An intracellular proton sensor commands lipid- and mechano-gating of the K⁺ channel TREK-1

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The 2P domain K⁺ channel TREK-1 is widely expressed in the nervous system. It is opened by a variety of physical and chemical stimuli including membrane stretch, intracellular acidosis and polyunsaturated fatty acids. This activation can be reversed by PKA-mediated phosphorylation. The C-terminal domain of TREK-1 is critical for its polymodal function. We demonstrate that the conversion of a specific glutamate residue (E306) to an alanine in this region locks TREK-1 in the open configuration and abolishes the cAMP/PKA down-modulation. The E306A substitution mimics intracellular acidosis and rescues both lipid- and mechano-sensitivity of a loss-of-function truncated TREK-1 mutant. We conclude that protonation of E306 tunes the TREK-1 mechanical setpoint and thus sets lipid sensitivity.

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Introduction

TREK-1 is a member of the 2P domain family of K⁺ channels (Fink *et al.*, 1996; Patel *et al.*, 1999, 2001; Lesage and Lazdunski, 2000; Patel and Honoré, 2001a,b). The biophysical and pharmacological properties of TREK-1 are similar to those described for the serotonin-sensitive K⁺ channel (S channel), which underlies presynaptic sensitization, a simple form of learning, in *Aplysia* (Belardetti and Siegelbaum, 1988; Hawkins *et al.*, 1993).

A variety of chemical factors stimulate TREK-1 activity. These include diverse cellular lipids such as long chain polyunsaturated fatty acids and lysophospholipids (Patel *et al.*, 1998; Maingret *et al.*, 2000b). In particular, TREK-1 is opened by arachidonic acid (AA) (Fink *et al.*, 1996; Patel *et al.*, 1998; Maingret *et al.*, 2000b). Since this activation occurs in excised patches, the effect of AA is believed to be either direct or via an interaction with the membrane bilayer (Patel *et al.*, 1998, 2001; Maingret *et al.*, 2000b). Inhalational anaesthetics including halothane and isoflurane are able to open TREK-1 (Patel *et al.*, 1999). The consequent neuronal hyperpolarization has been proposed to play an important functional role during general anaesthesia (Patel *et al.*, 1999; Patel and Honoré, 2001a).

Physical stimuli such as membrane stretch and cell volume expansion are also able to activate TREK-1 (Patel *et al.*, 1998; Maingret *et al.*, 1999b). TREK-1 mechano-gating occurs in the absence of a functional cytoskeleton (Patel *et al.*, 1998). It was recently proposed that modulation of plasma membrane thickness and/or curvature may directly influence TREK-1 gating (Patel *et al.*, 2001). Preferential insertion of anionic amphipaths, such as AA, in the external leaflet of the lipid bilayer has been proposed to mediate TREK-1 opening because of a membrane crenation effect (Patel *et al.*, 1998). The mechano-sensitive properties of TREK-1 are thus central to the activation by cellular second messengers of the phospholipase A₂ pathway (Patel *et al.*, 1998). At acidic internal pH (pH_i), TREK-1 opens in the absence of mechanical stimulation (Maingret *et al.*, 1999b). Intracellular acidosis gradually shifts the pressure–activation relationship of TREK-1 toward positive values, and ultimately leads to channel activation at atmospheric pressure (Maingret *et al.*, 1999b). Finally, TREK-1 is a heat-activated K⁺ channel with an exceptional Q10 value of 7, and is present in both dorsal root ganglia and hypothalamic neurons, where it is thought to play a role in cold sensing (Maingret *et al.*, 2000a).

At the pathophysiological level, TREK channels might play a significant role during brain ischaemia (Lauritzen *et al.*, 2000). AA release, cell swelling and intracellular acidosis, which occur during brain ischaemia, could contribute to open TREK-1. The consequent hyperpolarization may have a neuroprotective effect by decreasing glutamate release at the pre-synaptic level and by favouring the blockade of the NMDA receptor-associated channel by external Mg²⁺ at the post-synaptic level (Lauritzen *et al.*, 2000). Interestingly, TREK-1 is opened by the neuroprotective agent riluzole (Duprat *et al.*, 2000).

