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Anopheles gambiae TRPA1 is a heat-activated channel expressed in thermosensitive sensilla of female antennae

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Abstract

Heat sensitivity is a sensory modality that plays a critical role in close-range host-seeking behaviors of adult female *Anopheles gambiae*, the principal Afrotropical vector for human malaria. An essential step in this activity is the ability to discriminate and respond to increases in environmental temperature gradients through the process of peripheral thermoreception. Here, we report on the characterization of the anopheline homolog of the transient receptor potential (TRP) A1/ANKTM1 channel that is consistent with its role as a heat-sensor in host-seeking adult female mosquitoes. We identify a set of distal antennal sensory structures that specifically respond to temperature gradients and express *AgTRPA1*. Functional characterization of AgTRPA1 in *Xenopus* oocytes supports its role in the molecular transduction of temperature gradients in *An. gambiae*, providing a basis for targeting mosquito heat responses as a means toward reducing malaria transmission.

Keywords

coeloconic sensilla; temperature receptor; TRP channel

Introduction

The malaria vector *Anopheles gambiae* and other blood-feeding mosquitoes rely on temperature cues in addition to olfactory stimuli for host location (Bowen, 1991; Takken & Knols, 1999). Heat is emitted by mammalian hosts, serves as a universal attractant to many mosquito species (Bowen, 1991; Takken & Knols, 1999) and synergizes with host odor to increase the efficiency of host-seeking behaviors (Laarman, 1958; Schreck *et al*., 1990; Kline & Lemire, 1995). Furthermore, recent behavioral studies have indicated that heat alone has no effect on the landing response of female *An. gambiae*, but significantly

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synergizes the attraction to the host odor (J. Spitzen and W. Takken, unpublished observations).

Early antennal ablation studies suggested that the antenna might harbor the thermoreceptive neurons that underlie the heat-evoked behaviors of mosquitoes (Ismail, 1962). In addition, ultrastructural studies have characterized antennal small sensilla coeloconic (SC) and sensilla ampullacea as probable sites of temperature detection (McIver, 1973; Boo & McIver, 1975; McIver & Siemicki, 1976). In accord with these efforts, electrophysiological studies in the yellow-fever vector mosquito, *Aedes aegypti*, identified an antagonistic pair of thermoreceptive neurons within the small SC on the antennal tip (Davis & Sokolove, 1975; Gingl *et al*., 2005). Here, one thermoreceptive neuron is warm-sensitive and increases its spike frequency to temperature rises; the other is cold-sensitive and increases its spike frequency to temperature falls (Davis & Sokolove, 1975; Gingl *et al*., 2005).

Despite some knowledge of the physiology of antennal thermoreceptive neurons, little is known about genes that are involved in peripheral temperature detection in mosquitoes. In *Drosophila*, a family of 13 transient receptor potential (TRP) genes (Montell, 2005) encode six-transmembrane nonselective cation channels, several of which have been identified as central nervous system (CNS) thermoreceptors (Tracey *et al*., 2003; Lee *et al*., 2005; Rosenzweig *et al*., 2005; Hamada *et al*., 2008). Of these, *dTRPA1* is activated by high temperatures, with a threshold of ~27°C, and regulates thermotaxis (Viswanath *et al*., 2003; Rosenzweig *et al*., 2005; Hamada *et al*., 2008). Furthermore, *dTRPA1* has recently been shown to be activated indirectly through pathways coupled to phospholipase C to discriminate between the optimal temperature of 18°C and slightly higher temperatures (19–24°C) (Kwon *et al*., 2008). Not surprisingly, additional studies in *Drosophila* have revealed distinct TRP channels that are required for avoidance of cold (Rosenzweig *et al*., 2008).

