Thermosensitivity of the two-pore domain K^+ channels TREK-2 and TRAAK

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TREK-1, TREK-2 and TRAAK are members of the two-pore domain K^+ (K_{2P}) channel family and are activated by membrane stretch and free fatty acids. TREK-1 has been shown to be sensitive to temperature in expression systems. We studied the temperature-sensitivity of TREK-2 and TRAAK in COS-7 cells and in neuronal cells. In transfected COS-7 cells, TREK-2 and TRAAK whole-cell currents increased ∼20-fold as the bath temperature was raised from 24°C to 42°C. Similarly, in cell-attached patches of COS-7 cells, channel activity was very low, but increased progressively as the bath temperature was raised from 24°C to 42°C. The thresholds for activation of TREK-2 and TRAAK were ∼25°C and ∼31°C, respectively. Other K_{2P} channels such as TASK-3 and TRESK-2 were not significantly affected by an increase in temperature from 24°C to 37°C. When the C-terminus of TREK-2 was replaced with that of TASK-3, its sensitivity to free fatty acids and protons was abolished, but the mutant could still be activated by heat. At 37°C, TREK-1, TREK-2 and TRAAK were sensitive to arachidonic acid, pH and membrane stretch in both cell-attached and inside-out patches. In cerebellar granule and dorsal root ganglion neurones, TREK-1, TREK-2 and TRAAK were generally inactive in the cell-attached state at 24°C, but became very active at 37°C. In cell-attached patches of ventricular myocytes, TREK-1 was also normally closed at 24°C, but was active at 37°C. These results show that TREK-2 and TRAAK are also temperature-sensitive channels, are active at physiological body temperature, and therefore would contribute to the background K^+ conductance and regulate cell excitability in response to various physical and chemical stimuli.

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TREK-1, TREK-2 and TRAAK belong to the two-pore domain K^+ (K_{2P}) channel family and are expressed throughout the central nervous system and in many peripheral tissues (Medhurst *et al.* 2001; Talley *et al.* 2001). Unlike other members of the K_{2P} channel family such as TWIK, TASK, TRESK and TALK, TREK-1, TREK-2 and TRAAK are activated by free fatty acids and increased membrane tension (Patel *et al.* 2001; Kim, 2003). TREK-1 and TREK-2 are also activated by volatile anaesthetics and acidic solution, and inhibited by receptor agonists via G proteins Gs and Gq/11 (Patel *et al.* 1999; Lesage *et al.* 2000; Chemin *et al.* 2003; Enyeart *et al.* 2004). TRAAK is insensitive to volatile anaesthetics and G proteins, but is activated by alkaline solution (Kim *et al.* 2001*a*). These interesting properties of TREK-1 and TREK-2 suggest that they not only regulate cell excitability and thus synaptic transmission via receptor-mediated pathways, but may also become important during metabolically stressed conditions when intracellular levels of free fatty acids and protons are elevated, and cell swelling occurs. This idea is supported by a recent study that shows that TREK-1 null (*TREK-1*$^{-/-}$) mice have increased sensitivity to ischaemia and epileptogenic drugs (Heurteaux *et al.* 2004). TREK-2 may also be neuroprotective in certain regions of the brain, as it has properties similar to those of TREK-1.

In cultured and isolated neurones from rat brain, TREK-2 is normally in the closed state when recorded from cell-attached patches at room temperature (Kim & Clapham, 1989; Kim, 1992; Kim *et al.* 1995; Terrenoire *et al.* 2001; Han *et al.* 2002). They become active when a free fatty acid or negative pressure is applied to the membrane patches, or when inside-out patches are formed (Kim *et al.* 1995; Han *et al.* 2003). Similarly, TREK-1 expressed in

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cardiac myocytes is not open at room temperature, but becomes active when negative pressure or a free fatty acid is applied to the membrane (Kim, 1992; Terrenoire *et al.* 2001). Therefore, TREK-1 and TREK-2 are unlikely to contribute much to the net K^+ conductance at room temperature under unstressed conditions. However, in other peripheral tissues, such as adrenocortical cells and gastrointestinal smooth muscle cells, TREK-1 current was recorded at room temperature (Koh *et al.* 2001; Enyeart *et al.* 2002). This suggests that the basal activity of TREK-1 may depend on the cell type, possibly due to differences in the cytosolic environment.

Although TREK-1 expressed in oocyte and COS-7 cell showed low activity at room temperature, the activity increased greatly as heat was applied, and a maximal activation was observed near 37°C (Maingret *et al.* 2000). This suggests that TREK-1 is active at body temperature (37°C), and contributes significantly to the background K^+ conductance in the native system. Whether TREK-2 and TRAAK are also temperature-sensitive is important to know but has not yet been tested. In this study, we examined and compared the temperature sensitivity of all three members of the TREK/TRAAK family in COS-7 cells, as well as in neurones and cardiac myocytes that express these channels. It is not known whether temperature affects the single-channel kinetics of TREK and TRAAK. Therefore, we also studied the effect of heat on open-time duration, opening frequency and single-channel conductance of TREK-1, TREK-2 and TRAAK to determine which of these account for the heat-induced changes in K^+ current. Finally, we studied the role of the C-terminus of TREK-2 in temperature sensitivity, as this cytoplasmic domain is critical for the sensitivity to other activators including free fatty acids, protons and volatile anaesthetics (Patel *et al.* 1998; 1999; Kim *et al.* 2001*b*).

Methods

Transfection in COS-7 cells

Rat TREK-2 (GenBank accession number AF196965; Bang *et al.* 2000) and rat TRAAK (GenBank accession number AF302842; Kim *et al.* 2001*a*) were cloned previously in this laboratory (Bang *et al.* 2000; Kim *et al.* 2001*a*). Rat TREK-1 was cloned from cerebellum cDNA library screened with TREK-2 DNA using methods previously described (Bang *et al.* 2000). The C-terminus of TREK-2 was replaced with that of TASK-3 to obtain TREK-2/TASK-3C mutant as previously described using PCR methods (Kim *et al.* 2001*b*). TREK-1, TREK-2, TRAAK and TREK-2/TASK-3C were subcloned into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). COS-7 cells were seeded at a density of 2×10^5 cells per 35 mm dish 24 h prior to transfection in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (DMEM). COS-7 cells were cotransfected with a K_{2P} channel DNA in pcDNA3.1 and pcDNA3.1/GFP using LipofectAMINE and OPTI-MEM I Reduced Serum Medium (Life Technologies). Green fluorescence from cells expressing green fluorescent protein (GFP) was detected with the aid of a Nikon microscope equipped with a mercury lamp light source. Cells were used 2–3 days after transfection.

Cerebellar granule neurone culture and isolation

All animals were used in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health, Education and Welfare publication no. NIH85-23). The cerebellum was isolated from rapidly decapitated postnatal day 6–8 (P6–P8) rat pups ($n = 8$), and washed with oxygenated physiological buffer solution (PBS) at 4°C. The cerebellar cortex was cut into sections of 500 μm or less and incubated for 15 min in a solution containing papain (20 U ml^{-1}; Worthington, Lakewood, NJ, USA), and DL-cysteine (1 mM). After digestion, the tissue was washed twice with PBS solution and resuspended in a solution containing DNase I (1000 U ml^{-1}; Worthington). After gentle trituration of the solution using a fire-polished Pasteur pipette, the suspended cells were gently passed through a 25-gauge needle. The suspension was layered on top of sterilized fetal bovine serum and centrifuged at 1000 *g* for 10 min. The pellet was resuspended in plating medium that contains NeuroBasal Media (Gibco), supplemented with B-27 (10 μl ml^{-1}; Life Technologies, Rockville, MD, USA), glutamic acid (2.5 mM), glutamine (20 mM), gentamicin (50 μg ml^{-1}) and fungizone (2.5 μg ml^{-1}). The cells were plated on glass coverslips coated with poly-L-lysine at a density of 1×10^5 cells per 35 mm dish. After a 24-h period for cell attachment, the medium was changed every 3 days with new plating medium containing B-27 (20 μl ml^{-1}), glutamine, gentamicin and fungizone in NeuroBasal Medium. Cells were kept for 6 days at 37°C in a humidified incubator gassed with a 95% air−5% CO_2 mixture.