The cytosolic C-terminal domain of TREK-1 is critical for channel activation by physical stimulation including stretch, acidosis and heat (Patel *et al.*, 1998; Maingret *et al.*, 1999b, 2000a,b). For instance, progressive deletion of this region gradually shifts the pressure–activation relationship towards more negative values, and makes mutant channels resistant to stretch (Maingret *et al.*, 1999b). Chemical activation of TREK-1 is also largely dependent on the C-terminal region (Patel *et al.*, 1998, 1999; Maingret *et al.*, 2000b). Truncated mutants are resistant to AA and inhalational anaesthetics (Patel *et al.*, 1998, 1999; Maingret *et al.*, 2000b). Moreover, exchanging the C-terminus of TREK-1 with that of TASK-1, another 2P domain background K⁺ channel, transforms TREK-1 into a constitutively active K⁺ channel insensitive to AA and volatile anaesthetics (Patel *et al.*, 1998, 1999). The activity of the functional homologue TREK-2 is similarly dependent on the C-terminal region (Lesage *et al.*, 2000; Kim *et al.*, 2001b). Deletion of the

C-terminus, or its substitution with that of TASK-3, abolished the sensitivity to free fatty acids and to intracellular acidosis, reduced the sensitivity to pressure and is also critical for basal activity and opening in bursts (Kim *et al.*, 2001b). However, in TRAAK, the third AA- and stretch-sensitive 2P domain K⁺ channel, the C-terminal region is apparently not critical for channel function (Fink *et al.*, 1998; Maingret *et al.*, 1999a; Kim *et al.*, 2001a).

The *Aplysia* S-type K⁺ channel is closed by PKA-mediated phosphorylation (Belardetti and Siegelbaum, 1988). TREK-1 is also down-modulated by the cAMP/PKA pathway (Fink *et al.*, 1996; Patel *et al.*, 1998). Activation of serotonin receptors coupled to the cAMP/PKA pathway reverses TREK-1 activation by lipids (Patel *et al.*, 1998). The C-terminal domain is involved in this regulation. Phosphorylation of S333 located at the distal end of the C-terminus of TREK-1 is responsible for cAMP-mediated TREK-1 inhibition (Patel *et al.*, 1998). It has recently been proposed that phosphorylation/dephosphorylation of S333 may be responsible for the inter-conversion between voltage-dependent and leaky phenotypes of rat TREK-1 expressed in *Xenopus* oocytes (Bockenhauer *et al.*, 2001).

In the present study, using site-directed mutagenesis, we identified a C-terminal acidic residue E306, which mediates TREK-1 opening during intracellular acidosis. We demonstrate that protonation of this residue controls both TREK-1 mechano- and lipid-sensitivities. Functional coupling between mechanical and chemical stimulation has important implications for the role of TREK-1 in various physiological and pathophysiological conditions.

Results

TREK-1 was transiently transfected in COS cells, and channel activity was monitored using the whole-cell patch-clamp configuration. TREK-1 basal channel activity was strongly and reversibly stimulated by AA (18.8 ± 2.4-fold, *n* = 28) (Figure 1A). We evaluated the role of charged and polar residues in the proximal C-terminal region on TREK-1 channel activity. Each residue was substituted by an alanine (Figure 1B). The amplitude of the basal and AA-stimulated currents was measured for each mutant. We identified four mutants (S300, T303, E306 and E309) characterized by a significantly higher basal current amplitude (Figure 1B). When stimulated by AA, the currents generated by all mutants except E306A significantly increased in amplitude (Figure 1B and C). The amplitude of the basal E306A current was in fact comparable to that of currents generated by the wild type (WT) and other mutants in the presence of AA (Figure 1B).

