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Drosophila Painless Is a Ca^{2+}-Requiring Channel Activated by Noxious Heat

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Thermal changes activate some members of the transient receptor potential (TRP) ion channel super family. They are primary sensors for detecting environmental temperatures. The *Drosophila* TRP channel Painless is believed responsible for avoidance of noxious heat because *painless* mutant flies display defects in heat sensing. However, no studies have proven its heat responsiveness. We show that Painless expressed in human embryonic kidney-derived 293 (HEK293) cells is a noxious heat-activated, Ca^{2+}-permeable channel, and the function is mostly dependent on Ca^{2+}. In Ca^{2+}-imaging, Painless mediated a robust intracellular Ca^{2+} (Ca^{2+}_i) increase during heating, and it showed heat-evoked inward currents in whole-cell patch-clamp mode. Ca^{2+} permeability was much higher than that of other cations. Heat-evoked currents were negligible in the absence of extracellular Ca^{2+} (Ca^{2+}_o) and Ca^{2+}_i, whereas 200 nM Ca^{2+}_i enabled heat activation of Painless. Activation kinetics were significantly accelerated in the presence of Ca^{2+}_i. The temperature threshold for Painless activation was 42.6°C in the presence of Ca^{2+}_i, whereas the threshold was significantly increased to 44.1°C when only Ca^{2+}_o was present. Temperature thresholds were further reduced after repetitive heating in a Ca^{2+}-dependent manner. Ca^{2+}-dependent heat activation of Painless was observed at the single-channel level in excised membranes. We found that a Ca^{2+}-regulatory site is located in the N-terminal region of Painless. Painless-expressing HEK293 cells were insensitive to various thermosensitive TRP channel activators including allyl isothiocyanate, whereas mammalian TRPA1 inhibitors, ruthenium red, and camphor, reversibly blocked heat activation of Painless. Our results demonstrate that Painless is a direct sensor for noxious heat in *Drosophila*.

Key words: Drosophila; Painless; TRP channel; Ca^{2+} ion; temperature sensation; noxious heat

Introduction

Animals need to be able to distinguish between permissive habitats and those that could cause injury from excessive temperatures. Thus, the ability to sense environmental temperatures is a critical and fundamental trait. Recent studies have revealed that several ion channels belonging to the transient receptor potential (TRP) ion channel super family have an ability to sense temperature changes. In mammals, nine TRP channels have been reported to respond to a physiological range of temperatures, from cold to hot (Dhaka et al., 2006; Caterina, 2007; Tominaga, 2007). Some of these thermosensitive TRP channels are strongly expressed in nociceptive neurons, and a number of reports claim that they have critical roles in detection of temperatures in the noxious range. TRPV1 expressed in C-fibers is activated by heat (>43°C) (Caterina et al., 1997), and TRPV1-null mice are defective in sensing noxious heat (Caterina et al., 2000). TRPM8, which senses cold temperatures (<27°C) (McKemy et al., 2002; Peier et al., 2002), is expressed in nociceptive and non-nociceptive neurons and its loss causes deficiencies in cold sensation (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007, 2008; Takashima et al., 2007). TRPA1 is expressed in C-fibers, mostly accompanying TRPV1 (Story et al., 2003), and acts as a sensor for harmful stimuli, although its responsiveness to noxious cold is controversial (Jordt et al., 2004; Bautista et al., 2006; Kwan et al., 2006; Sawada et al., 2007). Thus, the thermosensitive TRP channels are implicated in the detection of harmful temperatures that might otherwise cause serious damage to the organism.

Detecting ambient temperatures is particularly important for small insects. Body temperature and locomotive activity of small insects predominantly depend on environmental temperatures because their large volume-to-weight ratios allow rapid heat exchange with the environment. In *Drosophila*, three ankyrin-repeat TRP (TRPA) channels are involved in temperature sensation. Temperatures >27°C activate dTRPA1 (Viswanath et al., 2003) and its knockdown by RNA interference revealed the importance of the channel for thermotaxis (Rosenzweig et al., 2005). Pyrexia is activated by heat (~40°C) and is proposed to protect flies from high-temperature stress (Lee et al., 2005). Another TRPA subtype, dubbed Painless, was reported to be responsible for avoidance of noxious heat >42°C in *Drosophila* larvae (Tracey et al., 2003) and adults (Xu et al., 2006). Wild-type larvae

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displayed distinct rolling behavior within 1 s when touched with a heated probe, whereas *painless* mutant larvae required more than 10 s to show such behavior. Painless appears to be distributed in a subset of sensory neurons of the peripheral nervous system, which resemble sensory neurons in mammals. Because the other two members of the TRPA subfamily possess the properties of temperature sensitivity, Painless has been regarded as a heat-activated channel. However, in contrast to dTRPA1 and pyrexia, no studies have proven that Painless itself responds to heat. In this study, we examined the heat sensitivity and activation properties of Painless in a heterologous expression system to assess whether Painless truly functions as a thermosensitive TRP channel.

Materials and Methods

Cloning of painless and construction of the expression vector. The full length of *painless* cDNA was isolated by RT-PCR with adult fly head RNA and two primers, 5′-TTTTTGCGGCCGCACCATGGACTTTAACAACTG-CGGCTTCATTGATCCG-3′ and 5′-TTTCTAGACTCTTCCGGT-CCTGGACCAGCTGTATTAATTGCTCCA-3′. The PCR product was digested with *Not*I and *Xba*I, and then cloned in a pAc5.1/V5-His B vector (Invitrogen) in which the *Drosophila* actin 5C promoter was replaced with a cytomegalovirus (CMV) promoter. The resulting construct (*painless*/V5 expression vector) was sequenced, and two base substitutions were found in the cDNA which did not cause amino acid substitutions. The vector containing full-length *painless* cDNA with stop codon (*painless* expression vector) or N356A, S357A, N363A, or D366A mutants were constructed by single amino acid substitutions using a modified Quikchange site-directed mutagenesis method (Stratagene). Briefly, PCR was performed using a *painless*/V5 expression vector as a template, two synthetic oligonucleotide primers containing a stop codon or specific mutations (*painless* expression vector-S and AS, 5′-ACGCAAATGGGCGGTAGG-3′ and 5′-CCTCTAGACGTCACTTC-CGGTCCTGGACCAGCTGTATTAATTGCTCCA-3′; N356A-S and AS, 5′-GCTTTGAATTGCTCATTgcCAGCGATCGCGTAGAC-3′ and 5′-GTCTACGCGATCGCTGgcAATGAGCAATTCAAAGC-3′; or S357A-S and AS, 5′-GAATTGCTCATTAACgcCGATCGCGTAGACATCAAC-3′ and 5′-GTTGATGTCTACGCGATCGgcGTTAATGAGCAATTC-3′; or N363A-S and AS, 5′-GATCGCGTAGACATCgcCGAAGCTGAT-TCCGG-3′ and 5′-CCGGAATCAGCTTCGgcGATGTCTACGCGATC-3′; or D366A-S and AS, 5′-CATCAACGAAGCTGcTTCCGGA-CGCCTG-3′ and 5′-CAGGCGTCCGGAAgCAGCTTCGTTGATG-3′), and Primestar HS DNA polymerase (TaKaRa). The PCR products were digested with *Dpn*I (New England BioLabs) at 37°C for 1 h, and transformed into DH5α competent cells. The entire sequence including desired substitution in the mutants was confirmed. The *painless*/V5 expression vector was used for immunostaining. The *painless* expression vector and the four mutant vectors were used for Ca^{2+}-imaging and/or patch-clamp methods.

Cell culture, transfection, and immunostaining. Human embryonic kidney-derived 293 (HEK293) cells were maintained in DMEM containing 4500 mg/L glucose (Wako Pure Chemicals), 10% FBS (Biological Industries) and penicillin/streptomycin (Invitrogen) at 37°C in 5% CO_2. For Ca^{2+}-imaging, 1 μg of *painless* expression vector and 0.1 μg of pCMV-DsRed-express cDNAs in OPTI-MEM medium (Invitrogen) were transfected to HEK293 cells cultured on 35 mm dishes using Lipofectamine Plus reagents (Invitrogen). For patch-clamp recording, 0.1 μg of pGreen Lantern 1 cDNAs were used instead of DsRed cDNAs. After incubating for 3 to 4 h, cells were reseeded on cover glasses and further incubated at 33°C in 5% CO_2. Cells were used for the experiments 20–40 h after transfection. For immunostaining, HEK293 cells were inoculated in eight-well chamber slides (Nunc), and *painless*/V5 expression vectors were transfected using Effectene reagent (Qiagen). Two days after transfection, the cells were fixed with 4% paraformaldehyde/PBS, and then permeabilized with 0.5% Triton X-100/PBS for 5 min followed by blocking with 5% normal donkey serum/PBS containing 0.1% Triton X-100. The cells were incubated with rabbit anti-V5 epitope antibody (Sigma) at a 1000-fold dilution for 2 h at room temperature, after which FITC-conjugated anti-rabbit IgG antibody (Millipore) was added at a 300-fold dilution as above.