Dorsal root ganglion neurone culture

Cultured dorsal root ganglion (DRG) neurones were prepared as previously described (Oh *et al.* 1996). Briefly, DRG neurones were dissected from both levels of thoracic and lumbar spinal cord of six 1- or 2-day-old neonatal rats. DRG neurones were collected in cold culture medium (4°C) containing DMEM–F-12 mixture (Sigma, St Louis, MO, USA), fetal bovine serum (10%; Life Technologies), 1 mM sodium pyruvate, 25 ng ml^{-1} nerve growth factor, and 100 U ml^{-1} of penicillin-streptomycin. Ganglia were washed three times with DMEM–F-12 medium and incubated for 30 min in the DMEM–F-12 medium containing 1 mg ml^{-1} collagenase (Type II; Worthington).

The ganglia were then washed three times with Mg^{2+}- and Ca^{2+}-free Hank's balanced salt solution (HBSS) and incubated with gentle shaking in the warm (37°C) HBSS containing 2.5 mg ml^{-1} trypsin (Life Technologies). The solution was centrifuged at 1000 *g* for 10 min, and the pellet was washed three times with the culture medium to inhibit the enzyme. The pellet was suspended in the culture medium and gently triturated with a fire-polished Pasteur pipette. The suspension was plated on poly-L-lysine-coated glass coverslips in culture dish. Cells were incubated at 37°C in a 95% air−5% CO_2 gas mixture. Cells were used 1–3 days after plating.

Isolated heart cell preparation

Single ventricular cell of adult rat heart was prepared by collagenase digestion. Five rats weighing between 200 and 300 g were anaesthetized with halothane. The heart was then isolated and retrogradely perfused via the aorta in a Langendorff apparatus with a bicarbonate-buffered, Ca^{2+}-containing solution for 5 min. The perfusate was then switched to a Ca^{2+}-free bicarbonate-buffered solution. Then, hearts were perfused the Ca^{2+}-free, bicarbonate-buffered solution containing 0.07% collagenase (Type II, Worthington) and 0.03% hyaluronidase for 30 min. Thereafter, collagenase was washed out from the heart with the bicarbonate-buffered solution. Ventricular tissues were then cut into small pieces and incubated on ice in a storage solution. Storage solution was bicarbonate-buffered solution containing 1% bovine serum albumin, and pH was adjusted to 7.3 by titrating with NaOH. Bicarbonate-buffered solution contained (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 1.8. Ventricular tissues were mechanically dissociated to obtain single cells in the recording chamber in the recording solution. All solutions were oxygenated with 95% O_2−5% CO_2. Healthy cells were rod-shaped without any sign of membrane blebs or contracture. All cells were used within 6 h.

Electrophysiological studies

Electrophysiological recording was performed using a patch-clamp amplifier (Axopatch 200, Axon Instruments, Union City, CA, USA). Single channel currents were digitized with a digital data recorder (VR10, Instrutech, Great Neck, NY, USA), and stored on videotape. The recorded signal was filtered at 2 kHz using 8-pole Bessel filter (−3 dB; Frequency Devices, Haverhill, MA, USA) and transferred to a computer (Dell) using the Digidata 1200 interface (Axon Instruments) at a sampling rate of 20 kHz. Threshold detection of channel openings was set at 50%. Whole-cell currents were recorded after cancelling the capacitive transients. Whole-cell and single channel currents were analysed with the pCLAMP program (Version 8). For single channel analysis, the filter dead time was 100 μs (0.3/cutoff frequency) such that events shorter than 50 μs in duration would be missed. Data were analysed to obtain a duration histogram, amplitude histogram and channel activity (NP_o, where N is the number of channels in the patch, and P_o is the probability of a channel being open). NP_o was determined from ~1–2 min of current recording. The single channel current tracings shown in the figures were filtered at 2 kHz. In experiments using cell-attached patch and excised patch, pipette and bath solutions contained (mM): KCl 150, $MgCl_2$ 1, EGTA 5 and Hepes 10. The pH was adjusted to desired values using HCl or KOH. In whole-cell recordings, bath solution contained (mM): NaCl 135, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, glucose 5 and Hepes 10. The pH was adjusted to 7.3 using HCl or NaOH. Heat was applied to the perfusion chamber using a temperature controller (Warner Instruments, Hamden, CT, USA), and the bath temperature was measured using a bead thermistor placed in the recording chamber. Threshold for channel activation was determined to be the temperature at which a significant increase in averaged channel activity occurred during a 10-s period compared to the previous 10-s period period. All other chemicals were purchased from Sigma Chemical Co (St Louis, MO, USA). For statistics, Student's t test was used with $P < 0.05$ as the criterion for significance. Data are represented as means ± S.D. unless specified.

Results

Temperature sensitivity of TREK-2 in COS-7 cells

To test whether TREK-2 is sensitive to heat, the response of TREK-2 expressed in COS-7 cells to changes in bath temperature ranging from 24°C to 42°C was studied. Cells were perfused gently with a physiological solution containing 5 mM KCl, and the whole-cell currents were recorded at the holding potential of 0 mV. A small but significant basal current was present at room temperature (24°C). A slow increase in bath temperature (~0.1°C s^{-1}) produced a gradual increase in current without much delay (Fig. 1*A*). Due to rupture of the gigaseal of the patch pipette to cell membrane, TREK-2 current could not be recorded accurately at temperatures above ~42°C. In COS-7 cells transfected with GFP alone, a rise in perfusion temperature did not produce a significant change in whole-cell current. In cells expressing TREK-2 and perfused with solution kept at 24°C, the whole-cell current generally remained unchanged or increased only slightly (Fig. 1*A*). A rapid rise or drop in bath temperature produced corresponding changes in whole-cell current, showing that TREK-2 responds quickly to temperature changes within the range that was tested (Fig. 1*B*).

In another set of experiments, cell membrane potential was held at −80 mV and then a voltage ramp (−120 to +60 mV; 1 s duration) was applied at desired bath temperatures. The current–voltage curves obtained at 24°C, 37°C and 42°C from a cell expressing TREK-2 are shown in Fig. 1*C*. The heat-induced increase in TREK-2 current was voltage-independent, as percentage increases in response to 37°C from 24°C were not significantly different at −60 mV (179 ± 14%), −20 mV (190 ± 10%) and +20 mV (197 ± 14%). Because the change in bath temperature was not perfectly linear, the data obtained in Fig. 1*A* were replotted in Fig. 1*D*. These results show that TREK-2 is sensitive to a normal range of environmental temperature, and that TREK-2 is likely to be active at rest at body temperature of 37°C. Within the 24–37°C range, a 14-fold change in activity per 10°C was present, indicating that TREK-2 is a highly temperature-sensitive K^+ channel.

The temperature sensitivity of TREK-2 was further studied in cell-attached patches at the single-channel level. Membrane potential of cell-attached patches was held at −40 mV, and cells were perfused with solution containing 150 mM KCl. As expected from whole-cell studies, an increase in temperature was closely associated with increased channel activity (Fig. 2*A*). The threshold for activation was 25°C, based on the criterion that averaged channel activity at this temperature was significantly greater than at 24°C. The change in TREK-2 activity was rapidly reversible when the temperature of the perfusion solution was changed quickly from 37°C to 28°C. In another set of experiments, the bath solution was warmed without perfusing the recording chamber. Cell-attached patches were formed on the same cell after setting the temperature to a desired level. Single channel openings observed from a typical cell at 24°C, 32°C, 37°C and 42°C are shown in Fig. 2*B*. At 24°C, the channel activity was very low despite the presence of many channels in the patch, as typical of TREK channels (Kim *et al.* 1995; Bang *et al.* 2000). The single-channel conductances at −40 mV were 113 ± 4 pS, 108 ± 6 pS and 110 ± 8 pS at 24°C, 32°C and 37°C, respectively, and thus were not significantly affected by temperature. Therefore, the increase in channel activity produced by heat was entirely due to the increase in the frequency of opening. Two other K_{2P} channels, TASK-3 and TRESK-2, showed very little change in channel activity in response to heat. Figure 2*C* plots the relationships between channel activity in cell-attached patches and the bath temperature, relative to that observed at 37°C. These results further confirm that TREK-2 is a temperature-sensitive channel, and is active at rest near the physiological temperature of 37°C.