We compared TREK-1 WT with the E306A mutant at the single-channel level (Figure 2). In the inside-out patch configuration, the basal channel activity of TREK-1 WT was low (Figure 2A and B) [NP_o (number of channels × open channel probability) = 5.0 ± 1.9, *n* = 13]. Addition of AA in the internal medium rapidly and reversibly produced a considerable stimulation of channel activity (Figure 2C) (NP_o = 56.8 ± 17.1, *n* = 13). The channel activity was extremely flickery and occurred in bursts. In a symmetrical K⁺ gradient, the single-channel conductance

Fig. 1. Alanine scanning of the proximal C-terminal region of TREK-1. (A) AA strongly potentiates WT TREK-1 current amplitude. (B) Current amplitudes were measured at rest and after stimulation for 2 min with 10 μM AA at a membrane potential of 0 mV in a physiological K⁺ gradient. The number of cells studied is indicated. The S300, T303, E306 and E309 mutants have a significantly higher basal current compared with TREK-1 WT (indicated by an asterisk). A total of 0.1 μg of DNA was transfected per plate for WT and mutants. (C) AA has a minor effect on the E306A mutant. In (A), (B) and (C), currents were measured with voltage ramps of 600 ms in duration from a holding potential of -80 mV in a physiological K⁺ gradient.

weakly rectified in the inward direction at positive potentials (Figure 2G). In the linear range of the relationship, we estimated a single-channel conductance of 130 pS (*n* = 9). The basal channel activity of E306A was significantly higher than WT TREK-1 (Figure 2D and E) (NP_o = 37.6 ± 17.6, *n* = 9). Again, channel activity of E306A was comparable to AA-activated TREK-1, and channel opening was flickery and occurred in bursts (Figure 2C and D). Addition of AA had only a very modest stimulatory effect on E306A channel activity (Figure 2F) (NP_o = 46.6 ± 20.4, *n* = 9). In the linear range of the I-V relationship (-120 to 20 mV), the single-channel conductance (104 pS) (*n* = 14) was slightly reduced compared with WT TREK-1 (Figure 2G).

In the inside-out macro patch configuration, WT TREK-1 channel activity was low at atmospheric pressure (Figure 3A) (*n* = 26). However, applying increasing negative pressure (-66 mm Hg) to the membrane produced a gradual and saturable opening of TREK-1 (Figure 3A and E). The behaviour of the E306A mutant was very different. The basal E306A channel activity at atmospheric pressure was very high (Figure 3B) (*n* = 14). Applying a negative pressure of -66 mm Hg had no significant effect on E306A channel activity (Figure 3B and E).

Fig. 2. Single-channel properties of TREK-1 WT and E306A mutant. (A) Single-channel activity was recorded at -80 mV in the excised inside-out patch configuration in a symmetrical K^+ gradient. In the basal condition, the channel open probability is extremely low ($P_o = 0.006$) and only very few brief openings are detected for TREK-1 WT. (B) The amplitude histogram constructed with a 10 s recording demonstrates that TREK-1 WT channels are essentially closed at rest. (C) After addition of $10 \mu M$ AA to the internal medium, robust channel activity is detected for TREK-1 WT ($P_o = 0.45$). (D) The basal channel activity of E306A recorded at -80 mV in a symmetrical K^+ gradient is extremely high compared with TREK-1 WT ($P_o = 0.81$). (E) The amplitude histogram constructed with a 10 s recording demonstrates that the open channel probability of E306A is elevated in the non-stimulated condition. (F) Addition of $10 \mu M$ AA fails to significantly affect E306A channel activity ($P_o = 0.84$). (G) Single-channel I-V curves of TREK-1 WT ($n = 9$) and E306A ($n = 14$). The single-channel amplitude of E306A is slightly smaller than TREK-1 WT at negative and positive potentials. The single-channel conductances estimated in the linear range of the I-V relationships are 130 and 104 pS for TREK-1 WT and E306A, respectively. A total of $0.005 \mu g$ of DNA was transfected per plate in these experiments. Currents were filtered at 5 kHz and sampled at 20 kHz. The external solution was free of divalent cation.