Ca^{2+} imaging. Transfected HEK293 cells on cover glasses were incubated in culture medium containing 5 μM fura-2 AM (Invitrogen) at 33°C for 1 to 2 h. The cover glasses were washed with a standard bath solution containing (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.4, adjusted with NaOH, and fura-2 fluorescence was measured in a standard bath solution. The cover glasses were mounted in a chamber (RC-26G; Warner Instruments) connected to a gravity flow system to deliver various stimuli and heated bath solutions. Thermal stimulation was applied by increasing the bath temperature with a preheated solution through an inline heater (SH-27B; Warner Instruments). A xenon lamp was used as an illumination source. Fura-2 loaded in the cells was excited with 340 and 380 nm wavelengths and emission was monitored at a wavelength of 510 nm with a CCD camera (CoolSnap ES; Roper Scientific/Photometrics) to obtain fluorescent intensities of Ca^{2+}-bound and Ca^{2+}-free fura-2, respectively. Data were acquired and analyzed by IPlab software (Scanalytics). Chamber temperature was monitored with a thermocouple (TA-29; Warner Instruments) and sampled with an analog-to-digital converter with pClamp software (Molecular Devices).

Electrophysiology. The standard bath solution containing 2 mM $CaCl_2$ for whole-cell patch-clamp experiments was the same as that used in Ca^{2+}-imaging experiments. The same solution was used as the standard pipette solution for inside-out patch-clamp recording. The $Ca^{2+}(-)$ bath solution for whole-cell patch-clamp and the $Ca^{2+}(-)$ pipette solution for inside-out patch-clamp contained (in mM) 140 NaCl, 5 KCl, 2 $MgCl_2$, 5 EGTA, 10 HEPES, and 10 glucose, pH 7.4, adjusted with NaOH. The Cs-Asp/$Ca^{2+}(-)$ pipette solution for whole-cell patch-clamp and the Cs-Asp/$Ca^{2+}(-)$ bath solution for the inside-out patch-clamp contained (in mM) 120 aspartate, 10 CsCl, 1 $MgCl_2$, 5 EGTA, 17 mannitol, and 10 HEPES, pH 7.4, adjusted with CsOH. CaBuf (http://www.kuleuven.be/fysio/trp/cabuf) was used to calculate the free Ca^{2+} concentration and $CaCl_2$ was added to the $Ca^{2+}(-)$ pipette solution. The final concentrations of free Ca^{2+} in $CaCl_2$ solution were as follows (in mM): 0.077 $CaCl_2$ for 1 nM free Ca^{2+}, 0.675 $CaCl_2$ for 10 nM free Ca^{2+}, 3.032 $CaCl_2$ for 100 nM free Ca^{2+}, 3.72 $CaCl_2$ for 200 nM free Ca^{2+}, 4.105 $CaCl_2$ for 300 nM free Ca^{2+}, 4.68 $CaCl_2$ for 1000 nM free Ca^{2+}, 4.977 $CaCl_2$ for 10,000 nM free Ca^{2+}, and 5.099 nM $CaCl_2$ for 100,000 nM free Ca^{2+}. The Cs-Asp/Ca^{2+} 200 nM bath solution for inside-out patch-clamp was the same as the Cs-Asp/Ca^{2+} 200 nM pipette solution for whole-cell patch-clamp recording. For cation permeability experiments, the bath solutions contained (in mM) 140 NaCl (or 140 CsCl), 10 glucose, and 10 HEPES, pH 7.4, adjusted with NaOH (or CsOH) (for monovalent cations), or 110 $MgCl_2$ (or 110 $CaCl_2$), 2 $Mg(OH)_2$ [or 2 $Ca(OH)_2$], 10 glucose, and 10 HEPES, pH 7.4, adjusted with HCl (for divalent cations). The KCl pipette solution used for the ion permeability assay contained (in mM) 140 KCl, 5 EGTA, and 10 HEPES, pH 7.4, adjusted with KOH. The KCl/200 nM Ca^{2+} pipette solution contained 3.868 mM $CaCl_2$ in the KCl pipette solution. $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ indicate 2 mM extracellular Ca^{2+} or 200 nM intracellular Ca^{2+}, respectively, unless specified otherwise. Whole-cell recording data were sampled at 10 kHz and filtered at 5 kHz for analysis (Axopatch 200B amplifier with pClamp software; Molecular Devices). Inside-out patch recording data were sampled at 10 kHz and filtered at 2 kHz for analysis.

Membrane potential was clamped at −60 mV, unless specified. Shifts in liquid junction potentials during heating (ΔJP_H) were measured directly in separate experiments, and significant changes during heating (>5 mV) were observed. Therefore, membrane potentials and reversal potentials were corrected by subtracting the average ΔJP_H from data. All of the patch-clamp experiments were performed at room temperature except during heat stimulation. Thermal stimulation was applied by increasing the bath temperature with a preheated solution through the inline heater (1°C/s around thresholds, with a maximum of 46°C for fast heat application, a maximum of 51°C for high heat application) or by heating stage (slow heat application, 0.2°C/s around thresholds). After whole-cell configuration was achieved, the cell was raised 50 μm and placed in the center of the chamber. Temperature was monitored with a