Thermosensitivity of TREK-1 and TRAAK in COS-7 cells

As reported previously (Maingret *et al.* 2000), TREK-1 whole-cell current was also activated by heat (Fig. 3*A*). Under identical conditions, TRAAK current was also activated by increase in bath temperature from 24°C to 42°C. In cell-attached patches of COS-7 cells continuously perfused with heated solution, activity of both TREK-1 and TRAAK increased progressively as the temperature was raised from 24°C to 37°C in every patch tested (Fig. 3*B*). The threshold for activation of TREK-1 and

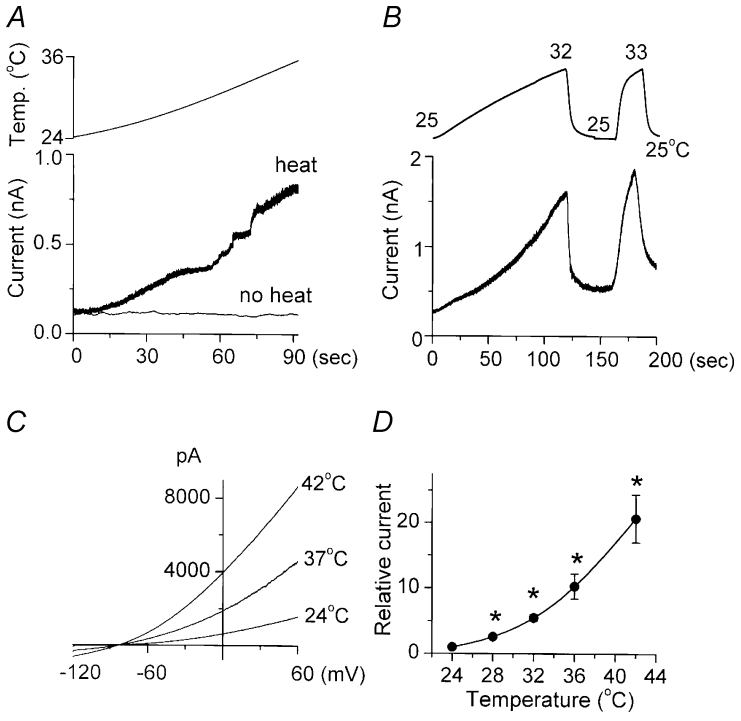

Figure 1. Heat activation of TREK-2 expressed in COS-7 cells
A, whole-cell currents were recorded from COS-7 cells transfected with DNA encoding TREK-2 and GFP. Cell membrane potential was held at 0 mV to record outward current. After 1–2 min of equilibration, the temperature of the perfusion solution was increased gradually from 24°C to 36°C, or set at 24°C (no heat). *B*, whole-cell currents were recorded in response to rapid cooling and heating within the 24°C to 37°C range. *C*, whole-cell currents were activated by a 1-s duration ramp pulse (–120 mV to +60 mV) at three temperatures as indicated. *D*, the graph shows the effect of temperature on the whole-cell current measured at 0 mV. Each point is the mean ± S.E.M. of 10 determinations. A smooth curve was fitted through the points. *Significant difference from the value observed at 24°C ($P < 0.05$). The value observed at 24°C was taken as 1.0.

TRAAK were 25 ± 1°C and 31 ± 2°C, respectively. In experiments in which the bath temperature was heated without superfusion of cells, TREK-1 and TRAAK activities in cell-attached patches was very low at 24°C, but increased as the bath temperature was raised (Fig. 3*C*). Typical single-channel openings observed at 24°C, 32°C, 37°C and 42°C from the same cell are shown in Fig. 3*C*. At −40 mV, the single-channel conductances were 108 ± 7 pS, 110 ± 10 pS and 110 ± 7 pS at 24°C, 32°C and 37°C, respectively, for TREK-1. It was difficult to determine accurately the single-channel conductance of TRAAK, due to variable amplitudes caused by extremely short openings. Nevertheless, it is clear from the tracings that heat augments both TREK-1 and TRAAK activities by increasing the opening frequency. The relationship between the bath temperature and single channel activity for TREK-1 and TREK-2 shows that the temperature sensitivity of TREK-1 and TREK-2 are similar in the temperature range tested (Fig. 3*D*). These results show that all three members of the TREK/TRAAK family are temperature-sensitive.

It has been reported that TREK-1 in the inside-out state is not sensitive to heat (Maingret *et al.* 2000). We tested whether this was also true for TREK-2 and TRAAK by forming inside-out patches and increasing the temperature of the perfusion solution from 24°C to 42°C. When the channel activity was stable in the inside-out patches, no significant changes in channel activity could be recorded with TREK-1, TREK-2 or TRAAK within this temperature range ($P > 0.05$; $n = 5$ each). Therefore, cell integrity is required for temperature sensitivity for all three K^+ channels.

Modulation of TREK-2 and TRAAK at 37°C

At room temperature, TREK-2 is activated by unsaturated free fatty acids, low intracellular pH and increased membrane tension (Patel *et al.* 2001; Kim, 2003). TRAAK is activated by high intracellular pH (Kim *et al.* 2001*a*). Experiments were conducted to test whether these properties of TREK-2 and TRAAK are preserved at 37°C. Bath temperature was maintained constant close to 37°C by perfusing with heated solution. Cell-attached patches were formed on COS-7 cells expressing TREK-1, TREK-2 and TRAAK. To lower intracellular pH, an acidic perfusion solution (pH 5.0) was applied to the bath. The pH of the pipette solution and therefore the extracellular solution bathing the outside of the patch membrane was 7.3. After approximately 1.5 min, TREK-1 and TREK-2 activity started to increase slowly and reached a peak at about 5 min. TRAAK was activated by application of an alkaline solution to the bath (pH 10.3). These effects of changes in pH of the bath solution were reversible. Application of negative pressure (−40 mmHg) to the cell-attached patches produced a rapid increase the activity of all three channels (Fig. 4*A*). Application of arachidonic acid (20 μM) to the bath solution initially showed no effect, but within ∼2 min, the activity of all three channels started to increase slowly, reaching a peak after ∼4 min ($n = 5$). Figure 4*A* shows the channel openings in cell-attached patches before and 4 min after activation of the channels by each of the activators. These results, summarized in Fig. 4*B*, show clearly that TREK-1, TREK-2 and TRAAK are sensitive to a change in pH_i, membrane stretch and free fatty acids in cell-attached conditions at 37°C.

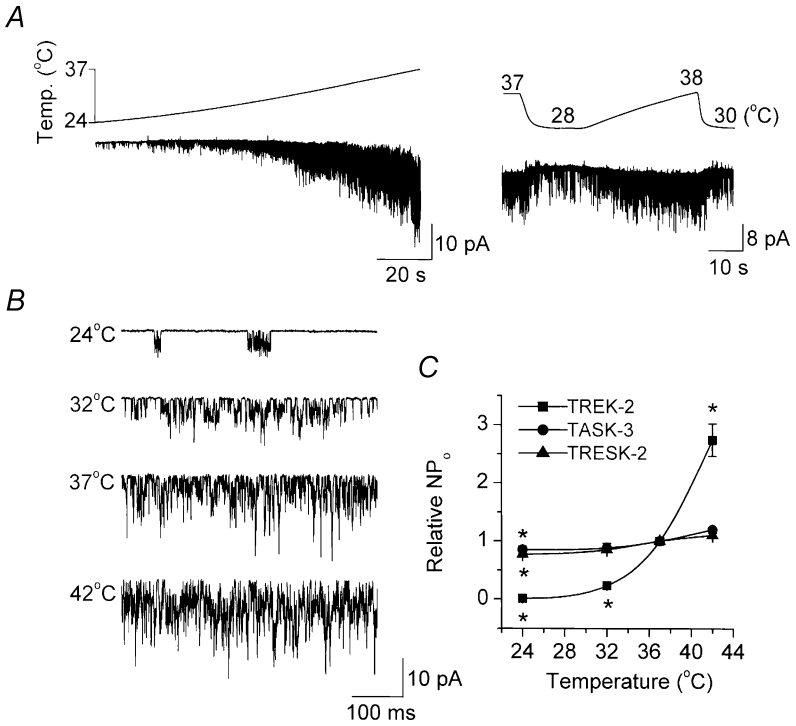

Figure 2. Activation of single channel currents of TREK-2 by heat
A, cell-attached patches were formed on COS-7 cells expressing TREK-2. Cell membrane potential was held at –30 mV. The temperature of the perfusion solution was increased gradually (left) or changed rapidly (right) as indicated by upper traces. Pipette and bath solutions contained 150 mM KCl. Single-channel current shown was filtered at 0.02 kHz. *B*, cell-attached patches were formed and single-channel currents recorded at –40 mV. The temperature of the bath solution was set at 24°C, 32°C, 37°C or 42°C. Current tracings typical of those from five experiments are shown. *C*, the graph shows the effect of temperature on the channel activity relative to that obtained at 37°C. Each point is mean ± S.E.M. of 10 determinations. Smooth curves were fitted through the points. *Significant difference from the corresponding control value obtained at 37°C ($P < 0.05$). Results of experiments using TASK-3 and TRESK-2 are also plotted.