In the absence of membrane stretch, TREK-1 WT could also be opened by intracellular acidosis (Figure 3C and E) ($n = 17$). Acidosis produced a reversible decrease in E306A channel activity (Figure 3D and E) ($n = 20$). The reduction in current amplitude at intracellular pH 5.0 is probably related to the decrease in single-channel conductance at intracellular acidic pH (Maingret *et al.*, 1999b). Finally, the addition of AA to the intracellular medium failed to affect E306A channel activity ($n = 9$), whereas it produced a dramatic increase in WT channel activity (Figures 2C, F and 3E) ($n = 13$).

A conserved glutamate is found at position 286 in the other AA- and stretch-sensitive 2P domain K^+ channel TRAAK (Fink *et al.*, 1998). E286 was substituted with an alanine and mutant channel sensitivity to AA, stretch and pH_i were compared with TRAAK WT. In outside-out patches, the basal [22 ± 4 and 60 ± 13 pA for TRAAK ($n = 6$) and E286A ($n = 8$), respectively] and AA-stimulated currents (343 ± 185 and 471 ± 166 pA) were not significantly different. In inside-out patches, both channels were stimulated by intracellular alkalinization from pH 7.2 [20 ± 16 and 329 ± 110 pA for TRAAK ($n = 10$) and E286A ($n = 11$), respectively] to pH 8.4 (1228 ± 351 and 1330 ± 387 pA), but in contrast to TREK-1, were not opened by intracellular acidification to pH 5.0 (43 ± 29 and 29 ± 21 pA). Finally, both TRAAK (2896 ± 628 pA, $n = 10$) and E286A (971 ± 278 pA,

$n = 11$) channels were opened by a membrane stretch of -73 mm Hg at pH 7.2 in the inside-out patch configuration.

E306 was substituted with several hydrophobic or charged residues in TREK-1. Alanine, cysteine and lysine substitutions produced the same increase in basal channel activity with a parallel suppression of the stimulatory effect of AA (Figure 4A). Unlike the previous substitutions, the conservative mutation E306D did not produce a gain of function (Figure 4A) ($n = 14$). E306D channels were closed at rest and could be opened upon stimulation with AA (Figure 4A) ($n = 14$). Intracellular Cd^{2+} completely and reversibly inhibited the E306C mutant, whereas it was without effect on the E306A mutant (Figure 4B and inset).

Deletion of the last 100 amino acids in the C-terminal region ($\Delta 100$ TREK-1) almost completely abolished TREK-1 mechano-sensitivity (Figure 5A, left panel, B, C and F). However, lowering intracellular pH from 7.2 to 5.0 strongly sensitized $\Delta 100$ TREK-1 to stretch (Figure 5A–C). Introduction of the E306A mutation into the $\Delta 100$ TREK-1 mutant ($\Delta 100$ TREK-1/E306A) rescued channel mechano-gating at intracellular pH 7.2 (Figure 5D–F). The relationship between TREK-1 WT channel activity and negative pressure was fitted with a Boltzmann function (Figure 5F). The pressure value required to elicit half channel activity ($P_{0.5}$) was -38 mm Hg for TREK-1 WT at intracellular pH 7.2 ($n = 21$)

Fig. 3. The E306A mutant is insensitive to membrane stretch, intracellular acidosis and AA. (A) Membrane stretch of -66 mm Hg reversibly opens TREK-1 WT channels in inside-out macro patches. (B) The same membrane stretch fails to affect E306A. (C) Intracellular acidosis from pH 7.2 to pH 5.0 reversibly opens TREK-1 in an inside-out patch. (D) Intracellular acidosis from pH 7.2 to pH 5.0 reversibly reduces E306A current amplitude. (E) Intracellular acidosis to pH 5.0, 10 μ M AA and stretch of -66 mm Hg strongly stimulate TREK-1 WT, while failing to enhance E306A current in inside-out patches. These experiments were performed in a physiological K^+ gradient at a holding potential of 0 mV. The number of patches studied is indicated and the effect of pH 5.0, AA and stretch on TREK-1 WT is significantly different from E306A (indicated by an asterisk).