Here we examine the molecular basis for peripheral heat sensitivity in adult female *An. gambiae*. We confirm that a pair of small SCs on the distal tip of female *An. gambiae* antennae contains neurons that specifically respond to a temperature rise with high sensitivity. We have screened the *An. gambiae* homologs of *Drosophila* TRPA channels for antennal expression and have identified *AgTRPA1* as a consequence of its specific localization to the distal antennal small SCs. Lastly, we have functionally characterized *AgTRPA1* heterologously expressed in *Xenopus* oocytes to demonstrate that it encodes a functional thermoreceptor. In these studies, *AgTRPA1* was activated by temperature increases from 25 to ~37°C, consistent with the hypothesis that *An. gambiae* relies on antennally expressed *AgTRPA1* to detect increasing temperature gradients derived from host body heat during crucial close-range, host-seeking behaviors.

Materials and methods

Insects

Anopheles gambiae sensu stricto Giles, originating from Suakoko, Liberia, were reared as described previously (Fox *et al*., 2001; Qiu *et al*., 2006a). Non-blood-fed, 5- to 7-day-old females were used for all experiments. All animal experiments followed the guidelines set by the Vanderbilt Institutional Animal Care and Use Committee (IACUC) to minimize the number of animals used. *Xenopus laevis* were from eNASCO (Modesto, CA, USA). Before surgery, animals were placed in a solution containing 2 g ethyl-3-amino-benzoate methanesulfonate salt from Sigma (in 1 L H_2O) for 20–30 min until completely anesthetized.

In vivo *single sensillum recordings*

Extracellular recordings of neuronal activity in female *An. gambiae* were made as described (Qiu *et al*., 2006b) using an USB-IDAC analog-to-digital conversion interface with AUTOSPIKE software for sampling, visualization and analysis (Syntech, Kirchzarten, Germany). Recordings were made from the paired small SCs at the distal tip of the 13th antennal segment. The recording electrode (tungsten, tip *c*. 1 μm) was placed into the pit of the sensillum and pierced through the cuticle of the peg located inside the pit.

An increase of air temperature was generated to study whether warm-sensitive neurons are located in these small SCs. A charcoal-filtered air stream was pumped out of the compensatory air pulse outlet of a stimulus controller (CS-55, Syntech) at 1.5 L/min and passed through a Pasteur pipette that was maintained at room temperature (*c*. 25°C). At discrete time intervals, the air stream was switched to the normal air pulse outlet of the stimulus controller and led through a Pasteur pipette heated by direct contact with hot water (85 ± 3°C). Each air stream was connected to one arm of a Y-shaped connector, the common outlet (inner diameter 2 mm) of which was positioned *c*. 5 mm from the tip of the mosquito antenna. Warmed air was led over the antenna during a period of 10 s and, subsequently, the air stream was switched back to the airflow at room temperature. This sequence was repeated twice. The temperature of the air stream increased from approximately 25–37°C and was constantly monitored at a distance of *c*. 1 mm from the sensilla using a type-K thermocouple and TC-08 data logger (Pico Technology, St Neots, UK).

Anterograde dye filling of distal antennal small SC neurons

Anterograde dye filling from the distal pair of antennal small SC structures was performed by slivering the sensilla with either electrodes or fine scissors, followed by immediate immersion in 2% Texas red or Lucifer yellow in PBS solution (w/v) in the glass electrode for 4 h at 4°C. At that point the electrodes were removed and the antennae were amputated from the mosquito head. Whole mounts of the antenna were washed in 0.1% PBST for 1–2 h and mounted on a glass slide using Vectashield (Vector Laboratories, Burlingame, CA, USA). Whole mounts of the stained mosquito antenna were observed by using an LSM 510 confocal microscope (Zeiss, Thornwood, NY, USA).