The above results observed in cell-attached patches indicate that TREK-1, TREK-2 and TRAAK would also be activated by the same modulators in the inside-out conditions at 37°C. To confirm this, inside-out patches were formed from COS-7 cells expressing TREK-1, TREK-2 or TRAAK. Applying a gentle negative pressure (−20 mmHg) to the patch membrane caused significant increases in the activity of all three channels at 37°C (Fig. 5*A*). The effects of arachidonic acid (20 μM) and altered pH (pH 5.8 or 8.3) were studied by applying them to the cytoplasmic side of the membrane. Arachidonic acid caused a marked activation of TREK-1, TREK-2 and TRAAK at 37°C (Fig. 5*A* and *B*). Acid produced a strong activation of TREK-1 and TREK-2, and alkali (pH 8.3) activated TRAAK. The results shown in Figs 4 and 5 show that the sensitivity of all three K_{2P} channels to various modulators are preserved at 37°C whether the patch condition was cell-attached or inside-out.

Thermosensitivity of TREK-2 expressed in cerebellar granule neurones

In cerebral neurones, the expression of TREK-1 and TREK-2 are generally low, compared with the over-expression system of COS-7 cells. Therefore, it is important to directly test whether these K_{2P} channels expressed in native cells are indeed sensitive to temperature. We studied three cell types that express one or more of TREK/TRAAK channels: cerebellar granule neurones, dorsal root ganglion neurones and cardiac myocytes.

Cerebellar granule neurones express TREK-2 as well as TASK-1 and TASK-3 (Millar *et al.* 2000; Han *et al.* 2002; Lauritzen *et al.* 2003). At 24°C, TASK-1 and TASK-3 were active at rest but TREK-2 was almost always closed in cell-attached patches. However when the bath temperature was kept at 37°C and 42°C, TREK-2

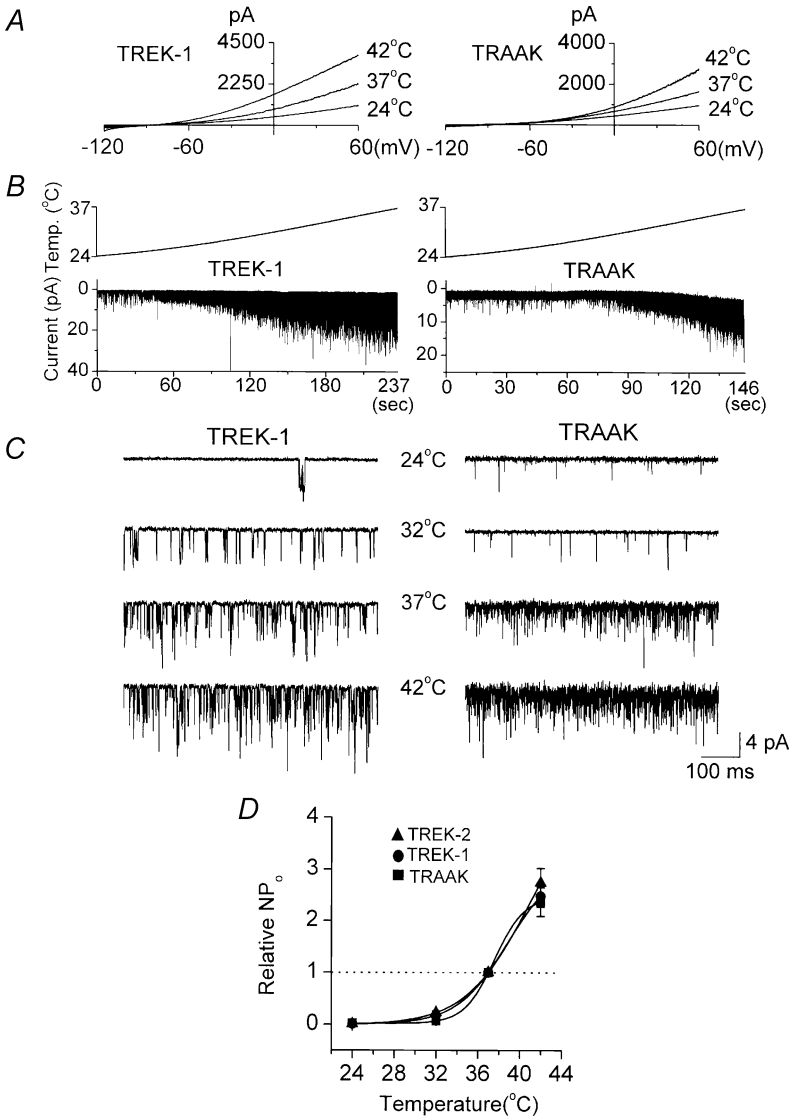

Figure 3. Effect of temperature on TREK-1 and TRAAK in COS-7 cells
A, whole-cell currents were recorded at three different temperatures. Currents were activated by ramp pulses (–120 mV to +60 mV) of 1-s duration. Bath solution contains 5 mM KCl. *B*, the temperature of the perfusion solution was increased gradually (left, TREK-1; right, TRAAK) as indicated in the top tracings. Pipette and bath solutions contained 150 mM KCl. Single-channel current shown was filtered at 0.2 kHz. *C*, channel openings in cell-attached patches from COS-7 cells incubated at different bath temperatures are shown for TREK-1 and TRAAK. Current tracings typical of those from five experiments are shown. *D*, the graph plots the relative channel activity of TREK-1, TREK-2 and TRAAK as a function of bath temperature. Data points were fitted to sigmoid curves.

became active at all membrane potentials tested (−80 to +80 mV) (Fig. 6*A* and *B*). At 37°C, the single-channel conductances of this K^+ channel at −40 mV and +40 mV were 112 ± 8 pS and 60 ± 8 pS, respectively, showing a mild inward rectification, similar to cloned TREK-2 expressed in COS-7 cells (Bang *et al.* 2000). To be sure that the K^+ channel was TREK-2, inside-out patches were formed to test for activation by arachidonic acid, low pH and negative pressure. As shown in Fig. 6*C* and summarized in Fig. 6*D*, all three manoeuvres produced strong activation of the K^+ channel. These results therefore show that TREK-2 expressed in cerebellar granule neurones are temperature-dependent and active near the physiological temperature of 37°C.

Thermosensitivity of TREK-1 expressed in cardiac myocytes

In isolated ventricular cells, cell-attached patches showed no opening of TREK-1 at 24°C, although its presence in the patch was confirmed by its activation by application of negative pressure or arachidonic acid in the inside-out state. At the bath temperature of 37°C, a K^+ channel with kinetic properties similar to TREK-1 was active in the cell-attached state (Fig. 7*A* and *B*). The identity of the cardiac K^+ channel was further tested using three well-known manoeuvres that are known to modulate TREK-1. At 37°C, the cardiac K^+ channel was further activated by arachidonic acid, low pH solution (pH 5.8) and negative pressure (−20 mmHg) applied to the membrane (Fig. 7*C* and *D*). At 37°C, the single channel conductance of the cardiac K^+ channel at −40 mV and +40 mV was 115 ± 8 pS and 104 ± 7 pS, respectively. These values are not significantly different from those of the cloned TREK-1 expressed in COS-7 cells (Kim, 2003). The noisy open state and relatively linear current–voltage relationship are also typical properties of TREK-1 (Maingret *et al.* 1999; Patel & Honore, 2002).