(Figure 5F). The Δ 100 mutant was hardly affected by membrane stretch at pH_i 7.2, while the $P_{0.5}$ value was shifted to -48 mm Hg at pH_i 5.0 ($n = 4$) (Figure 5C). This latter value is comparable to the $P_{0.5}$ value of -49 mm Hg for the Δ 100 TREK-1/E306A mutant at pH_i 7.2 (Figure 5F) ($n = 5$). While the stretch response of the Δ 100 TREK-1 mutant was significantly stimulated at pH_i 5.0, that of the Δ 100 TREK-1/E306A mutant was, on the contrary, slightly inhibited (Figure 5A-E).

The protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) is often used to produce chemical ischaemia (Hyllienmark and Brismar, 1996; Murai *et al.*, 1997; Buckler and Vaughan-Jones, 1998). FCCP inhibits mitochondrial ATP synthesis and produces intracellular acidosis. FCCP strongly stimulated TREK-1 current amplitude, although it failed to affect mock-transfected COS cells (Figure 6A and B). Other metabolic

Fig. 4. The side chain at position 306 is critical for TREK-1 gating. (A) Substitution of E306 with hydrophobic, positively and negatively charged residues differentially affects channel opening. For each mutant, the current was recorded in the absence and in the presence of 10 μ M AA. The currents were recorded in the whole-cell configuration and the amplitude was measured at 0 mV in a physiological K^+ gradient. (B) The E306C, but not the E306A mutant is reversibly blocked by intracellular Cd^{2+} in the inside-out patch configuration. The free concentration of Cd^{2+} in the intracellular medium was 100 μ M. Voltage ramps of 600 ms in duration were applied from a holding potential of -80 mV in a physiological K^+ gradient (inset). The currents were recorded in inside-out macro patches, and the mean current amplitude was measured at 0 mV. The number of patches studied is indicated and statistical difference is indicated with an asterisk.

inhibitors such as cyanide and rotenone, which block oxidative phosphorylation by acting on the electron transfer chain, were however ineffective (Figure 6C). FCCP produced a dose-dependent, reversible activation of TREK-1 with an EC_{50} of 2.1 μ M (Figure 6D). This stimulatory effect of FCCP was not observed with the Δ 100, E306A and Δ 100/E306A mutants (Figure 6E). In contrast, AA (10 μ M) significantly stimulated the Δ 100 TREK-1/E306A mutant (7.3 ± 0.8 , $n = 10$), although it failed to affect the Δ 100 and E306A mutants (Figure 6F).

TREK-1 was down-modulated by PKA-mediated phosphorylation (Figure 7). Both basal and stimulated TREK-1 channel activities were strongly inhibited by the addition of the membrane-permeant derivative chlorophenylthio-cAMP (CPT-cAMP) (Figure 7A and B). For instance, the TREK-1 current stimulation mediated by HCO_3^- -induced intracellular acidosis was reversed by CPT-cAMP (Figure 7A). However, the replacement of E306 by an alanine residue, which provided constitutive activation, impaired the key regulation of the channel via cAMP and PKA phosphorylation of S333 (Figure 7B). E306A was, like the PKA mutant S333A, resistant to the cAMP/PKA-mediated inhibition (Figure 7B and C) ($n = 15$). In contrast, the mutant with the conservative substitution