Reverse transcriptase PCR (RT-PCR)

Mosquitoes were cold anesthetized and 100 antennae were dissected by hand on a chilled table. RNA preparation and cDNA synthesis were carried out as described previously (Kwon *et al*., 2006; Lu *et al*., 2007). Negative control samples without reverse transcriptase were included in each cDNA synthesis and subsequent PCR analysis. PCR cDNA template levels were monitored for integrity and amount using the ubiquitously expressed *An. gambiae* ribosomal protein S7 gene (*rps7*) (Salazar *et al*., 1993) as an internal control. PCR was performed using a DNA Engine Dyad (GMI, Ramesy, MN, USA) under the following conditions: 94°C for 2 min; 32–35 cycles of 94°C for 15 s, 60°C for 20 s and 72°C for 20 s; and 72°C for 5 min. Primer pairs that span introns were used to distinguish cDNA amplicons from those amplified from remaining genomic DNA. The complete set of *AgTRP* primers can be accessed at http://www.cas.vanderbilt.edu/zwiebel/primers3.htm. PCR amplification products were run on a 1.5% agarose gel and verified by DNA sequencing.

In situ *hybridization and immunolabeling*

Riboprobes for *in situ* hybridization comprising approximately 900 bp of *AgTRPA1* coding sequence were prepared as follows. *AgTRPA1* sequences were amplified from *An. gambiae* antenna cDNA samples utilizing the following PCR primers: AgtrpA1-F 5′-CTATTCGGCGGCTTCAATAAC-3′ and AgTrpA1r 5′-

TCATTTGCCAATAGATTTGTTGAAGC-3′. PCR products were ligated to pCRII-Topo (Invitrogen, Carlsbad, CA, USA), and digoxigenin-labeled RNA probes were generated for sense and antisense utilizing SP6 and T7 RNA polymerase, respectively. Detailed procedures for probe preparation and *in situ* hybridization on *An. gambiae* olfactory tissues were described previously (Kwon *et al*., 2006; Lu *et al*., 2007). Cell nuclei were labeled using YOYO-1 (Invitrogen) and anti-horseradish peroxidase (hrp) immuno-labeling was used to mark neuronal axon and dendrites (Pitts *et al*., 2004). Antennal sections were mounted in Vectashield (Vector Laboratories) and visualized using an LSM510 confocal microscope (Carl Zeiss).

AgTRPA1 *expression in* X. laevis oocytes and two-electrode, voltage-clamp electrophysiological recording

Full-length coding sequences of *AgTRPA1* were PCR amplified from female *An. gambiae* antennal cDNA under the following conditions: 94°C for 2 min; 32 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 3 min 30 s; and 72°C for 10 min. The PCR product was first cloned into pENTR/D-TOPO (Invitrogen) and then sub-cloned into pSP64DV by means of the Gateway LR reaction (Lu *et al*., 2007). Complementary RNA (cRNA) was synthesized from linearized vectors with mMESSAGE mMACHINE (Ambion).

Mature healthy oocytes (stage V–VII) were treated with 2 mg/mL collagenase S-1 in washing buffer [96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$ and 5 mM HEPES (pH 7.6)] for 1–2 h at room temperature. Oocytes were later microinjected with 50 nL *AgTRPA1* cRNA. After injection, oocytes were incubated for 5–7 days at 18°C in 1 × Ringer's solution [96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 0.8 mM $CaCl_2$ and 5 mM HEPES (pH 7.6)] supplemented with 5% dialysed horse serum, 50 mg/mL tetracycline, 100 mg/mL streptomycin and 550 mg/mL sodium pyruvate. Whole-cell currents were recorded from the injected *Xenopus* oocytes with a two-electrode voltage clamp. Temperature control was achieved with a combination of an HE-104R thermal stage and an HCC-100A temperature controller (Dagan Instruments, Minneapolis, MN, USA) and constant perfusion of cooled/heated solution. Currents were recorded using an OC-725C oocyte clamp (Warner Instruments, Hamden, CT, USA), and pCLAMP8.2 software suite (Axon Instruments, Sunnyvale, CA, USA).