Thermosensitivity of TREK-1, TREK-2 and TRAAK expressed in dorsal root ganglion neurones

In DRG neurones, K^+ channels with single channel properties similar to those of TREK-1, TREK-2 and

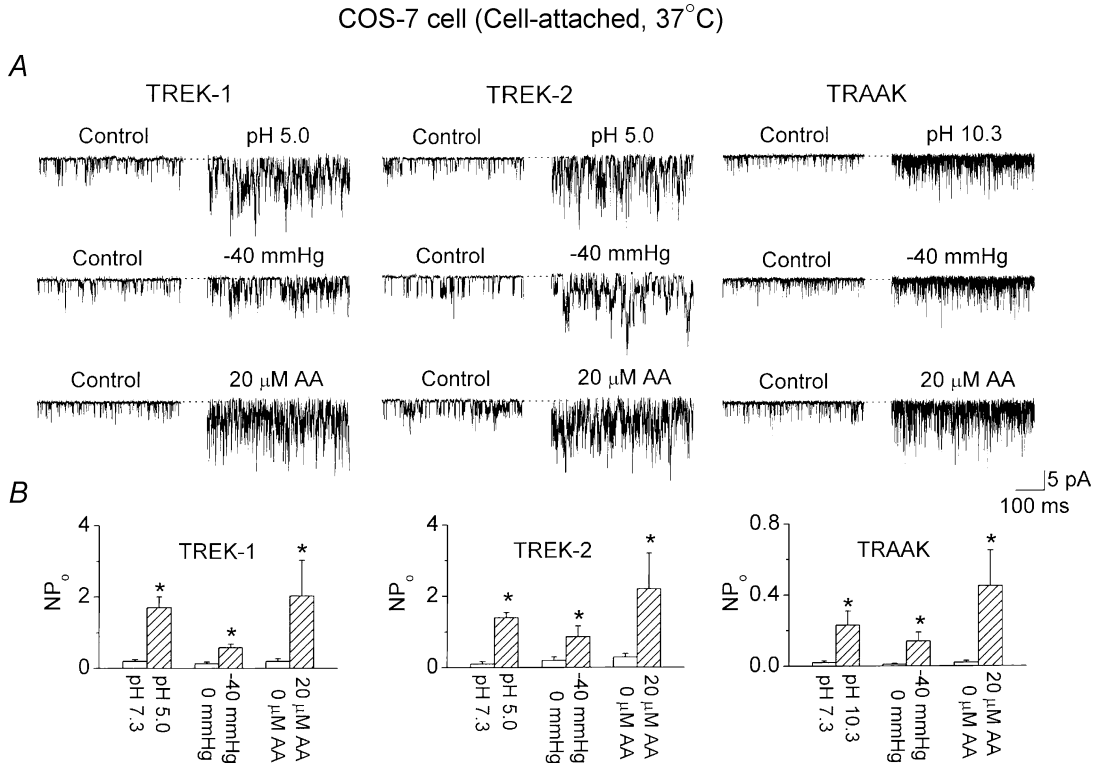

Figure 4. Activation of TREK-1, TREK-2 and TRAAK by pH, pressure and arachidonic acid at 37°C in cell-attached patches

A, cell-attached patches were formed, and negative pressure (–40 mmHg), arachidonic acid (AA; 20 μM), or desired pH solution (pH_o, 5.0 for TREK-1 and TREK-2; pH_o, 10.3 for TRAAK) was applied to the bath. Activation by the three stimuli was reversible in every patch. The holding potential was −40 mV. *B*, bar graphs show channel activities before and after application of each activator on the TREK-1, TREK-2 and TRAAK expressed in COS-7 cell. Each bar represents the mean ± S.D. of five determinations. *Significant difference from the respective control value in the open bar ($P < 0.05$).

TRAAK were recorded in cell-attached and inside-out patches. The single-channel conductances of the three K^+ channels in DRG neurones were 104 ± 8 pS, 110 ± 7 pS, and 95 ± 18 pS at -40 mV similar to those of cloned TREK-1 (108 ± 7 pS), TREK-2 (113 ± 4 pS) and TRAAK (92 ± 16 pS), respectively, expressed in COS-7 cells. TREK-2 could be clearly distinguished from TREK-1, because the conductance of TREK-2 at depolarized potentials (52 pS, +60 mV) is about half of that of TREK-1 (108 pS), and the open state of TREK-2 is much noisier than that of TREK-1 (Bang *et al.* 2000; Kim, 2003). The identity of these K^+ channels was further confirmed by their sensitivity to pH, membrane stretch and arachidonic acid (see Fig. 8*D*). The 92-pS channel in DRG neurones was determined to be TRAAK as it was activated by alkaline solution (pH 8.8), as well as arachidonic acid and negative pressure. Of the three channels, TREK-2 was recorded most frequently (40 of 143 patches; 28%). TREK-1 and TRAAK were observed in 13% and 8% of patches, respectively (Fig. 8*C*). The density of TREK-2 per patch was also higher than that of either TREK-1 or TRAAK, as multiple openings of TREK-2 were present in ~50% of patches after its activation by arachidonic acid or protons. For TREK-1 or TRAAK, only one level of opening was present in all patches studied.

At room temperature (24°C), only a very low level of activity (open probability less than 0.0002) was recorded for each of the three K^+ channels in cell-attached patches (Fig. 8*A*). When no TREK-1, TREK-2 or TRAAK channel openings were present in cell-attached patches, inside-out patches were formed and arachidonic acid applied to verify whether TREK-1, TREK-2 or TRAAK were present in the patch. When DRG neurones were maintained at 37°C and cell-attached patches were formed, TREK-1, TREK-2 or TRAAK were active at rest in all patches that contained TREK-1, TREK-2 or TRAAK (Fig. 8*A*). These results obtained in the cell-attached state at 24°C and 37°C are summarized in Fig. 8*B*. The high activity of TREK-2 at 37°C compared with TREK-1 and TRAAK is probably due to the relatively high density of TREK-2 in DRG neurones. In Fig. 8*D*, activation of TREK-2 in inside-out patches by arachidonic acid (20 μM), negative pressure (−20 mmHg) and acid solution (pH 5.8) are shown. In inside-out patches incubated at 37°C, TREK-1 and TREK-2 were easily activated by low pH, arachidonic acid and negative pressure, whereas TRAAK was activated by alkaline solution. In cell-attached patches containing TREK-2 at 37°C, application of arachidonic acid (20 μM) to the bath solution and negative pressure (−40 mmHg) also caused 11.3 ± 3.5-fold and 3.5 ± 1.5-fold ($n = 5$) increases in channel activity, similar to that observed in COS-7 cells. These findings in native excitable cells show that TREK-1, TREK-2 and TRAAK are active at 37°C, and would be expected to contribute significantly to the net background K^+ conductance.

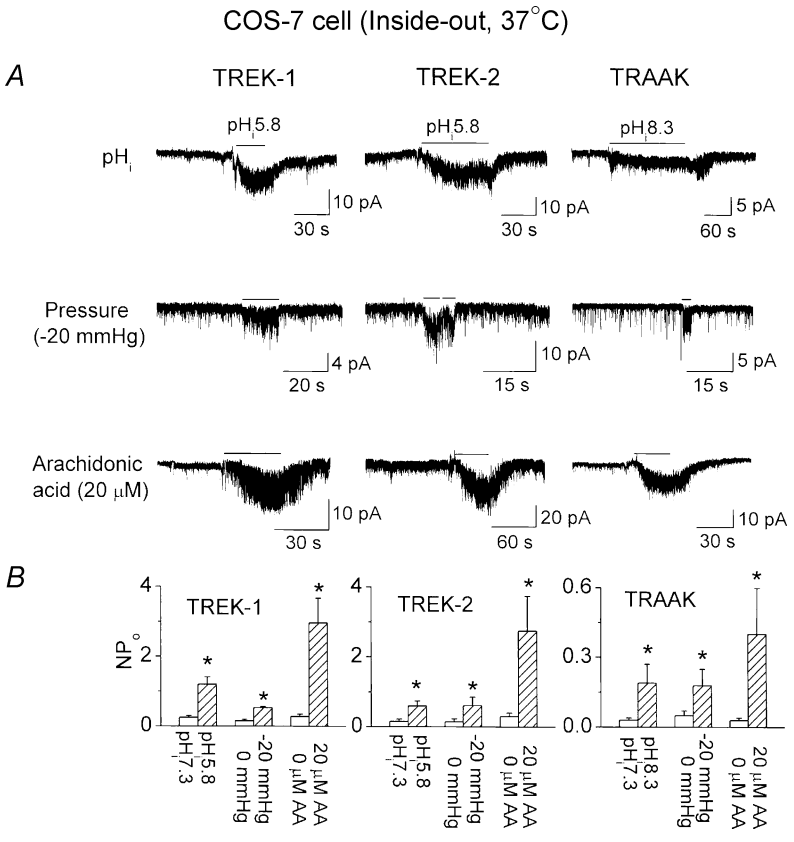

Figure 5. Activation of TREK-1, TREK-2 and TRAAK by pH, pressure and arachidonic acid at 37°C in inside-out patches
A, inside-out patches were formed, and negative pressure (−20 mmHg), arachidonic acid (AA; 20 μM), or pH solution (pH$_i$, 5.8 for TREK-1 and TREK-2; pH$_i$, 8.3 for TRAAK) was applied to the patch membrane. Activation by the three stimuli was reversible in every patch. The holding potential was −40 mV. *B*, bar graphs show channel activities before and after application of each activator on the TREK-1, TREK-2 and TRAAK expressed in COS-7 cell. Each bar represents the mean ± S.D. of five determinations. *Significant difference from the respective control value in the open bar ($P < 0.05$).