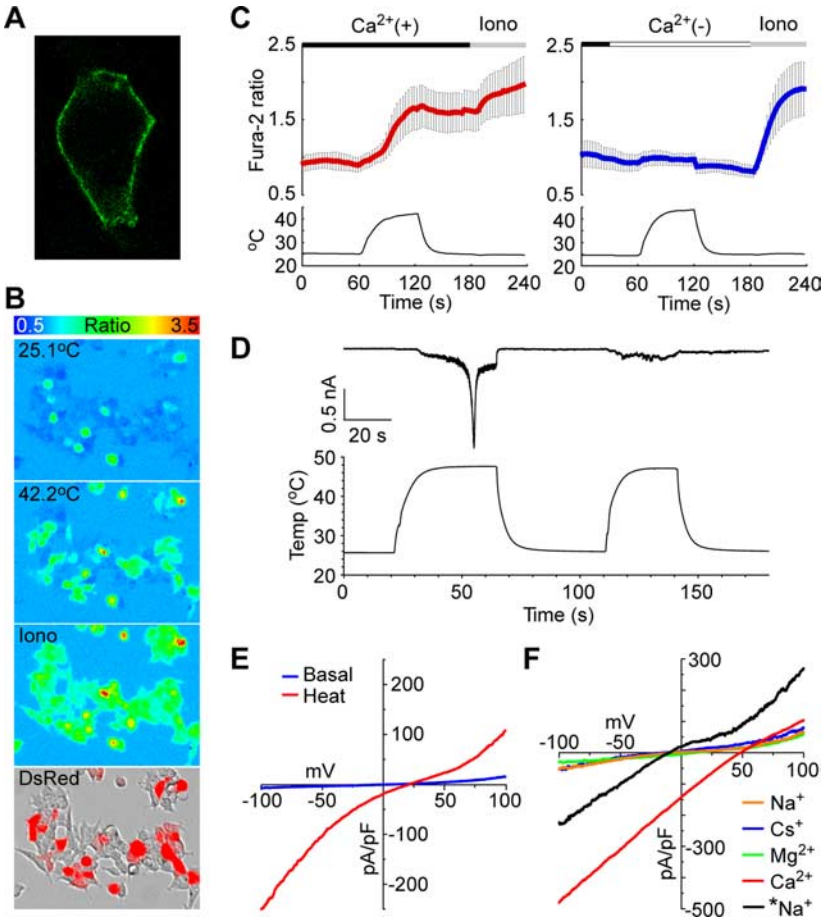

Figure 1. Heat-evoked activation and ion selectivity of Painless. ***A***, A representative image of a Painless-expressing HEK293 cell immunostained with anti-V5-tag antibody. ***B***, Representative fura-2 Ca^{2+} imaging shows $[Ca^{2+}]_i$ increase in Painless-expressing HEK293 cells during heating (~42°C). Pseudocolor indicates intensity of the fluorescence ratio of 340/380 nm. Ionomycin (5 μM) with 10 mM $CaCl_2$ (Iono) was applied to confirm cell viability. DsRed (red color) indicates Painless-expressing cells. ***C***, Quantitative changes in the fura-2 ratio in Painless-expressing cells during heating. A clear $[Ca^{2+}]_i$ increase was observed in the presence [$Ca^{2+}(+)$, red bar], but not in the absence [$Ca^{2+}(-)$, blue bar] of 2 mM extracellular $CaCl_2$. Average traces from 111 cells (left) or 73 cells (right) ±SD are shown in the top panels. Bottom panels show temperature changes. ***D***, Heat elicits inward current activation in a Painless-expressing HEK293 cell at −60 mV holding potential in a whole-cell patch-clamp mode ($n = 12$). A standard bath solution and Cs-Asp/$Ca^{2+}(-)$ pipette solution were used. ***E***, Current–voltage relationship of heat-evoked current exhibits dual-rectification with positive reversal potential. Heat-dependent shift of the liquid junctional potentials (ΔJP_H) were not corrected in the plot. The reversal potential was 28.8 ± 1.0 mV after compensation of ΔJP_H ($n = 8$). A standard bath solution and Cs-Asp/$Ca^{2+}(-)$ pipette solution were used. ***F***, Heat-evoked currents exhibit high Ca^{2+} permeability (n = 6–7). NaCl, CsCl, $MgCl_2$, or $CaCl_2$ bath solutions and KCl pipette solution were used. Note that $*Na^+$ (black trace) was obtained using NaCl bath and KCl/200 nM Ca^{2+} pipette solution. Basal traces were subtracted but ΔJP_H values were not compensated in the plot. After compensation of ΔJP_H values of the reversal potentials, permeability ratios were calculated (P_{Na}:P_{Cs}:P_K:P_{Mg}:P_{Ca} = 1:0.84:1.13:4.87:41.66).

thermocouple (TA-30; Warner Instruments) placed within 100 μm of the patch-clamped cell. The current–voltage (I–V) relationship during heating was obtained by using voltage ramps (−100 to +100 mV in 100 ms). Permeability ratios for cations were calculated as described previously (Adams et al., 1980; Caterina et al., 1997). In brief, the reversal potential was measured using voltage ramps (−100 to +100 mV in 100 ms). The permeability ratio for monovalent cations to K (P_X/P_K) was calculated as follows: $P_X/P_K = \exp(\Delta V_{rev}F/RT)$, where V_{rev} is the reversal potential, F is Faraday's constant, R is the universal gas constant, and T is absolute temperature. For measurement of divalent cation permeability, P_Y/P_K was calculated as follows: $P_Y/P_K = 3[K^+]_i \exp(\Delta V_{rev}F/RT)\ (1 + \exp(\Delta V_{rev}F/RT))/4[Y^{2+}]_o$, where bracketed terms are activities. Assumed activity coefficients are 0.75 for monovalent ions and 0.25 for divalent ions. Temperature profiles and Arrhenius plots were calculated using Origin software (Microcal). The temperature coefficient Q_{10} was used to characterize the temperature dependence of the membrane current. The absolute current values were plotted on a log scale against the reciprocal of the absolute temperature (T) (Arrhenius plot). Q_{10} values were calculated from $Q_{\Delta T} = (Q_{10})^{\Delta T/10}$ for an arbitrary temperature ΔT.

Statistical analysis. Multiple comparisons were performed using Tukey's method. Values are shown as mean ± SEM, unless indicated. A value of $p < 0.05$ was considered significant.

Results

Painless is a heat-activated, Ca^{2+}-permeable channel

We cloned *painless* cDNA from adult *Drosophila* heads and constructed expression vectors for mammalian cells. Abundant expression of Painless in the plasma membrane was observed when the plasmid was transiently transfected into HEK293 cells (Fig. 1*A*). Because the majority of the thermosensitive TRP channels show high conductance for Ca^{2+}, we first performed Ca^{2+}-imaging experiments using fura-2 to test whether Painless could raise intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) with heat stimulation. A robust $[Ca^{2+}]_i$ increase was observed in Painless-expressing cells when the temperature was raised >40°C (Fig. 1*B*,*C*, left). The $[Ca^{2+}]_i$ increase was completely abolished in the absence of extracellular Ca^{2+} ($Ca^{2+}{}_o$) (Fig. 1*C*, right), indicating that it was caused by an influx from extracellular space through Painless.

Next, we conducted patch-clamp experiments to clarify the electrophysiological properties of Painless. In the whole-cell configuration, Painless appeared to show transient inward currents during heating at a holding potential of −60 mV (Fig. 1*D*). The currents developed gradually when temperature was raised to >45°C, followed by fast activation and inactivation. The activation temperature was similar to that observed in heat-sensitive neurons in *Drosophila* larva (Tracey et al., 2003). After the activated currents were almost desensitized, the second heat stimulation could no longer elicit large currents and the inactivation state lasted for >20 min (data not shown). The I–V relationship of Painless showed dual rectification with an apparent positive reversal potential of 28.8 ± 1.0 mV (Fig. 1*E*), suggesting that Painless has high permeability to either of the cations included in the bath solution. We measured the reversal potentials in various extracellular monovalent or divalent cation solutions with the KCl pipette solution (Fig. 1*F*). The reversal potentials obtained with extracellular NaCl, CsCl, or $MgCl_2$ shifted modestly from zero, whereas that obtained with extracellular $CaCl_2$ showed a distinct positive shift (−3.37 ± 0.84 mV for NaCl, −7.94 ± 1.56 mV for CsCl, 14.34 ± 1.64 mV for $MgCl_2$, and 47.43 ± 1.31 mV for $CaCl_2$). Calculated relative permeability ratios indicated that Painless is a cation channel with extremely high Ca^{2+} permeability (P_{Na}:P_{Cs}:P_K:P_{Mg}:P_{Ca} = 1:0.84:1.13:4.87:41.66). The P_{Ca}/P_{Na} value was

similar to that for the TRP channel of *Drosophila* (Liu et al., 2007). We did not observe large currents when $CaCl_2$ was absent in extracellular and intracellular solutions, but larger currents were obtained when physiological intracellular Ca^{2+} ($Ca^{2+}{}_i$) was present in the pipette solution (Fig. 1*F*) (see below, Heat-evoked Painless activation requires Ca^{2+} ions). The reversal potential and the relative permeability ratio obtained with extracellular NaCl and intracellular KCl/200 nM free-$Ca^{2+}{}_i$ were similar to those obtained without $Ca^{2+}{}_i$ (-6.15 ± 0.33 mV and P_{Na}:P_K = 1:1.25). From these results, we conclude that Painless is a heat-sensitive TRP channel with high Ca^{2+} permeability.

Heat-evoked Painless activation requires Ca^{2+} ions

The activation properties of the thermosensitive TRP channels are modified by Ca^{2+} in various ways (Mohapatra et al., 2003; Strotmann et al., 2003; Chuang et al., 2004; Nagata et al., 2005; Talavera et al., 2005; Nilius et al., 2006; Tong et al., 2006; Doerner et al., 2007; Zurborg et al., 2007; Xiao et al., 2008). Because Painless displayed high Ca^{2+} permeability, we tested heat responsiveness under different combinations of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. In the absence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$, heat-evoked currents were almost negligible (<50 pA at −60 mV) (Fig. 2*A*). Conversely, as already shown in Figure 1, large currents were observed in the presence of $Ca^{2+}{}_o$ in the same cell. Therefore, we applied higher heat (~50°C) to test whether the heat responsiveness of Painless decreased without Ca^{2+}. Activation currents were slightly increased with higher heat in the absence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$; however, they were still much smaller than those in the presence of $Ca^{2+}{}_o$ (supplemental Fig. 1*A*, available at www.jneurosci.org as supplemental material). Painless was also activated by heat in the presence of physiological levels of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ (Fig. 2*B*). Interestingly, the presence of $Ca^{2+}{}_i$ was sufficient to elicit heat activation without $Ca^{2+}{}_o$ (Figs. 1*F*, 2*C*). The inactivation occurred regardless of $Ca^{2+}{}_o$ in the presence of $Ca^{2+}{}_i$, followed by small currents in the second heat application (Fig. 2*B*,C). These results suggest that Painless requires $Ca^{2+}{}_i$ to generate large currents during heating, and desensitization of the channel is not caused by $Ca^{2+}{}_i$ increase.