Results

Physiological responses to temperature rise in female antennae

As is the case for several other mosquito species, each antenna of *An. gambiae* adult females bears approximately seven small SC structures (Fig. 1A) (McIver, 1982; Pitts & Zwiebel, 2006). A set of paired small SCs are located on the distal tip of the 13th segment, three are located on the distal edge of the first segment and one each on segments 12 and 13 (Fig. 1). Extracellular recordings were made from the distal-most small SC pair and identified a specific neuron that displayed both excitatory responses to swift increases of temperature and the largest spike amplitude of the 20 sensilla from which successful recordings were obtained (Fig. 2A). The impulse frequency of the heat-sensitive small SC neuron increased and decreased instantly with the corresponding temperature rise and fall, respectively (Fig. 2D and E). In contrast, the impulse frequency of a control olfactory receptor neuron (ORN) recorded extracellularly from a nearby antennal trichoid sensillum remained unchanged regardless of the temperature rise or fall (Fig. 2C). Furthermore, the change in impulse frequency relative to the spontaneous frequency at room temperature showed a linear correlation with the change in temperature (ΔT) (*F*-test, $P < 0.001$, Fig. 2F). The differential sensitivity, represented as the difference in impulse frequency per degree centigrade of change in temperature, was 6.36 spikes/s. Although application of multiple chemical odorants known to elicit antennal ORN activity (Qiu *et al*., 2006b) failed to evoke any

alteration in the activity of the heat-sensitive small SC (data not shown), stimulation with high humidity (100% dH_2O-saturated air) elicited significant increases in impulse frequency of a neuron with large spike amplitude (see Supporting Information Fig. S4).

Expression of AgTRPA1 *in adult female antennae*

We next investigated whether TRPA channels, which had been directly linked to heat reception in *Drosophila*, play significant roles in this peripheral temperature detection in *An. gambiae*. With the notable exception of *painless* (Tracey *et al*., 2003), three of the four *Drosophila* TRPA channels (Viswanath *et al*., 2003) have close homologs in the genome of *An. gambiae*. In the present study, putative *AgTRPA* genes were first screened for antennal expression using standard RT-PCR protocols. These semi-quantitative studies indicated that *AgTRPA1* is robustly expressed in antennae, while *AgTRPA2* is expressed at much lower levels and *AgTRPA3* is not expressed at all (Fig. S1A). Additional RT-PCR studies were used to examine the expression pattern of *AgTRPA1* in sensory tissues of adult female *An. gambiae* mosquitoes. The resulting data indicated that *AgTRPA1* is highly expressed in the female antenna as well as at lower levels in the head, maxillary palp and proboscis relative to constitutively expressed *rps7* controls (Fig. S1B). In contrast, *dTRPA1* expression is restricted to the proboscis and a set of warmth-activated anterior cell (AC) neurons in the *Drosophila* CNS (Hamada *et al*., 2008).

The antennal expression of *AgTRPA1* was confirmed and elaborated upon with fluorescence *in situ* hybridization (FISH) coupled with immunolabeling. These data indicated that *AgTRPA1* expression is restricted to a very limited number of antennal neurons. Indeed, in paraffin sections, we consistently observed only one neuron in the first and second antennal segments and typically two neurons in the distal-most region of the 13th segment, which were labeled by antisense *AgTRPA1* riboprobes (Fig. 3). The 10 mid-segments (from the third to the 12th antennal segments) were entirely devoid of *AgTRPA1* signals. Furthermore, the position of each *AgTRPA1*-positive neuron cell body correlated with the small SC localization reported previously in morphological ultrastructural studies (Ismail, 1964; Pitts & Zwiebel, 2006). Lastly, anterograde dye-filling studies (Fig. S2) indicated *AgTRPA1* labeling in the 13th antennal segment closely matched the position of the cell bodies associated with paired small SCs on the antennal tip, which we have shown to harbor thermoreceptive neurons in *An. gambiae* and which have previously been implicated in heat sensitivity in *Ae. aegypti* (Davis & Sokolove, 1975; Gingl *et al*., 2005).