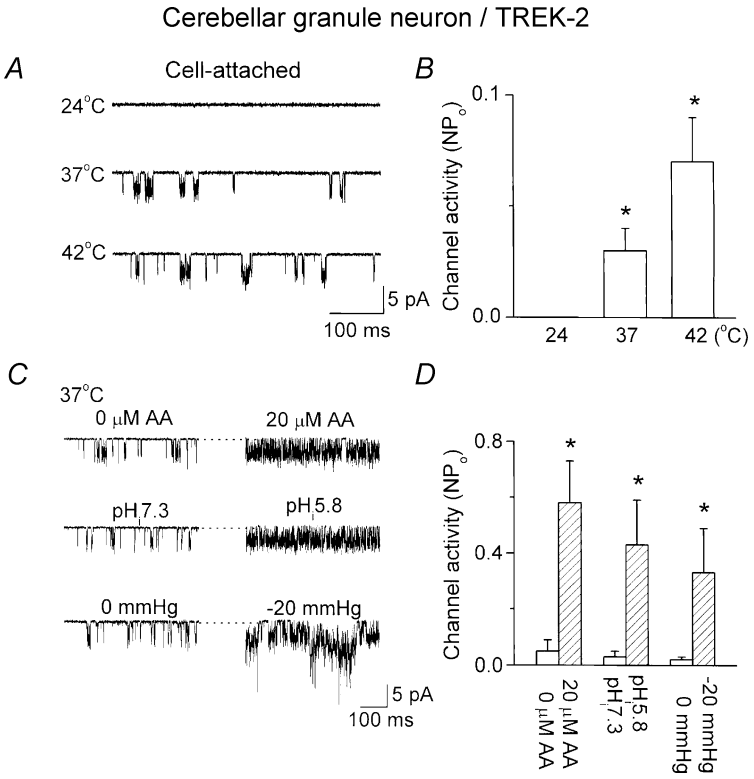

Figure 6. Temperature sensitivity of TREK-2 in cerebellar granule neurones
A, cell-attached patches were formed on cultured cerebellar granule neurones kept at 24°C and 37°C. The cell membrane potential was held at −40 mV. Pipette and bath solutions contained 150 mM KCl. *B*, bar graphs show channel activity determined at −40 mV. *Significant difference from the value obtained at 24°C in cell-attached patches ($P < 0.05$). Each bar is the mean ± S.D. of 10 determinations. *C*, at 37°C, inside-out patches were formed, and arachidonic acid (AA; 20 μM), intracellular acidic condition (pH_i 5.8), and negative pressure (−20 mmHg) were applied to the patch membrane. The holding potential was −40 mV. *D*, the bar graph shows channel activity before and after application of each activator. *Significant difference from the corresponding control value ($P < 0.05$).

Carboxy terminus of TREK-2 is not critical for thermosensitivity

The carboxy terminus of TREK-2 is critical for transducing the activation by arachidonic acid and protons at 24°C (Kim *et al.* 2001*b*). To test whether the carboxy terminus is involved in the temperature sensitivity of TREK-2, a chimera was constructed in which the C-terminus of TREK-2 was replaced with that of TASK-3, which is temperature-insensitive, and expressed in COS-7 cells. To be sure that this mutant (TREK-2/TASK-3C) has lost its sensitivity to lipids and protons at 37°C, inside-out patches were formed and low pH solution (pH 5.8) and arachidonic acid were applied to the bath solution. As shown in Fig. 9*A*, TREK-2/TASK-3C was insensitive to low pH solution and arachidonic acid at 37°C. However,

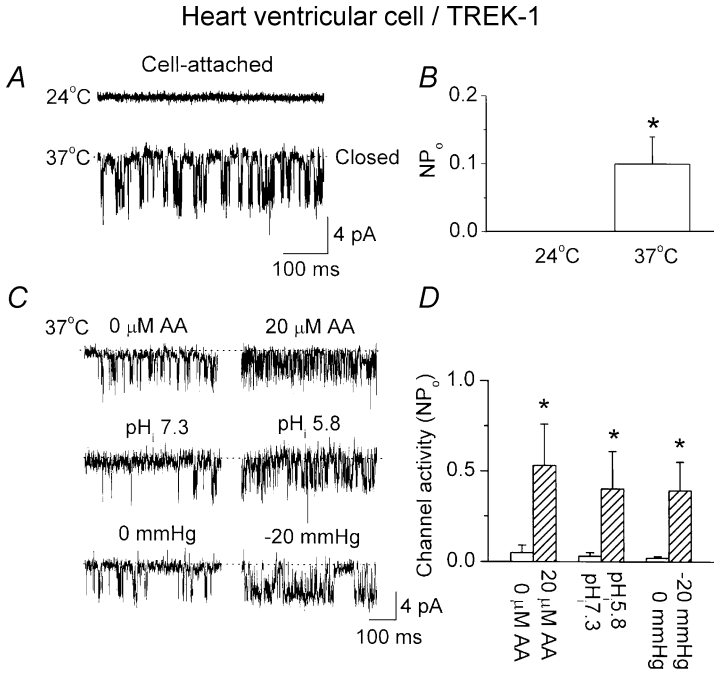

Figure 7. Temperature sensitivity of TREK-1 in ventricular cells
A, cell-attached patches were formed on isolated ventricular myocytes kept at 24°C and 37°C. The cell membrane potential was held at −40 mV. Pipette and bath solutions contained 150 mM KCl. *B*, the bar graph shows channel activity determined at −40 mV. *Significant difference from the value at obtained at 24°C ($P < 0.05$). Each bar is the mean ± S.D. of five determinations. *C*, at 37°C, inside-out patches were formed and arachidonic acid (AA; 20 μM), intracellular acidic condition (pH_i 5.8), and negative pressure (−20 mmHg) were applied to the patch membrane. The holding potential was −40 mV. *D*, the bar graph shows channel activity before and after application of arachidonic acid (AA; 20 μM), low pH (pH_i 5.8), and negative pressure (−20 mmHg). *Significant difference from the corresponding control value ($P < 0.05$).

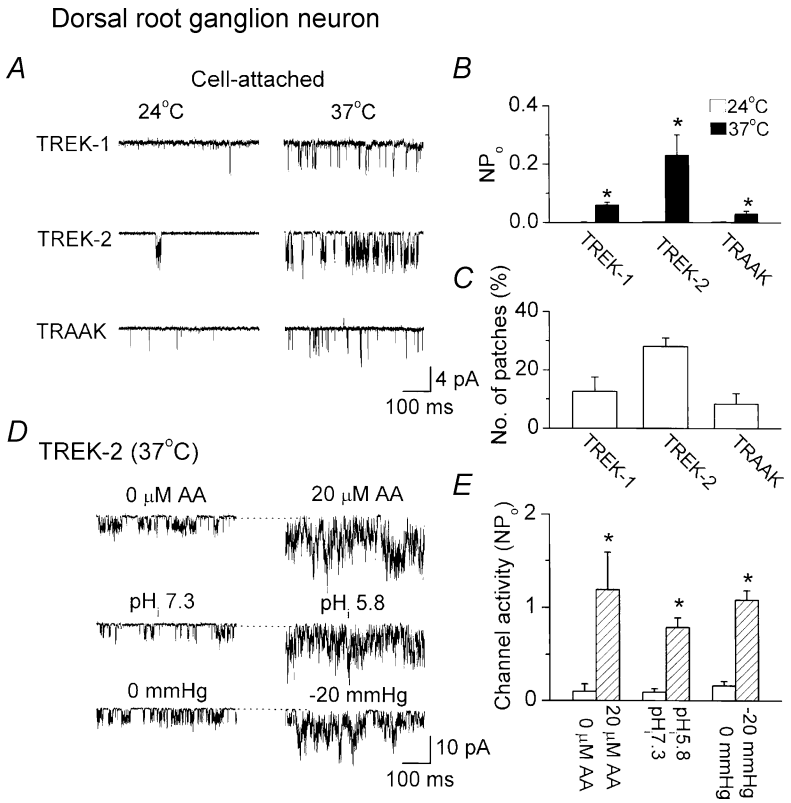

Figure 8. TREK-1, TREK-2 and TRAAK in DRG neurones
A, cell-attached patches were formed on DRG neurones maintained at either 24°C or 37°C. Single-channel openings of TREK-1, TREK-2 and TRAAK at 37°C are shown. *B*, a graph shows the channel activities at 24°C or 37°C for the three channels. Each bar represents the mean ± S.D. of five determinations. *Significant difference ($P < 0.05$). *C*, the percentage of patches containing TREK-1, TREK-2 and TRAAK in cell-attached patches of DRG neurones are plotted. *D*, inside-out patches containing TREK-2 were identified and maintained at 37°C. Single-channel openings before and after application of arachidonic acid, negative pressure or low pH solution are shown. *E*, a summary graph shows the degree of activation of TREK-2 at 37°C by arachidonic acid, negative pressure or low pH solution in the inside-out patches. Each bar represents the mean ± S.D. of five determinations. *Significant difference ($P < 0.05$).