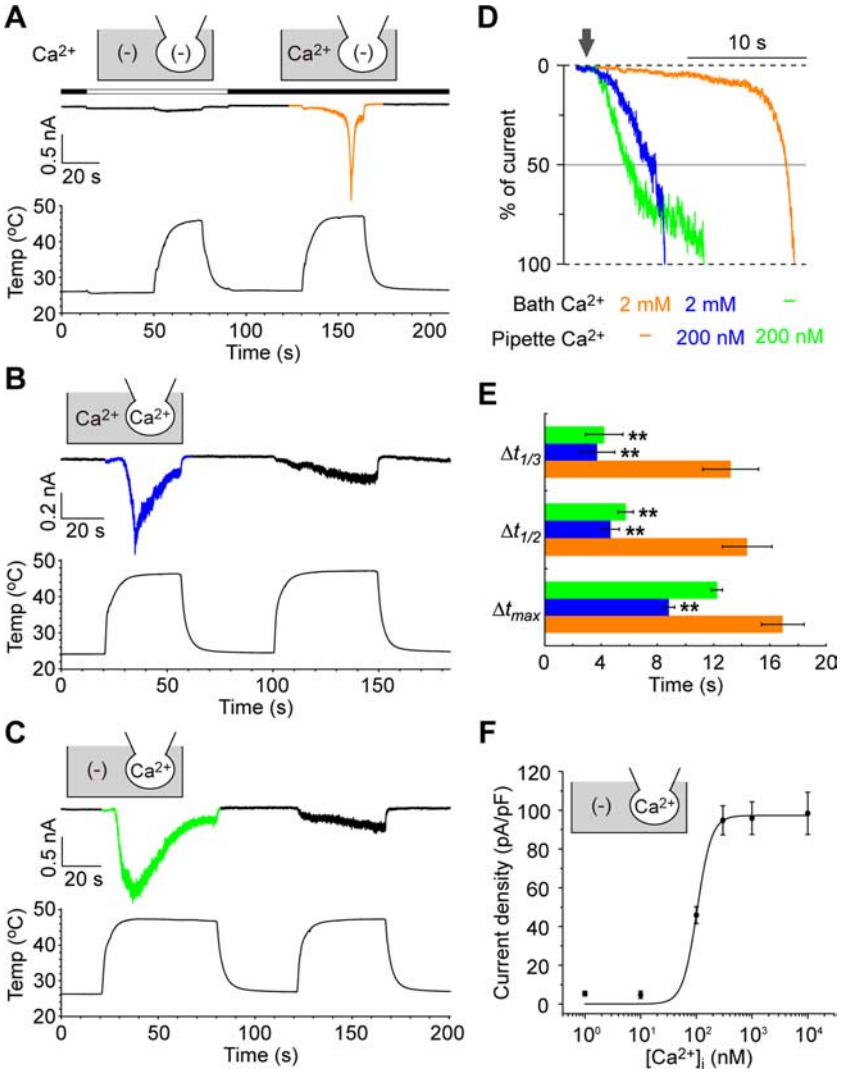

Figure 2. Ca^{2+}-requiring activation of Painless. ***A***, Painless exhibits only faint currents during heating in the absence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. Cs-Asp/Ca^{2+}(−) pipette solution [(−) in the pipette] and Ca^{2+}(−) bath solution [(−) in gray area] were used. Functional Painless expression was confirmed by heat application in standard bath solution (Ca^{2+} in gray area). ***B***, Heat elicits inward currents in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. After inactivation, Painless shows small currents after a second heating. Cs-Asp/200 nM Ca^{2+} pipette solution (Ca^{2+} in the pipette) and standard bath solution (Ca^{2+} in gray area) were used. ***C***, Heat elicits inward currents in the absence of $Ca^{2+}{}_o$ and the presence of $Ca^{2+}{}_i$. After inactivation, Painless shows small currents after a second heating. Cs-Asp/200 nM Ca^{2+} pipette solution (Ca^{2+} in the pipette) and Ca^{2+}(−) bath solution [(−) in gray area] were used. Currents in ***A–C*** are typical examples in a whole-cell patch-clamp mode. ***D***, $Ca^{2+}{}_i$ facilitates activation kinetics of heat-evoked currents. Colored traces correspond to the ones shown in ***A–C***. The arrow indicates the initial points of the currents (0%). One hundred percent of the current means maximal activation. Half of the maximal current is indicated as a gray line. ***E***, Quantification of the time required for 33% ($\Delta t_{1/3}$), 50% ($\Delta t_{1/2}$), and 100% (Δt_{max}) of maximal activation of Painless by heat. Colored bars correspond to the ones shown in ***A–C***. Data represent mean ± SEM. $^{**}p < 0.01$ ($n = 9–14$). ***F***, Heat responsiveness of Painless depends on $Ca^{2+}{}_i$ concentration. Cs-Asp pipette solution including 1–10^4 nM Ca^{2+} (Ca^{2+} in the pipette) and Ca^{2+}(−) bath solution [(−) in the gray area] were used. Maximal values of current density were obtained and fitted to Hill plots. The $Ca^{2+}{}_i$ concentration required for eliciting half of the maximal current was 103.4 ± 6.2 nM (Hill coefficient of 3.4 ± 1.8). Data represent mean ± SEM ($n = 8–18$). The background currents were taken in each $[Ca^{2+}]_i$ by applying heat to mock-transfected HEK293 cells and were subtracted from each point.

We also found that the activation kinetics varied depending on different Ca^{2+} conditions. In the presence of $Ca^{2+}{}_o$ alone, heat-evoked currents developed gradually (Fig. 2*A*,*D*, orange), whereas $Ca^{2+}{}_i$ elicited rapidly developing currents (Fig. 2*B–D*, blue, green). Indeed, the time required to generate 1/3 ($\Delta t_{1/3}$) or one-half ($\Delta t_{1/2}$) of the maximal activation of Painless by heat was significantly shorter in the presence of $Ca^{2+}{}_i$ than that in the presence of $Ca^{2+}{}_o$ alone (Fig. 2*E*). The inactivation rate during heat application also seemed to differ between the absence and presence of $Ca^{2+}{}_i$ (Fig. 2*A–C*). However, the time required for full inactivation varied and sometimes inactivation never occurred in the presence of $Ca^{2+}{}_i$. Therefore, we did not pursue the difference in inactivation kinetics. We next evaluated the $Ca^{2+}{}_i$ requirement for Painless during heating by changing $Ca^{2+}{}_i$ concentration ($[Ca^{2+}]_i$) (Fig. 2*F*). The $[Ca^{2+}]_i$ required for half ac-

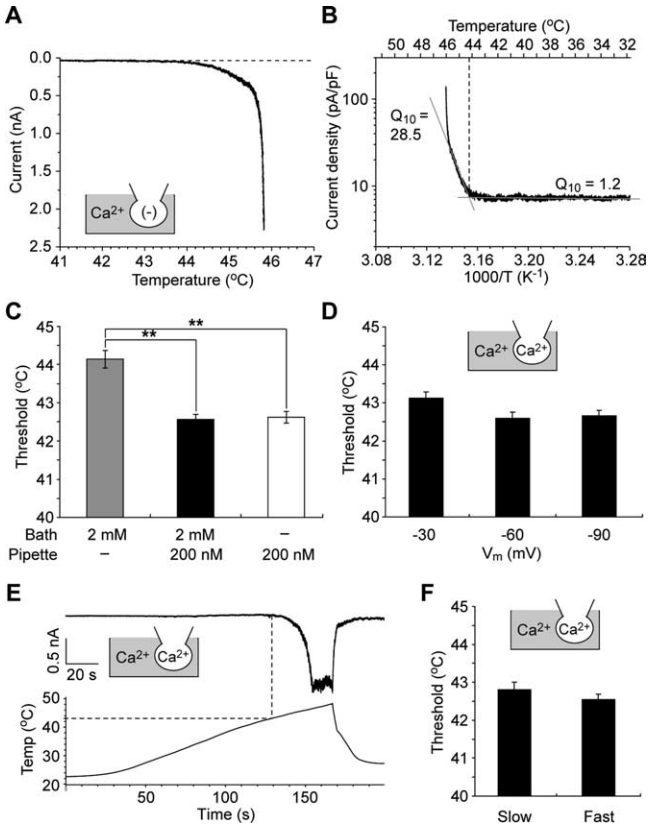

Figure 3. Temperature thresholds of Painless activation. ***A***, A representative temperature-response profile for heat-evoked Painless current in the presence of $Ca^{2+}{}_o$ alone at −60 mV holding potential. The dotted line indicates basal level. ***B***, An Arrhenius plot for heat-evoked Painless current shows a clear flex point on temperature dependency (data in ***A*** were converted). The crossing point of the two linear-fitted lines (a flex point) was defined as a temperature threshold for Painless activation. The Q_{10} value was calculated for each line (see Materials and Methods). ***C***, Temperature thresholds for Painless activation are significantly lower in the presence of $Ca^{2+}{}_i$. Data represent mean ± SEM. $^{**}p < 0.01$ ($n = 9–14$). All of the values were obtained from the same cells analyzed in Figure 2*E*. ***D***, Temperature thresholds do not depend on membrane potential in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. Data represent mean ± SEM ($n = 9–11$). ***E***, A representative trace shows activation currents of Painless elicited by slow heat application (0.2°C/s) in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. The dotted line indicates a temperature threshold. ***F***, Temperature thresholds do not depend on heat application rate in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. Painless was stimulated with slow (0.2°C/s) or fast (1°C/s) heat application. Data represent mean ± SEM ($n = 12–14$).

tivation by heat was 103.4 ± 6.2 nM; a Hill coefficient of ~3.4. Ca^{2+} alone was not sufficient to activate Painless because we never observed visible currents in the presence of 2 mM $Ca^{2+}{}_o$ and 1 mM $Ca^{2+}{}_i$ (supplemental Fig. 1*B*, available at www.jneurosci.org as supplemental material). Together, Painless activity is coordinated by physiological levels of $Ca^{2+}{}_i$, permitting it to function as a heat sensor.