AgTRPA1 *is activated by increasing temperatures*

To investigate further its activation by increasing temperatures, *AgTRPA1* was heterologously expressed in *Xenopus* oocytes and functionally characterized using two-electrode, voltage-clamp physiology. A temperature increase from room temperature (22°C) to ~37°C consistently elicited a large inward current of over 500 nA in *Xenopus* oocytes injected with cRNA encoding *AgTRPA1*, and no significant adaptation was observed when the stimulus was repeated (Fig. 4B). The thermal response continuously increased along with the temperature from 25 to ~37°C (Fig. 4C), reaching a maximal response at ~40°C (Fig. S5). Importantly, control oocytes injected with buffer alone failed to manifest any response to temperature increases from 22 to ~37°C (Figs 4A and S5). Additional controls included oocytes coexpressing *AgOR28* and *AgOR7* which have previously been shown to support odorant-induced currents in response to 6-methyl-5-hepten-2-one and other chemical stimuli (Lu *et al*., 2007). In this case AgOR28/AgOR7 oocytes failed to respond to a temperature shift from 25 to ~37°C (Fig. S3). These results correlate with the response properties of the warm-sensitive neuron in our *in vivo* single-sensillum recording (SSR) analyses and are consistent with similar independent studies (Hamada *et al*., 2008). Taken

together, these data support the hypothesis that *AgTRPA1* is responsible for peripheral thermoreceptive responses as a consequence of its expression in small SC neurons.

Discussion

We have characterized the electrophysiological response to changes in ambient air temperature of distal small SC-associated neurons on the antennae of adult female *An. gambiae*. Using *in vivo* SSR analyses, we have confirmed the presence of thermoreceptive neurons within small SC structures that specifically respond with excitation to an increase in air temperature. In *Ae. aegypti*, a heat-excited neuron was also found in a similarly located small SC (peg in-pit sensilla) at the antennal tip segment (Gingl *et al*., 2005). It is noteworthy that the differential sensitivity we detected in this study for *An. gambiae* was nearly twice that reported previously for *Ae. aegypti*. We have tested 11 compounds and one synthetic mixture representing known ligands of various antennal ORNs of female *An. gambiae* (Qiu *et al*., 2006b) and none elicited responses by neurons innervating SC. Interestingly, an increase in humidity in the air stream did elicit an excitatory response by one of the neurons in the SCs (Fig. S4). It is possible that the heat-sensitive neuron is also responding to the change in humidity, as both heat and humidity stimuli evoked similar spike amplitudes. Bimodal thermo-/hygroreceptor neurons have indeed been observed in other insects (Loftus & Corbiere-Tichane, 1981; Alter & Loftus, 1985).

To begin to address the molecular basis for peripheral thermoreception functionally, we mapped a member of the TRP gene family of related channel proteins in *An. gambiae*, *AgTRPA1*, to neurons associated with distal thermoreceptive antennal small SCs. In addition to significant homology to *Drosophila dTRPA1* and other TRP channel homologs, *AgTRPA1* conferred responses to a temperature increase from room temperature to ~40°C when functionally expressed in *Xenopus* oocytes. This agrees well with a previous study (Hamada *et al*., 2008) and indicates that AgTRPA1, like dTRPA1, functions as a warm-sensitive thermoreceptor. AgTRPA1 has close homologs in several other mosquito species, including *Ae. aegypti* and *Culex pipiens* (Hamada *et al*., 2008), suggesting that it is likely to function as a peripheral heat sensor in those mosquito species as well.

Taken together, these data support the hypothesis that *AgTRPA1* is expressed in the antenna where it functions as a peripheral thermoreceptor in *An. gambiae*. This is in contrast to the situation in *Drosophila*, in which *dTRPA1* is thought to function as an internal heat detector in the brain (Rosenzweig *et al*., 2005; Hamada *et al*., 2008); and although peripheral *dTRPA1* expression is noted in the proboscis, ablation studies have failed to support a role for this expression in warmth-avoidance paradigms (Hamada *et al*., 2008). The expression of a peripheral thermoreceptor in the major olfactory organ of *An. gambiae* provides a basis for the observation that temperature, an important sensory cue in host location, is a *bona fide* antennal stimulus. Indeed, temperature sensitivity may be encoded along with chemosensory information, and the two stimulus modalities may be integrated through post-synaptic events in the antennal lobe and higher brain centers (Zeiner & Tichy, 2000). Consistent with this view, behavioral studies have demonstrated that elevated source temperatures of ~32–37°C synergize to increase the attraction to host-derived odorants in host-seeking *An. gambiae* females (J. Spitzen and W. Takken, unpublished observations). Indeed, it will be of interest to elucidate the mechanism by which the two types of sensory inputs are coupled and transformed into an ultimate sensory percept of the host.