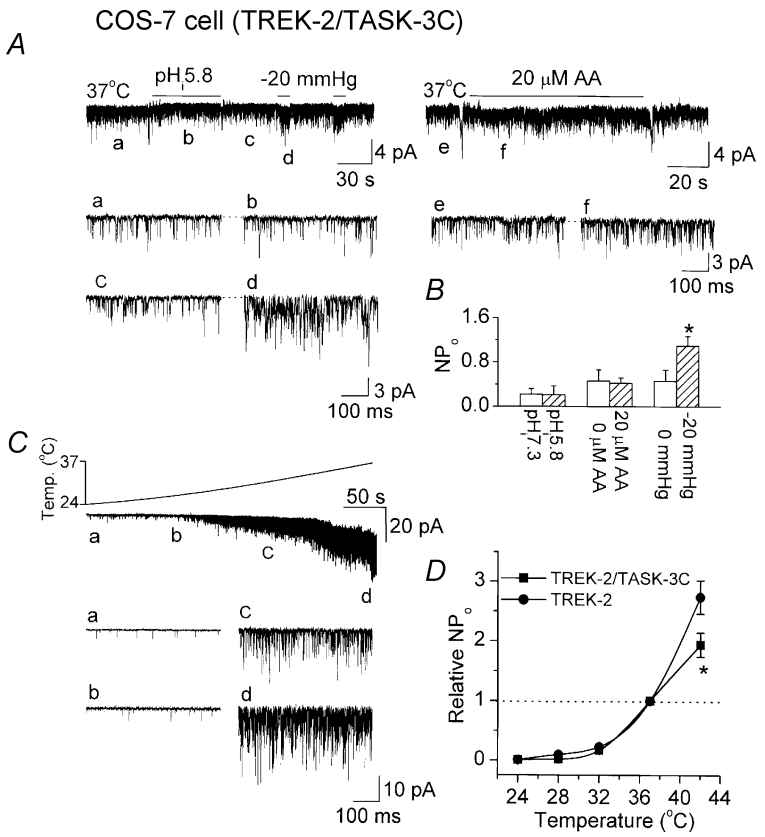

Figure 9. Effect of acid, membrane stretch and arachidonic acid on TREK-2/TASK-3C mutant
A, bath solution was kept at 37°C. Inside-out patches were formed, and acid solution (pH 5.8) and arachidonic acid (AA) was applied to the cytoplasmic side of the membrane. Negative pressure was applied via the pipette. Channel openings at expanded time scale are also shown, as indicated. Cell membrane potential was held at −30 mV. Pipette and bath solutions contained 150 mM KCl. *B*, the bar graph shows channel activity before and after application of each activator. Each bar is the mean ± S.D. of six determinations. *Significant difference from the corresponding control value ($P < 0.05$). Note that the mutant is insensitive to acid and AA. *C*, cell-attached patches were formed and the temperature of the perfusion solution increased slowly from 24°C to 37°C. The cell membrane potential was held at −40 mV. Pipette and bath solutions contained 150 mM KCl. Channel openings at expanded time scale are also shown. The threshold temperature was 28 ± 3°C. *D*, the graph shows the effect of temperature on channel activity relative to that obtained at 37°C. The relative channel activity of TREK-2 is also shown for comparison. *Significant difference at 42°C between TREK-2 and mutant ($P < 0.05$). Each bar is the mean ± S.E.M. of five determinations.

TREK-2/TASK-3C was still mechanosensitive at 37°C. These results are summarized in Fig. 9*B*.

To test for temperature-sensitivity of TREK-2/TASK-3C, temperature of the perfusion solution was increased gradually and channel activity recorded in the cell-attached state (Fig. 9*C*). It is interesting that no increase in channel activity was observed until about 28°C. Then, a strong temperature-dependent activation was observed that was similar to that observed with TREK-1 and TREK-2, and this effect was also reversible. Compared to channel activity at 24°C, the activity at 37°C was 74-fold greater ($n = 5$). When TREK-2/TASK-3C response to heat was normalized to the value observed at 37°C and compared with that of TREK-2, the sensitivity of the mutant was lower than the wild-type at warm temperatures (37°C– 42°C).

Heat shortens the open-time duration

In the course of the above studies, it was observed that in addition to increasing the frequency of opening, heat also shortened the open-time duration of both TREK-1 and TREK-2 in many cell-attached patches. This is illustrated more clearly in Fig. 10*A* and *B* where open-time histograms were obtained from channel openings at three different temperatures in COS-7 cells. A single exponential function was sufficient to fit the open-time histograms, indicating the presence of one major open state for both TREK-1 and TREK-2. In a majority of patches (85%), the long open bursts (mean burst duration greater than 50 ms) that are usually observed at room temperature were observed infrequently at higher temperatures (37°C and 42°C). In the remaining 15% of patches, however, the mean open time did not change significantly after heating to 37°C and 42°C, and typical open bursts were still present. In cerebellar granule neurones, heat also shortened the mean open time of TREK-2, but the effect was not as great as that observed in COS-7 cells. In cerebellar granule neurones, the mean open times were 1.1 ± 0.1 ms, 0.8 ± 0.1 ms and 0.7 ± 0.1 ms at 24°C, 37°C and 42°C, respectively.

Discussion

This study shows that TREK-2 and TRAAK are thermosensitive and that they are active near the physiological temperature of 37°C. The range of their temperature sensitivity is rather wide, starting near room temperature (∼24C°) to > 42°C. Such temperature sensitivity of TREK-2 and TRAAK is similar to that of TREK-1, which has been studied previously in oocytes and COS cells (Maingret *et al.* 2000). Therefore, all three K_{2P} channels should contribute to the leak or background K^+ conductance that helps to stabilize the resting membrane

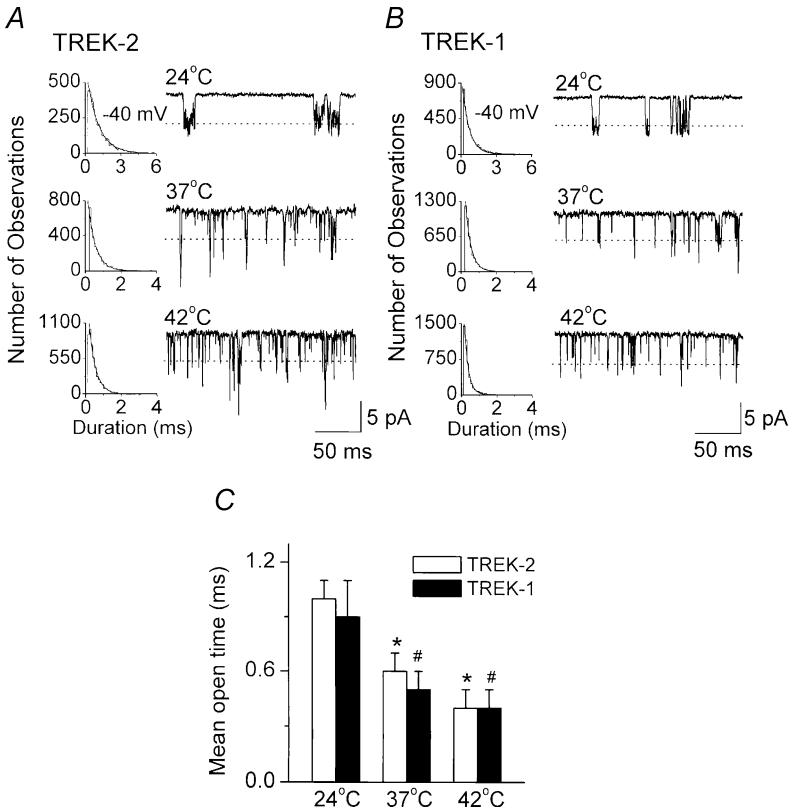

Figure 10. Heat shortens the open -ime duration of TREK-1 and TREK-2 in COS-7 cells
A, channel openings at 24°C, 37°C and 42°C were recorded from COS-7 cells expressing TREK-2. Open-time histograms were obtained and fitted with a single exponential function, as shown on the left of each trace. *B*, same as in *A* except that COS-7 cells expressing TREK-1 were used. *C*, the bar graph shows averaged mean open times obtained at three different temperatures. *Significant difference from the corresponding value obtained at 24°C ($P < 0.05$).

potential and suppress excitability in those cells that express them. As receptor agonists cause inhibition of TREK-1 and TREK-2 via G proteins (Lesage *et al.* 2000; Chemin *et al.* 2003; Enyeart *et al.* 2004), these K_{2P} channels would also be involved in the regulation of agonist-mediated synaptic transmission at physiological temperatures in the absence of any other activators.

