nocifensive escape behavior observed in the adult flies in response to heat was also rescued by an expression of Subdued in the nociceptive neurons (Fig. 5B). Taken together, this evidence indicates that Subdued is required for thermal nociception in sensory nociceptive cells.

Genetic Interaction between Subdued and Thermo-TRPs—Thermo-TRP channels (Painless and dTRP1A) are expressed in class IV md neurons and are required for thermal nociception, as is Subdued. Thermo-TRP channels are calcium-permeable and, therefore, could increase the intracellular calcium ions when activated by noxious temperatures. Subdued is activated by intracellular calcium ions (13), so it can be activated after the thermo-TRP channels are activated by heat. We examined this possibility using genetic means. Heterozygotes of subdued showed normal thermal nociception, as did the heterozygotes of painless and dTrpA1 (Fig. 6, A–C). However, the transheterozygotes of subdued with either painless or dTrpA1 showed a defect in its heat nociceptive response comparable with homozygotes of dTrpA1, painless, and subdued at larval (Fig. 6, D–F) and adult stages (Fig. 4D), suggesting that the Subdued channel could magnify the depolarization initiated by Painless and dTRP1A channels in thermal nociception.

Subdued Is a Calcium- and Heat-activated Chloride Channel—Subdued has been shown recently to mediate chloride conductance, which is activated by intracellular calcium ions (13). Because our genetic analysis suggests that Subdued participates in thermal transduction, we examined the Subdued channel for its response to calcium ions and temperatures. Whole-cell voltage clamp recordings were performed on HEK293 cells expressing Subdued. All bath and pipette solutions contained 140 mM CsCl. At intracellular Ca2+ concentrations of 1 μM and an ambient temperature of 43°C, robust ionic currents were recorded in HEK293 cells that expressed Subdued (Fig. 7A). When mouse Atoh4, a heat-insensitive, Ca2+-activated Cl− channel (3), was tested as a control, no significant current was evoked by the temperature increase in HEK293 cells that were expressing Atoh4 under identical experimental conditions (Fig. 7A). Treatment with niflumic acid (100 μM), a potent chloride channel inhibitor, resulted in a strong reduction in the ionic currents induced by both heat and intracellular calcium (Fig. 7B).
indicating that Subdued-mediated conductance occurs via chloride current.

We then investigated the effects of intracellular Ca\(^{2+}\) concentration and temperature on the activation of Subdued currents. Subdued currents were elicited, even in the absence of intracellular Ca\(^{2+}\), by increasing the bath temperature (Fig. 8A, top panel). Also, the Subdued currents were potentiated by increasing the intracellular Ca\(^{2+}\) concentration at a set temper-
The average current-voltage relationships were plotted for three different concentrations of intracellular Ca\(^{2+}\) (0, 0.1, and 1 M). The synergistic effects of Ca\(^{2+}\) and heat were evident in the current-voltage relationships (Fig. 8C). These electrophysiological results indicate that Subdued is a Cl\(^{-}\) channel that is considerably activated when both intracellular Ca\(^{2+}\) and heat are present.

**DISCUSSION**

*Drosophila* larvae sense noxious temperatures and show nociceptive behaviors when touched with a heated substance. These behaviors are mediated by class IV md neurons, which fire at temperatures above 40 °C (4, 7). We showed that Subdued channels opened at temperatures above 40 °C, matching the threshold temperature of nociceptive neuronal activation. Subdued RNAi reduction in class IV md neurons was found to impair the larval nociceptive behavior in response to a heated probe. The same was true for Subdued-null mutants. This finding suggests that chloride conduits through the Subdued channel are excitatory to sensory nociceptors, similar to mammalian dorsal root ganglion neurons (3). The expression of Subdued in sensory nociceptive neurons rescues the subdued mutant phenotype. Consistently, the subdued enhancer-trap (c240-Gal4) was expressed in class IV md neurons. Taken together, these data suggest that Subdued functions in sensory nociceptive neurons to transduce noxious thermal stimuli.

The thermo-TRP ion channels Painless and dTRPA1 are required for thermal nociception by class IV md neurons (5, 10, 11). Similarly, we show that the Subdued channel is activated by noxious temperatures and is required in sensory nociceptors for heat-induced nociceptive behaviors. This evidence suggests that nociceptors utilize both the thermo-TRPs and the anoctamin ion channel. Because the Painless and dTRPA1 ion channels are highly calcium-permeable upon activation, and because robust Subdued activation requires both calcium ions and heat, it is possible that activation of the Painless and dTRPA1 channels strengthens the subsequent activation of Subdued channels. Transheterozygotes of *subdued* with either *painless* or *dTrpA1* exhibited defects in thermal nociceptive response, supporting the possibility that Subdued collaborates...
with thermo-TRPs in thermal transduction. Subdued participation in nociceptor depolarization, along with the thermo-TRP channels, may therefore be necessary for the efficient activation of high-threshold nociceptors in response to noxious temperatures.

The subdued mutants have defects in their immunity to ingested virulent bacteria and consume less food (13). Expression of Subdued, as monitored by an expression of c240-Gal4, is not visible in the gut but is visible in the proventriculus and renal tube (data not shown). The proventriculus is a valve that regulates food transport to the midgut, so Subdued might play a role in food transit through the proventriculus, which might then account for the subdued knockout phenotype of consuming less food. The renal tubule plays a role in the immune response (19), so the subdued defect in the immunity to virulent bacteria may be associated with subdued expression in the renal tubule. Subdued is expressed in the epithelial cells of the oviduct that regulates egg release from the ovary to the uterus (20) and also in the spermathecal secretory cells that play a role in sperm storage and its release into the uterus (21). The female sterility observed in subdued mutant flies might be due to a failure to release either egg or sperm from the ovary or spermatheca, respectively, and that possibility needs to be explored further.

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