In previous physiological studies in *Ae. aegypti*, it has been demonstrated that antennal small SCs respond far more sensitively to convective than to radiant heat (Davis & Sokolove, 1975; Gingl *et al*., 2005). It is likely that heat, emitted from a host's breath and body, is incorporated into host odor blends, resulting in an odor/heat plume that is detected by the

mosquito antenna. As heat is a universal stimulus for mosquitoes and most other blood-feeding insects, the identification of a peripheral mosquito thermoreceptor provides a potential target for the design of useful insect repellents. Such an advance would facilitate the ongoing fight against malaria by reducing the vectorial capacity of *An. gambiae* mosquitoes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AgTRPA1	*Anopheles gambiae* transient receptor potential A1
CNS	central nervous system
FISH	fluorescent *in situ* hybridization
ORN	olfactory receptor neuron
SC	sensilla coeloconic
SSR	single-sensillum recording
TRP	transient receptor potential

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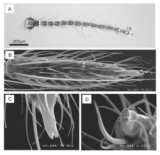

Fig. 1.

The ultrastructure of small coeloconic sensilla. (A) *An. gambiae* antenna showing the positions of small coeloconic sensilla (white dots) according to Pitts & Zwiebel (2006). (B) Scanning electron micrograph showing the small coeloconic sensilla on the 13th antennal segment. Small coeloconic sensilla are indicated by arrowheads. (C, D) Small coeloconic sensilla on the tip of the antenna.

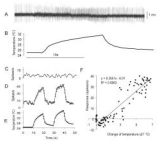

Fig. 2.

Single-sensillum electrophysiological recordings from small coeloconic sensilla. (A) Extracellular recording from a small coeloconic sensillum at the tip of the antenna (segment 13) of female *An. gambiae* showing the excitatory response of a neuron to an increase in air temperature. (B) Time course of air temperature applied during the recording shown in A. Time scale of A and B are the same and indicated by the bar under B. (C) The impulse frequency of a control olfactory receptor neuron in an antennal trichoid sensillum remains unchanged in response to temperature alterations. (D) Mean impulse frequency (spikes/s ± SEM) of the heat-sensitive neuron in response to two increase/decrease cycles of air temperature ($n = 7$). (E) Time course of air temperature (mean ± SEM) applied during the experiment shown in C ($n = 7$). (F) Linear regression of the response intensity of the heat-sensitive neuron to changes in air temperature, ΔT. For the first stimulation cycle of temperature, ΔT = temperature at time x minus mean room temperature; for the second stimulation cycle, ΔT = temperature at time x minus average temperature during the last 3 s before the onset of the temperature rise. Neuron response = impulse frequency at time x minus impulse frequency at room temperature. Data in D–F originate from seven of the 20 distal small coeloconic sensilla from which recordings were taken. Although responses to temperature were observed in all 20 sensilla, either due to low signal-to-noise ratios or different stimuli intensity and duration used, data from only seven sensilla were summarized.