Fig. 5. E306A mutation rescues the mechano-sensitivity of a loss-of-function C-terminal truncated TREK-1 mutant. (A) Excised inside-out patches expressing the $\Delta 100$ mutant (the last 100 amino acids in the C-terminal region have been deleted) were stimulated by a membrane stretch of -66 mm Hg at both intracellular pH 7.2 (left panel) and pH 5.0 (right panel). (B) Effects of an intracellular acidification from pH 7.2 to pH 5.0 on mean current amplitude recorded at both atmospheric pressure (white) and at -66 mm Hg (black) in inside-out patches expressing the $\Delta 100$ TREK-1 mutant ($n = 14$). The difference between stretch-activated channel activity at pH 7.2 and pH 5.0 is significant (indicated by an asterisk). (C) Relationships between $\Delta 100$ TREK-1 ($n = 4$) mean current amplitude and membrane negative pressure (mm Hg). The experimental data were fitted with a Boltzmann function. The intracellular pH was lowered from 7.2 (open symbols) to 5.0 (filled symbols). The pressure value required to elicit 50% of channel activity is estimated as -48 mm Hg at pH 5.0. (D) Effect of an intracellular acidification from pH 7.2 (left panel) to pH 5.0 (right panel) on the $\Delta 100$ /E306A mutant at both atmospheric pressure and at -66 mm Hg (stretch). (E) Effects of an intracellular acidification from pH 7.2 to pH 5.0 on mean current amplitude recorded at both atmospheric pressure (white) and at -66 mm Hg (black) in inside-out patches expressing the $\Delta 100$ /E306A TREK-1 mutant ($n = 9$). (F) Relationships between TREK-1 WT ($n = 21$), $\Delta 100$ TREK-1 ($n = 5$) and $\Delta 100$ TREK-1/E306A ($n = 10$) mean current amplitude and membrane negative pressure (mm Hg). The experimental data were fitted with a Boltzmann function. The pressure values required to elicit 50% of channel activity were -38 and -49 mm Hg for TREK-1 WT and $\Delta 100$ TREK-1/E306A, respectively. In these experiments, the holding potential was 0 mV and currents were recorded in a physiological K^+ gradient.

E306D was identical to TREK-1 WT and was inhibited by the addition of cAMP (Figure 7B).

Discussion

Constitutive opening of the mechano-gated K^+ channel TREK-1 by a gain-of-function mutation

TREK-1 is opened by a variety of physical and chemical stimuli including stretch, intracellular acidification and lipids (Patel *et al.*, 1998; Maingret *et al.*, 1999b, 2000a,b). We identified a charge cluster (D294–E309) in the proximal cytoplasmic C-terminal domain of TREK-1 that is critical for channel activation (Patel *et al.*, 1998, 1999; Maingret *et al.*, 1999b, 2000a,b, 2002). In the present report, we identify four mutants (S300, T303, E306 and E309) in this region, characterized by a significantly high basal current in the absence of physical and chemical stimulation. Analysis of the C-terminal sequence using DNA star software indicates that the proximal C-terminal domain is likely to be composed of long stretches of α -helices (Figure 8A). S300 and T303 are putative phosphorylation sites, and thus are potentially negatively charged when phosphorylated. The four mutations that we identified are located on the same side of the α -helix. Interestingly, an AA-responsive sequence (KFI-SEQTKKE) has recently been identified in the cytosolic N-terminal region of the AA-activated cationic channel

LTRPC2 (Hara *et al.*, 2002). This AA-responsive sequence is conserved in the proximal C-terminal domains of both TREK-1 (R297–E305) and TREK-2 (RVISKK-TKE) (Fink *et al.*, 1996; Patel *et al.*, 1998; Lesage *et al.*, 2000).

The E306 residue is critical for TREK-1 gating, as substitution by an alanine produces a constitutively active K^+ channel. Mutant channels at this position are already active at rest and thus become insensitive to AA, stretch and acidosis. However, in the LTRPC2 channel, an arginine is found at the same position in the AA-responsive sequence (Hara *et al.*, 2002). Our results demonstrate that E306A in TREK-1 acts as a gain-of-function mutation. The side chain of E306 is important as the replacement of this glutamic acid residue, by either a hydrophobic or positively charged one, leads to TREK-1 opening, while a conservative substitution with an aspartic acid residue has no effect. Interestingly, constitutive opening by replacement of E306 with a cysteine residue (E306C) can be selectively and reversibly blocked by Cd^{2+} , indicating that E306 is accessible from the intracellular side.