Temperature threshold for Painless activation is Ca^{2+} dependent

We determined the temperature threshold for Painless activation under different combinations of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. The temperature-response profile of Painless showed that currents were activated over ~44°C in the presence of $Ca^{2+}{}_o$ alone (Fig. 3*A*). To determine the temperature thresholds more precisely, we generated Arrhenius plots, which displayed an explicit flex point during heating (Fig. 3*B*). The basal Q_{10} value was 1.2, but it rapidly increased to 28.5 above the flex point. Because thermosensitive TRP channels are known to have Q_{10} values >10 (regarded as a thermosensitive reaction), we defined the flex point as a Q_{10} value sufficiently >10 as the temperature threshold for Painless activation, which was 44.1 ± 0.2°C when only $Ca^{2+}{}_o$ was present (Fig. 3*C*, left). The temperature threshold in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ was 42.6 ± 0.1°C, and the threshold in the presence of $Ca^{2+}{}_i$ alone was 42.6 ± 0.2°C (Fig. 3*C*, middle, right). These values were significantly lower than the temperature threshold in the presence of $Ca^{2+}{}_o$ alone. Thus, physiological levels of $Ca^{2+}{}_i$ reduce the temperature threshold for Painless activation.

It has been shown that mammalian thermosensitive TRP channels such as TRPV1 and TRPM8 have voltage dependency, and their temperature thresholds strongly depend on voltage (Voets et al., 2004). To test whether the temperature threshold for Painless has such a voltage dependency, the thresholds were determined under different membrane potentials (Fig. 3*D*). There was no significant difference in the thresholds among holding potentials of −30, −60, and −90 mV (thresholds were 43.1 ± 0.2°C at −30 mV, 42.6 ± 0.2°C at −60 mV, and 42.7 ± 0.1°C at −90 mV, respectively). The current sizes varied among samples probably because of different expression levels of Painless protein, but there was no correlation between current sizes and the temperature thresholds defined by our method in each membrane potential (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Additionally, we examined whether heat application rate affected the temperature threshold of Painless, because some thermosensitive TRP channels have been known to show such dependency, and, especially, some neurons in *Drosophila* have been reported to display increasing neural activity at temperatures as low as 28°C after slow increases in temperature (Tracey et al., 2003). Interestingly, however, slow heat application (~0.2°C/s) activated Painless at 42.8 ± 0.2°C, which was not significantly different from that of fast heat application (42.6 ± 0.1°C, 1°C/s) (Fig. 3*E*,*F*). Together, it is likely that our method to determine the thresholds is suitable for this analysis, and the temperature thresholds for Painless activation are dependent on $Ca^{2+}{}_i$, but not on either membrane potential or heating rate.

Ca^{2+} sensitizes Painless with repeated heat stimulation

As described above, second heat application failed to activate Painless when the channel was fully inactivated during the first heat stimulation (Figs. 1*C*, 2*B*,*C*). However, multiple heat-evoked currents were attainable when heat stimulation was terminated at the early state of current development (Fig. 4*A*). Thermal stimulation elicited current activation more than three times in this repeated heating protocol. Utilizing such a protocol, we next asked whether Painless was sensitized after repetitive exposure to heat. In the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$, the temperature thresholds for activation on the second and third heat application were significantly reduced compared with the first one in the same cell (thresholds in the first, second, and third heat applications were 42.5 ± 0.1°C, 41.3 ± 0.3°C, and 41.2 ± 0.3°C, respectively) (Fig. 4*B*,*D*,*F*). In contrast, in the presence of $Ca^{2+}{}_i$ alone, the temperature thresholds were not changed through the repeated heat application (thresholds in the first, second, and third heat applications were 42.7 ± 0.2°C, 42.5 ± 0.1°C, and 42.5 ± 0.2°C, respectively) (Fig. 4*C*,*E*,*G*). This result indicates that Painless is sensitized after repeated heat exposure under physiological extracellular and intracellular Ca^{2+} conditions.

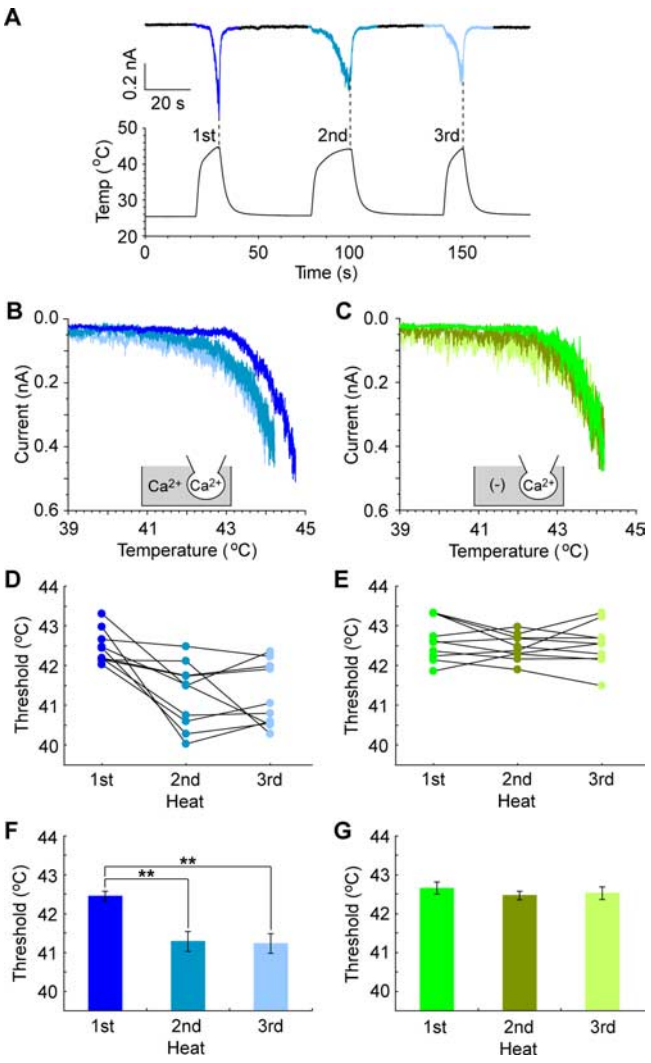

Figure 4. Temperature thresholds in repeated exposure to heat. ***A***, Multiple currents are observed in the repeated heating protocol (see Results). Representative heat-evoked currents in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ are shown. Heat application was terminated when the current reached ~0.5 nA (dotted lines). More than three responses at equivalent currents could be observed without desensitization. ***B***, ***C***, Temperature thresholds are reduced after repeated heat with (***B***), but not without (***C***), $Ca^{2+}{}_o$. Standard (Ca^{2+} in gray area) or Ca^{2+}(−) bath solution [(−) in gray area] and Cs-Asp/200 nM Ca^{2+} pipette solution (Ca^{2+} in the pipette) were used. ***D***, ***E***, Raw data of temperature thresholds for the first to third heating cycle are shown in individual cells ($n = 10$). Standard (***D***) or Ca^{2+} (−) bath solution (***E***) and Cs-Asp/Ca^{2+} 200 nM pipette solution were used. Thresholds were calculated as in Figure 2*C*. ***F***, ***G***, The temperature thresholds are significantly reduced after repeated heating in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ (***F***), but not in the presence of $Ca^{2+}{}_i$ alone (***G***). Data represent mean ± SEM. $^{**}p < 0.01$ ($n = 10$). All of the colored traces, dots, and bars correspond to the first, second, and third heat applications indicated in ***F*** and ***G***.