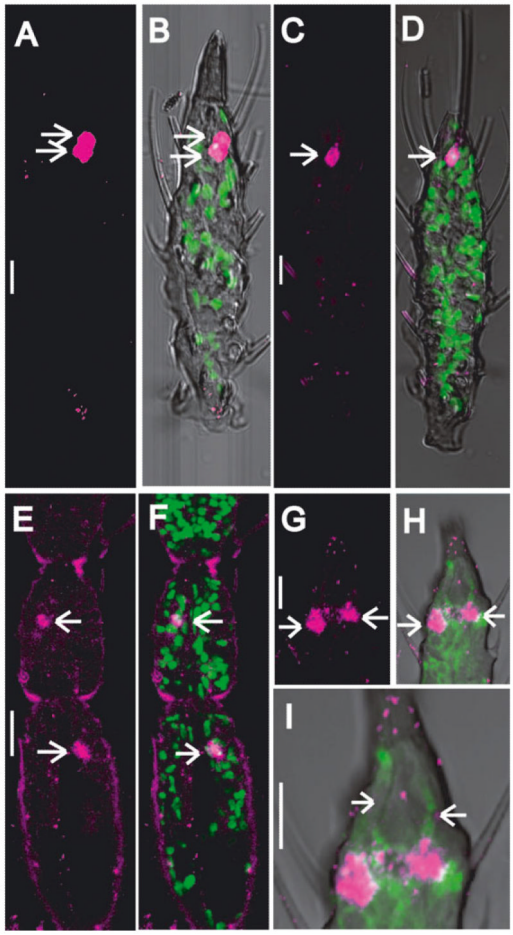

Fig. 3.

Expression of the *AgTRPA1* gene in the antenna. Panels A–B and C–D are single and double (merged) channel visualizations of replicate lateral optical sections through the distal-most (13th) antennal segment, respectively. Panels E–I are single and double (merged) channel visualizations of medial sections through the first and second (E, F) and 13th (G–I) antennal segments. A, C, E and G display Cy-3 (red-see colours in the on-line graphic) immunofluorescence indicative of *AgTRPA1*-positive cells (arrows). In the distal most (13th) antennal segment there are two *AgTRPA1*-positive cells (arrows, A–D and G–H, respectively). There is one *AgTRPA1*-positive cell in the first and second antennal segments (arrows, E and F, respectively). Panels B, D, F, H and I represent merged images where the green immunofluorescence in B, D and F display cell nuclei labeled using YOYO-1 (Invitrogen) and in H and I display anti-HRP immunolabeling to mark neuronal axons and dendrites. Panel I is a high-magnification Z-series image stack illustrating that the two *AgTRPA1*-positive neurons extend their dendrites into the tip of the antenna (arrowheads, anti-HRP labeling of neuronal dendrites). The scale bar represents 10 μm.

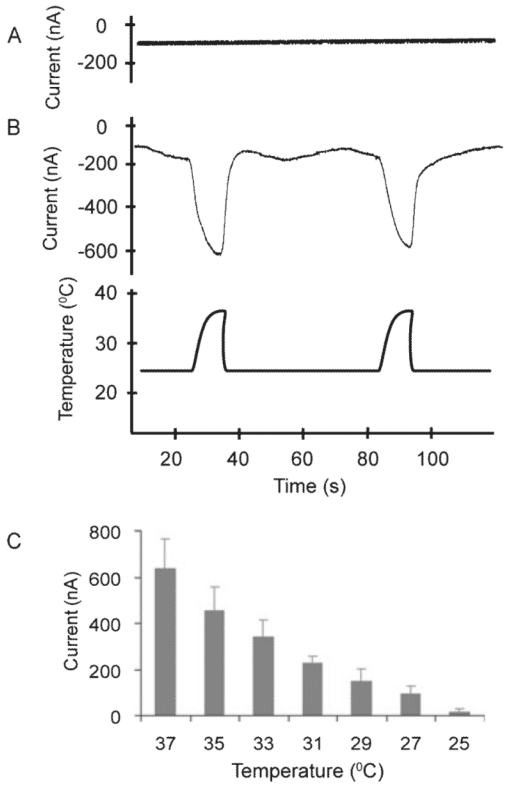

Fig. 4.

AgTRPA1 is activated by increasing temperature. (A) Response of control oocytes injected with buffer alone to heat stimuli. Inset: higher resolution of the inter-stimulus interval to demonstrate the viability of the control oocyte. (B) Inward currents of the oocytes expressing *AgTRPA1* to heat stimuli. (C) The relationship between temperature and response of oocytes expressing *AgTRPA1* (n = 5; error bars indicate SEM).