Thermosensitivity of TREK and TRAAK

Most electrophysiological experiments are routinely performed at room temperature (23–25°C). Therefore, the properties of many ion channels including those of K_{2P} channels at more physiological temperatures (24–37°C) are not well known. Many K_{2P} channels expressed in oocytes and mammalian cells form functional K^+ channels, as judged by their whole-cell or single-channel current measurements performed at ∼24°C. For example, TASK-1, TASK-2, TASK-3, TALK-1, TALK-2, TRESK-1 and TRESK-2 are among those K_{2P} channels that show spontaneous openings at physiological membrane potentials at room temperature (Patel & Honore, 2001; Kim, 2003; Kang *et al.* 2004; Kang & Kim, 2004). Therefore, it seems reasonable to expect that these K^+ channels would still be active at rest at 37°C. Indeed, our studies show that TASK channels expressed in cerebellar granule neurones and COS-7 cells and TRESK channels expressed in COS-7 cells are still active at 37°C and are minimally affected by changes in temperature (24–37°C).

Earlier studies in neurones and cardiac myocytes have shown that TREK-1 and TREK-2 are generally inactive at room temperature (Kim, 1992; Kim *et al.* 1995; Terrenoire *et al.* 2001). Even in COS-7 cells over-expressed with TREK-1 or TREK-2, cell-attached patches show very little activity, i.e. one or two openings every few seconds such that the open probability is less than 0.01. This is despite the fact that sometimes more than 10 channels are present in the patch, as judged by their subsequent activation by free fatty acids and membrane stretch. Thus, these K_{2P} channels require a gating molecule (e.g. protons, fatty acids, halothane) to activate them, similar to the opening of G protein-gated K^+ channels by $G\beta\gamma$ or phosphatidylinositol 4,5-bisphosphate (PIP_2). They could normally be kept inactive by an inhibitor residing in the membrane. As forming inside-out patches tends to increase the activity of both TREK-1 and TREK-2, certain factors in the cell are apparently keeping the channels closed. It has been suggested that cytoskeletal proteins may play that role, as agents that disrupt these proteins tended to augment channel activity (Patel *et al.* 1998, 2001). Temperature could be one of the factors that affect lipid–cytoskeletal interaction, leading to increased channel activity.

Because TREK-1 and TREK-2 are not active at rest in cardiac and neuronal cells at ∼24°C, we initially assumed that these K_{2P} channels would not contribute to the background K^+ conductance. However, the study by Maingret *et al.* (2000) showing that TREK-1 was activated by heat prompted us to re-assess the role of TREK-1, TREK-2 and TRAAK. Our study shows clearly that all three members of the TREK/TRAAK family are temperature-sensitive such that they are active near warm temperatures. What causes heat to open these channels? Because the channel activity closely follows the rapid changes in temperature, the heat-sensitive factor is unlikely to be newly synthesized heat-inducible proteins. As the temperature sensitivity can be observed in whole-cells dialysed with pipette solution, small soluble molecules and proteins are unlikely to be the intracellular factor that confers temperature sensitivity to TREK and TRAAK. For TREK-1, TREK-2 and TRAAK, the temperature sensitivity was observed in the absence of any external Ca^{2+}, indicating that Ca^{2+}, a small molecule that would also be easily dialysed, is probably not the mediator. It was also determined that the temperature-sensitivity of TRPV1 (VR1), a capsaicin-activated non-selective ion channel, does not to require Ca^{2+} (Cesare *et al.* 1999; Numazaki *et al.* 2003; Voets *et al.* 2004). As suggested previously, the temperature sensor could be a molecule that is closely associated with TREK-1, TREK-2 and TRAAK (Maingret *et al.* 2000). However such a molecule has not yet been identified.

A recent study indicates that the temperature sensitivity of various TRP ion channels is due to the shift in the voltage-dependent activation (Voets *et al.* 2004). For example, the heat-activated TRPV1 is highly active at 24°C at +100 mV, whereas it only starts to become active at ∼35°C at −100 mV. Similarly, voltage-dependent shift in the activation curve is present for TRPM8 that is activated by cold. However for TREK-1 and TREK-2, the temperature sensitivity was rather insensitive to voltage, indicating that the mechanism of temperature sensitivity of TREK-1 and TREK-2 differs from that of TRP ion channels.

It has been reported that TREK-1 mutant in which most of the carboxy terminus was deleted except the proximal 10 amino acids was weakly sensitive to heat (Maingret *et al.* 2000). A chimera in which the TREK-1 carboxy terminus was replaced with that of TASK-1 was also found to be rather insensitive to heat (Maingret *et al.* 2000). This suggests that the carboxy terminus of TREK-1 plays an important role in providing the temperature sensitivity. However, for TREK-2, our results show that, although the threshold for activation was shifted to a higher temperature by a few degrees, the TREK-2/TASK-3C chimera could still be activated by heat, although to a lesser extent than that observed with the wild-type TREK-2. If a molecule associated with TREK-2 confers temperature sensitivity, the results suggest that it can still associate with the mutant channel.

The reduction of open-time duration in addition to the increase in opening frequency of TREK channels by heat is an interesting phenomenon that has not been reported previously. This effect of temperature on the open-time duration of TREK-1 and TREK-2 may simply be due to a general increase in thermal energy provided to the channel, as open-time duration of other channels such as TASK-1 and TASK-3 were also shortened by heat. The reason for the greater effect of heat on the open-time duration of TREK-2 in COS-7 cells than in cerebellar granule cells is not clear, but may be due to difference in membrane lipid composition. Whether temperature also affects open-time duration of TRP ion channels is not yet known.

Potential role in sensory transduction

The sensitivity of TREK-1 and TREK-2 to temperature suggests that they may be involved in temperature regulation and nociception, as they are expressed in the hypothalamus and DRG neurones (Maingret *et al.* 2000; Fioretti *et al.* 2004). Other nociceptive sensory neurones such as trigeminal neurones also express the TREK channels (Matsumoto *et al.* 2001). TREK-1 mRNA is highly expressed in cell bodies of DRG neurones, but it is not known whether TREK-1 is expressed in nerve terminals of DRG neurones and keratinocytes in the skin that actually sense chemical, mechanical and thermal stimuli. Functional expression of TREK-1, TREK-2 and TRAAK in DRG neurones and their temperature sensitivity clearly suggest that they would regulate excitability of these neurones and thereby help modulate sensory information.

It is well known that DRG neurones express various TRP ion channels that sense hot, warm and cold temperatures (Oh *et al.* 1996; Caterina *et al.* 1997; McKemy *et al.* 2002; Clapham, 2003; Tsuzuki *et al.* 2004). For example, TRPM8 senses cold (threshold, $<25^{\circ}$C), TRPV3 and TRPV4 sense warm ($>30^{\circ}$C), and TRPV1 senses hot ($>43^{\circ}$C) temperatures and is activated by capsaicin and acid (McKemy *et al.* 2002; Smith *et al.* 2002). Because TREK-1 and TREK-2 are affected by temperature in the physiological range (25°C–42°C), they are likely to sense both warm and cool temperatures. With an increase in temperature, TREK-1 and TREK-2 would tend to suppress excitation produced by TRP channels. Perhaps TREK-1 and TREK-2 act as a brake to prevent over-stimulation of TRP ion channels during heat exposure. Cool temperatures would reduce TREK-1, TREK-2 and TRAAK current and increase excitability. Indeed, cold transduction in DRG neurones has been reported to occur in part via inhibition of a background K^+ conductance with properties of TREK (Reid & Flonta, 2001; Viana *et al.* 2002). Therefore, both TRP and TREK ion channels may work in concert to transduce the thermal signal into cell excitability.

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