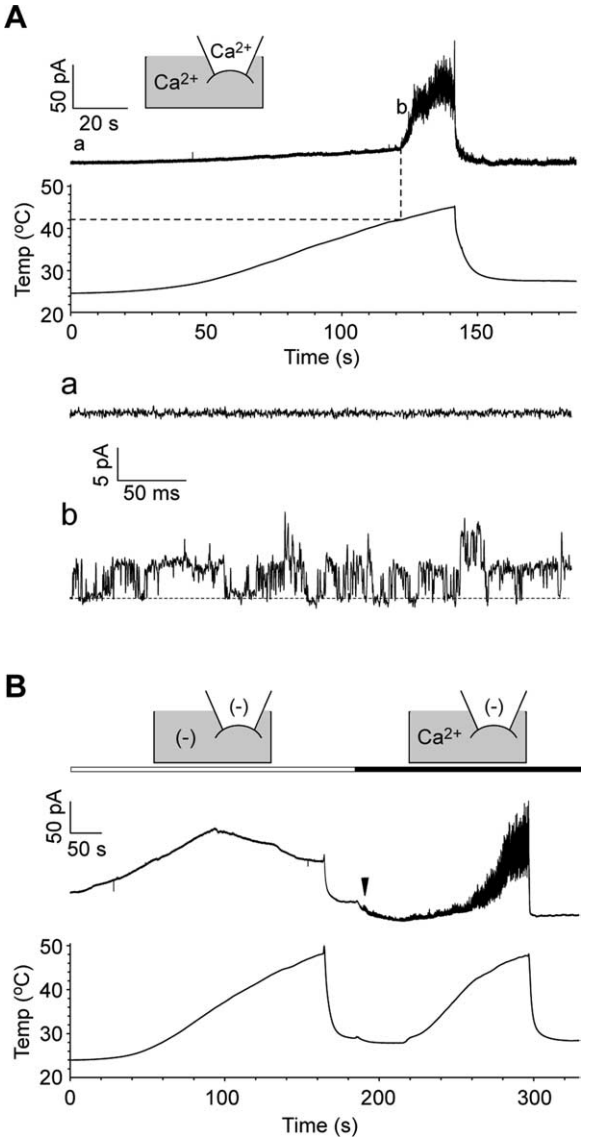

Figure 5. Single-channel activation of Painless during heating. ***A***, The top trace shows activation currents in Painless-expressing excised membrane during slow heating (0.2°C/s) in an inside-out patch-clamp mode at +60 mV holding potential ($n = 4$). The dotted line indicates an initiation point of the currents. Standard pipette solution (Ca^{2+} in the pipette) and Cs-Asp/200 nM Ca^{2+} bath solution (Ca^{2+} in gray area) were used. The basal trace in ***a*** and single-channel currents in ***b*** are magnified from corresponding lines in the top trace. The dotted line in ***b*** indicates the closed-channel level. ***B***, Painless is robustly activated by heat in the presence of Ca^{2+} on the cytoplasmic side ($n = 6$). Ca^{2+}(−) pipette solution [(−) in the pipette] and Cs-Asp/Ca^{2+}(−) bath solution [(−) in gray area] or Cs-Asp/200 nM Ca^{2+} bath solution (Ca^{2+} in gray area) were used. Note that movements in basal lines including leak always occurred in the absence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$, but those are clearly different from single-channel currents observed in the presence of $Ca^{2+}{}_i$. Moreover, small but apparent single-channel currents were evoked as soon as Ca^{2+} was applied to the cytoplasmic side before heat application (arrowhead).

Painless is activated by heat in a membrane-delimited manner

To clarify whether Painless is directly activated by heat, we tried to observe the heat activation currents at the single-channel level. We chose a slow heat application protocol (~0.2°C/s) (Fig. 3*E*), because the basal line fluctuated during fast heat application (~1°C/s). In an inside-out patch-clamp configuration, heat-evoked single-channel currents were observed during heating with an apparent threshold of 42°C in Painless-expressing cells, and its unitary conductance was 49.6 ± 3.5 pS (Fig. 5*A*). Furthermore, Painless was rarely activated during heating in the absence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$, whereas Painless responded to heat when Ca^{2+} was applied to the cytoplasmic side in the same membrane (Fig. 5*B*). Interestingly, we sometimes observed Painless-mediated single-channel currents at innocuous temperatures (<30°C) (Fig. 5*B*, arrowhead). These results indicate that Painless is activated by heat in a membrane-delimited manner, and intracellular Ca^{2+} is required for its activation.

Cytoplasmic regulatory region for Ca^{2+} in Painless

The results shown above demonstrate that intracellular Ca^{2+} is critical for the responsiveness of Painless to heat and it seems to

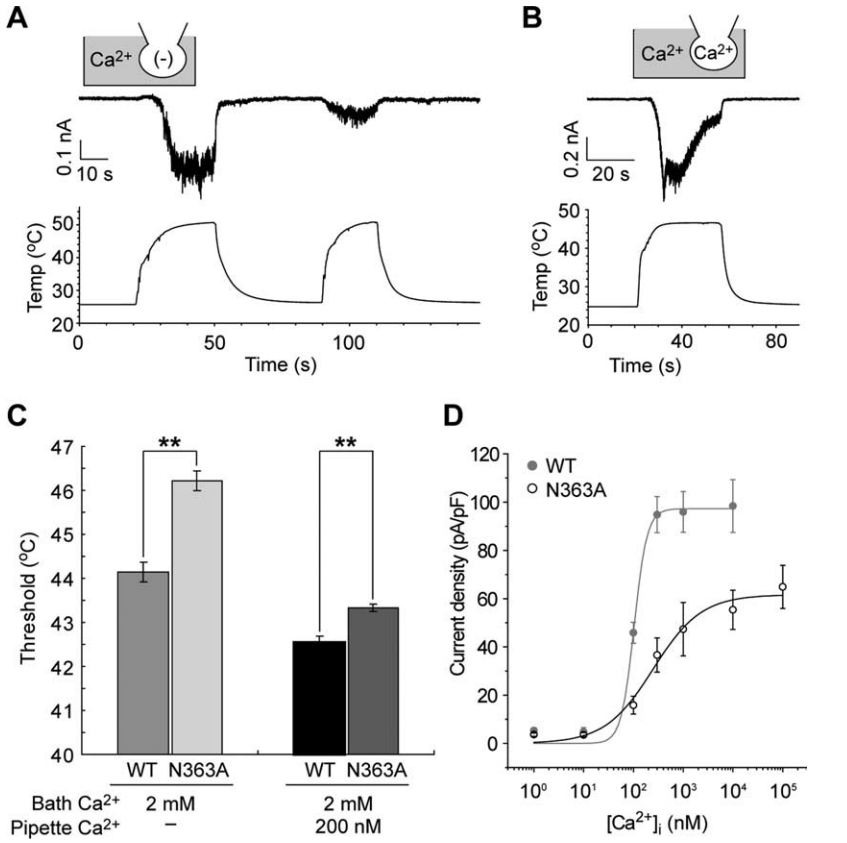

Figure 6. Effects of N-terminal mutation on heat responsiveness of Painless. ***A***, ***B***, Mutant Painless N363A shows small currents in the presence of $Ca^{2+}{}_o$ alone (***A***), but exhibits large currents in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ (***B***). Standard bath solution (Ca^{2+} in gray area) and Cs-Asp/Ca^{2+}(−) pipette solution [(−) in the pipette] (***A***) or Cs-Asp/Ca^{2+} 200 nM pipette solution (Ca^{2+} in the pipette) (***B***) were used. Current traces are typical examples in a whole-cell patch-clamp mode ($n = 10–14$). ***C***, Temperature thresholds for mutant Painless N363A are significantly higher than those for wild-type Painless in the presence of $Ca^{2+}{}_o$ alone or in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$. Data represent mean ± SEM. $^{**}p < 0.01$ ($n = 9–14$). ***D***, $[Ca^{2+}]_i$ sensitivity of mutant Painless N363A was decreased. Cs-Asp pipette solution including $1–10^5$ nM Ca^{2+} and Ca^{2+}(−) bath solution were used. Maximal values of current density were obtained and fitted to Hill plots. Gray points and the fitted line indicate wild-type Painless (see also Fig. 2*F*). $[Ca^{2+}]_i$ required for eliciting half of the maximal current was 249.3 ± 59.8 nM (Hill coefficient of 0.9 ± 0.2). Data represent mean ± SEM ($n = 9–15$). The background currents were taken in each $[Ca^{2+}]_i$ by applying heat to mock-transfected HEK293 cells and were subtracted from each point.

directly regulate the activity of the channel. Intracellular Ca^{2+} directly activates mammalian TRPA1 via binding to its putative EF-hand-like motif in the cytoplasmic N-terminal region (Doerner et al., 2007; Zurborg et al., 2007). We compared the amino acid sequences of the N-terminal cytoplasmic region between Painless and mammalian TRPA1 and focused on a region in Painless that corresponds to the EF-hand-like motif in the mammalian TRPA1 (supplemental Fig. 3*A*, available at www.jneurosci.org as supplemental material). This region is located in the ankyrin repeat domain in Painless. We then investigated the possibility that this region is a Ca^{2+}-regulatory region for Painless. Painless mutants bearing N356, S357, N363, or D366 substitutions to alanine were constructed, because each amino acid is functionally important in EF-hand-like motif in TRPA1 (Doerner et al., 2007; Zurborg et al., 2007). Heat was applied to HEK293 cells expressing the mutant Painless with or without $Ca^{2+}{}_i$, and the temperature thresholds were evaluated. Interestingly, in the presence of $Ca^{2+}{}_o$ alone, the N363A mutant elicited small currents, whereas the other three mutants showed large currents during heating (Fig. 6*A*) (data not shown). All of the mutants including N363A exhibited heat-evoked currents similar to wild-type Painless in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ (Fig. 6*B*, supplemental Fig. 3*B–D*, available at www.jneurosci.org as supplemental material). Additionally, the temperature threshold for activation of Painless N363A was 46.2 ± 0.2°C in the presence of $Ca^{2+}{}_o$ alone, and 43.3 ± 0.1°C in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$, both of which were significantly higher than those of wild-type Painless (44.1 ± 0.2°C in the presence of $Ca^{2+}{}_o$ alone, 42.6 ± 0.1°C in the presence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$) (Fig. 6*C*). In contrast, temperature thresholds for activation of Painless N356A, S357A, and D366A were not different from those of wild-type Painless in the presence or the absence of $Ca^{2+}{}_i$ (supplemental Fig. 3*E*, available at www.jneurosci.org as supplemental material). The $[Ca^{2+}]_i$ required for half activation of Painless N363A by heat was 249.3 ± 59.8 nM, with a Hill coefficient of ~0.9 (Fig. 6*D*), both of which were different from those of wild-type Painless (Fig. 2). Thus, this region may play a role as a Ca^{2+}-regulatory site for heat activation of Painless.

Painless is insensitive to thermosensitive TRP channel activators in HEK293 cells

Painless was reported to be important for avoidance of wasabi as well as noxious heat (Al-Anzi et al., 2006). Wild-type flies tended to avoid food containing allyl isothiocyanate (AITC), which is the main ingredient of wasabi and mustard, in a dose-dependent manner. In contrast, *painless* mutant flies were defective in such avoidance behavior. The fact that Painless is most closely related to mammalian TRPA1, which is activated by AITC, implies that Painless might be a direct sensor for AITC. Surprisingly, however, data from Ca^{2+}-imaging and patch-clamp experiments indicated that Painless-expressing HEK293 cells were completely insensitive to AITC (Fig. 7*A*, supplemental Fig. 4, available at www.jneurosci.org as supplemental material). We did not observe a positive response in Painless-expressing HEK293 cells up to 2 mM AITC. We also examined other TRPA1 activators, such as cold stimulus (~10°C), cinnamaldehyde, allicin, icilin, acrolein, formalin, methyl paraben, menthol, and 2-APB. All failed to activate Painless expressed in HEK293 cells (Fig. 7*B*, supplemental Fig. 4, available at www.jneurosci.org as supplemental material) (data not shown). Typical thermosensitive TRP channel activators such as capsaicin, camphor and 4α-phorbol-12,13-didecanoate also failed to activate Painless (supplemental Fig. 5, available at www.jneurosci.org as supplemental material) (data not shown).

Painless mutant flies reportedly display an increased threshold for mechanical stimulation compared with wild-type (Tracey et al., 2003). However, we failed to detect any intracellular Ca^{2+} increase or current activation in Painless-expressing HEK293 cells in response to super hypotonic (160 mOsm) or hypertonic (432 mOsm) stimulus, or by direct touch with a glass pipette tip (supplemental Fig. 5, available at www.jneurosci.org as supple-

mental material) (data not shown). To determine whether Painless responds to other hazardous stimuli or bitter substances, acidic/alkaline solution (pH 5 or 10) or caffeine were applied. None of them elicited activation of Painless (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). Together, the only activator thus far identified for Painless is heat.

We next tested whether mammalian TRPA1 channel blockers affected heat-evoked Painless activation. Ruthenium red, a broad blocker for some TRP channels including TRPA1, reversibly inhibited heat-evoked activation of Painless (Fig. 7*C*). Camphor and menthol, which are well known agonists for TRPV3 and TRPM8, respectively, have been reported to inhibit mammalian TRPA1 activation (Xu et al., 2005; Macpherson et al., 2006; Karashima et al., 2007). Camphor (3 mM) could partially block heat-evoked currents of Painless, whereas 2 mM camphor could not suppress the heat activation of Painless (Fig. 7*D*) (data not shown). Heated solutions containing >3 mM camphor consistently broke the gigaohm seal so that we did not evaluate the effects of higher concentrations of camphor. However, menthol (2 mM) failed to block heat activation of Painless (data not shown). Problems similar to those experienced with heated camphor were encountered with heated menthol. Thus, we were unable to determine whether higher concentrations of menthol inhibited Painless activation. These results indicate that Painless shares some properties with the mammalian TRPA1.

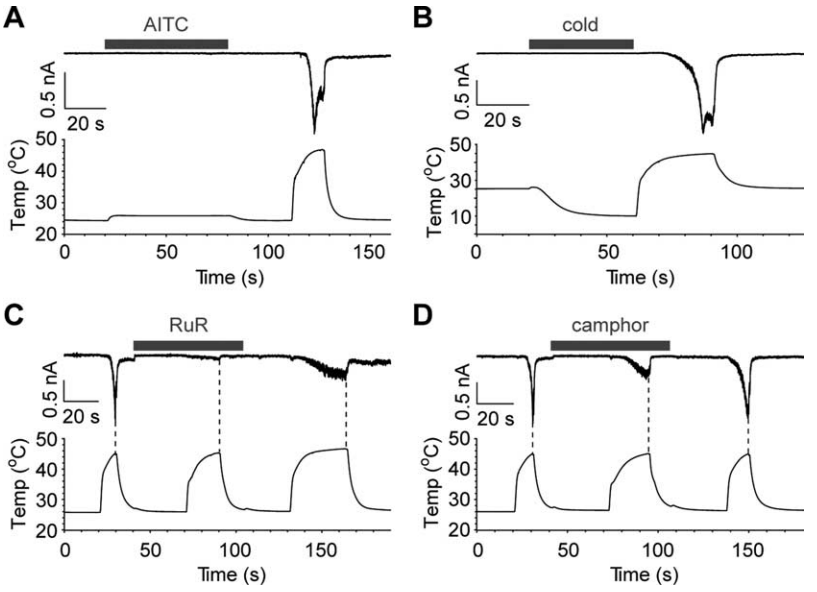

Figure 7. The effects of mammalian TRPA1 agonists and antagonists on Painless. ***A***, ***B***, Painless-expressing cells are not activated by AITC (2 mM; ***A***) or cold stimulation (~10°C; ***B***) ($n = 3–5$). Standard bath solution and Cs-Asp/200 nM Ca^{2+} pipette solution were used. Functional Painless expression was confirmed by heat application. ***C***, ***D***, Heat activation of Painless was reversibly blocked by ruthenium red (RuR, 10 μM; ***C***) or camphor (3 mM; ***D***) treatment ($n = 6–8$). Standard bath solution and Cs-Asp/200 nM Ca^{2+} pipette solution were used. The repeated heating protocol (Fig. 4) was used to validate the function of Painless before and after the treatment with antagonists. Heat termination points are indicated as dotted lines. Cells were treated with each antagonist, followed by application of heated bath solution containing antagonist. The inhibitory effects of ruthenium red were incompletely reversed. Higher concentrations of camphor could not be applied because camphor severely damaged the gigaohm seal.

Discussion

Painless is a direct heat sensor

The present study provides direct evidence that the *Drosophila* TRP channel Painless is a heat sensor. Although the biophysical properties of Painless have yet to be elucidated, members of the *Drosophila* TRPA subfamily, dTRPA1 and pyrexia, show temperature sensitivity both *in vitro* and *in vivo*, from which it has been inferred that Painless might also be a heat-sensitive channel (Montell, 2005). Indeed, a robust Ca^{2+} influx and current activation via Painless was observed during heating in our heterologous expression system (Fig. 1). The temperature threshold for Painless activation (~42.6°C) was consistent with the temperature that causes avoidance behavior *in vivo* (Tracey et al., 2003). Painless was activated by heat in a membrane-delimited and Ca^{2+}-dependent manner (Fig. 5), indicating that Painless can detect heat directly by using $Ca^{2+}{}_i$, but not intracellular signaling pathways. Thus, Painless itself may act as a primary heat detector to facilitate neural activity *in vivo*.

In vivo and *in vitro* analyses revealed different temperature thresholds. Previous work reported that there were two types of Painless-expressing neurons with low (~28°C) and high (~39°C) temperature thresholds (Tracey et al., 2003). However, the temperature thresholds of Painless in our system were 41 ~ 44°C, rather than <40°C, in various heating conditions such as $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$ concentrations, membrane potentials, or heat application rates. These differences might be due, in part, to the methods determining the thresholds. We defined each threshold as a reflex point with an increasing Q_{10} value >10 in Arrhenius plots. In this case, minuscule current development ($Q_{10} < 10$) was not regarded as the onset of Painless activation. However, magnitude of the background current does not affect the thresholds, because there was no correlation between current sizes and thresholds (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). We sometimes observed single-channel currents of Painless initiated at ~28°C, a temperature close to the value at which neural activity differed between wild-type and the *painless* mutant (Tracey et al., 2003), during slow heat application (0.2°C/s) (Fig. 6). This result suggests that Painless has an ability to respond to lower temperature *in vitro*, which might contribute to neural excitability. Alternatively, there could be biological differences between *in vitro* and *in vivo*. The lipid composition of the mammalian plasma membrane may affect Painless activity differently than it would in its native environment. In fact, the functions of TRP channels, including thermosensitive ones, are regulated by a series of lipids (Hardie, 2007). Temperature threshold for activation of mammalian TRPV1, which is activated by noxious heat (>43°C) like Painless, has been reported to be reduced with phosphorylation by PKC (Numazaki et al., 2002), and it is also known that TRPV1 function is regulated through binding with specific accessory proteins (Kim et al., 2006, 2008). Such physiological regulation may also exist to alter Painless property *in vivo*.

Painless activation is regulated by extracellular and intracellular Ca^{2+}

We determined four essential roles of Ca^{2+}. First, Ca^{2+} enables Painless to respond to heat. Whereas higher heat (~50°C) elicited only faint currents in the absence of $Ca^{2+}{}_o$ and $Ca^{2+}{}_i$, 200 nM

Ca^{2+}_i was sufficient for heat activation (Fig. 2), suggesting that Painless requires Ca^{2+}_i for functionality. Thermosensitive TRP channels such as TRPM4, TRPM5, and TRPA1 are activated by Ca^{2+}_i (Talavera et al., 2005; Nilius et al., 2006; Doerner et al., 2007; Zurborg et al., 2007), whereas Painless demands Ca^{2+} as a coagonist for heat. A similar concept has been reported in TRPM8, in that Ca^{2+}_i supports robust icilin-evoked responses (Chuang et al., 2004). $[Ca^{2+}]_i$ required for half activation of Painless was ~103 nM (Fig. 2), a concentration close to the reported value in the terminal and dorsal organ of the larval head (Liu et al., 2003).

Second, Ca^{2+} accelerates the activation kinetics of Painless. The longer time required for activation in the presence of Ca^{2+}_o alone (Fig. 2) is probably explained by a necessity for Ca^{2+}_o to first enter the cytoplasm until $[Ca^{2+}]_i$ reaches the threshold for maximal activation, which results in gradual current development, followed by accelerated activation. In the presence of Ca^{2+}_i, Painless is activated quickly because sufficient Ca^{2+}_i for full activation exists. Indeed, fly larvae moved away from a heated probe within 0.4 s (Tracey et al., 2003), supporting this idea. Moreover, inactivation rates of Painless seemed to be affected by Ca^{2+} conditions (Fig. 2). Ca^{2+}_i might delay the inactivation; however, Ca^{2+}_i should increase during activation also in the presence of Ca^{2+}_o alone, which shows rapid inactivation. Nevertheless, Ca^{2+} is apparently important for regulating the activation kinetics.

Third, Ca^{2+} reduces temperature thresholds. Painless is "ready for activation" in the presence of Ca^{2+}_i, so that it may respond to heat at its reduced temperature threshold of ~42.6°C (Fig. 3). However, the temperature threshold was ~44.1°C in the presence of Ca^{2+}_o alone, where heat should initiate the gating of Painless in a Ca^{2+}_i-independent manner. Quick avoidance from a heated probe occurred at ~42°C in fly larvae (Tracey et al., 2003); therefore, the *in vivo* temperature threshold is close to that obtained in the presence of Ca^{2+}_i. Thermosensitive TRP channels such as TRPV1 and TRPM8 have strong voltage dependencies in their temperature thresholds, whereas Painless did not (Fig. 3). This is not surprising because dual rectified *I–V* relationship of Painless is apparently different from outward rectified *I–V* relationship of TRPV1 and TRPM8.

Finally, Ca^{2+} contributes to sensitization of Painless during repetitive heating (Fig. 4). Significant reduction in the temperature thresholds during repeated heating was observed in the presence of Ca^{2+}_o and Ca^{2+}_i, but not in the presence of Ca^{2+}_i alone (Fig. 4), suggesting that Ca^{2+}_o and/or Ca^{2+} influx may be involved in the sensitization. This would be physiologically important, because flies are able to escape from a hazardous heat source in less time after second exposure. Sensitization during repetitive heating is a common feature in TRPV1, TRPV2, and TRPV3 (Caterina et al., 1999; Xu et al., 2002), although the underlying mechanism is still unknown including the requirement for Ca^{2+}.

N-terminal region of Painless is involved in Ca^{2+}-dependent heat activation

Recently, mammalian TRPA1 was reported to be activated by Ca^{2+} (Doerner et al., 2007; Zurborg et al., 2007). Ca^{2+}_i-dependent activation significantly deteriorated when the EF-hand-like motif in the N-terminal region was mutated. We compared amino acid sequences between Painless and TRPA1 and determined the candidate region for Ca^{2+}_i regulation in Painless, which was located in the ankyrin repeat domain (Fig. 6, supplemental Fig. 3, available at www.jneurosci.org as supplemental material). The Painless N363A mutant displayed small heat-evoked currents, increased temperature thresholds, and higher $[Ca^{2+}]_i$ requirement for half activation with a reduced Hill coefficient (Fig. 6). These features could be explained if the "Ca^{2+}-regulatory region" in Painless included N363, and its affinity to Ca^{2+}_i was reduced by mutation. Thus, the mutant channel requires increasing $[Ca^{2+}]_i$ to be fully activated, which results in a higher temperature threshold and small size in currents. These results suggest that N363 is a key residue in Ca^{2+} sensitivity of Painless, although the function is different from the EF-hand-like motif in mammalian TRPA1. Painless is not activated by high $[Ca^{2+}]_i$ alone (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and requires much less $[Ca^{2+}]_i$ for its regulation, whereas TRPA1 requires Ca^{2+} at a micromolar level for activation. Furthermore, mutation of N356, S357, or D366 did not affect the heat responsiveness of Painless, whereas the corresponding amino acids in the EF-hand-like motif are necessary for Ca^{2+}_i-dependent TRPA1 activation.

Painless is insensitive to thermosensitive TRP channel activators in HEK293 cells

Thermosensitive TRP channels can be activated by various stimuli and one stimulant sometimes activates multiple thermosensitive TRP channels (Dhaka et al., 2006; Ramsey et al., 2006; Tominaga, 2007). Accordingly, Painless could mediate several stimuli such as AITC or a mechanical stimulus (Tracey et al., 2003; Al-Anzi et al., 2006). However, Painless-expressing HEK293 cells did not respond to a range of possible activators (Fig. 7, supplemental Figs. 4, 5, available at www.jneurosci.org as supplemental material). Recently, AITC was reported to activate mammalian TRPA1 through covalent modification (Hinman et al., 2006; Macpherson et al., 2007). However, several cysteines important for covalent modification were not present in Painless (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), which might explain its insensitivity to AITC, cinnamaldehyde, allicin, acrolein, and formalin. Thus, Painless is not likely to be a direct receptor for these cysteine modifiers. Alternatively, interaction with accessory protein(s), formation of heteromeric channel, and/or splice variants might alter properties of Painless *in vivo*. These possibilities remain to be addressed. Painless is expressed in the CNS as well as peripheral sensory neurons (Tracey et al., 2003; Al-Anzi et al., 2006), where Painless could be activated by endogenous ligands. Heat activation of Painless was inhibited by ruthenium red and camphor, indicating that Painless shares some properties with mammalian TRPA1. Camphor, a wood derivative from camphor laurel, has been used as a repellent for pests and proved to be effective for mosquitoes (Gillij et al., 2007). The repellent may inhibit the noxious heat sensor, perhaps interfering with the normal sensing ability of flies. It would therefore be intriguing to test the effects of camphor at the behavioral level.

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