It is important to note that E306 is also present in TREK-2 and TRAAK, the two other AA-sensitive and mechano-gated 2P domain K^+ channels, but absent from all the other mammalian 2P domain K^+ channels (Fink *et al.*, 1998; Lesage *et al.*, 2000). However, as previously

Fig. 6. Opening of TREK-1 by the protonophore FCCP critically depends on the presence of E306. (A) In a mock-transfected COS cell, 5 μ M FCCP does not activate a K⁺ current, but occasionally activates a small non-selective current reversing at a reversal potential of about -15 mV. (B) FCCP (5 μ M) strongly activates TREK-1. In (A) and (B), the holding potential was -80 mV and cells were stimulated by voltage steps from -160 to 120 mV with a 40 mV increment. (C) FCCP (3 μ M) opens TREK-1, whereas blockers of the mitochondrial electron transport chain [cyanide (3 and 10 mM) and rotenone (10 μ M)] do not significantly affect TREK-1. (D) FCCP opens TREK-1 dose dependently and the EC₅₀ is 2.1 μ M. (E) Effects of 5 μ M FCCP on TREK-1, Δ 100, E306A and Δ 100/E306A mutants. The effect of FCCP on mutant channels is not significant compared with mock-transfected cells. (F) Effects of 10 μ M AA on TREK-1, Δ 100, E306A and Δ 100/E306A mutants. The difference between the Δ 100 and the Δ 100/E306A mutants is significant (indicated by an asterisk). Currents were recorded in the whole-cell configuration at a membrane potential of 0 mV in a physiological K⁺ gradient.

reported, we found that TRAAK is opened by intracellular alkalinization rather than acidification and, furthermore, the E286 residue is not involved in this regulation (Kim *et al.*, 2001a). This result demonstrates further that the molecular gating of TRAAK is clearly different from TREK-1 (Kim *et al.*, 2001a).

The C-terminal glutamate E306 acts as an intracellular proton sensor

Intracellular acidification opens TREK-1 at atmospheric pressure with a pK_a value of 6.0 (Maingret *et al.*, 1999b). Moreover, lowering the intracellular pH gradually shifts the pressure-effect relationship of TREK-1 towards positive values. In other words, intracellular acidification sensitizes TREK-1 to stretch (Maingret *et al.*, 1999b). When the C-terminal region of TREK-1 is deleted, as for instance in the Δ 100 mutant, the mechano-sensitivity is strongly depressed (Maingret *et al.*, 1999b). However, lowering the intracellular pH is able to partially rescue the mechano-sensitivity of the mutant, while it has no significant effect on the basal current at atmospheric pressure. Similarly, substituting E306 with an alanine rescues the mechano-sensitivity of the truncated mutant Δ 100 and mimics the effect of intracellular acidosis. Moreover, the Δ 100/E306A activity, in contrast to Δ 100,

Fig. 7. E306A mutation impairs cAMP-mediated down-regulation of TREK-1. (A) Extracellular addition of the membrane-permeant CPT-cAMP derivative (0.5 mM) produces a rapid and strong inhibition of whole-cell TREK-1 currents elicited in the presence of a HCO₃⁻-rich solution (superfused for 2 min). The holding potential was -80 mV and the cell was depolarized to 100 mV. (B) Basal as well as stimulated (AA, HCO₃⁻) TREK-1 channel activities are down-regulated by CPT-cAMP. However, E306A and S333A are resistant to this treatment. The conservative mutation E306D is as sensitive to cAMP as TREK-1 WT. Currents were recorded in the whole-cell configuration at a membrane potential of 0 mV in a physiological K⁺ gradient. (C) E306A mutant is resistant to CPT-cAMP (0.5 mM). Voltage ramps of 600 ms in duration were applied from a holding potential of -80 mV in a physiological K⁺ gradient.

as expected, becomes resistant to intracellular acidification. The residue E306 is, therefore, a likely candidate for the TREK-1 intracellular proton sensor. The normal pK_a value of the carboxylic side chain of glutamate is 4.5, a value much lower than the estimated apparent pK_a value of 6.0 determined for the pH_i sensitivity of TREK-1 (Maingret *et al.*, 1999b). Hydrophobic pockets, hydrogen bonds and/or neighbouring charges are known to shift pK_a values of titrable groups relative to the free amino acids. Considering that E306 is located in the middle of the charge cluster D294-E309, it is possible that this specific ionic environment may shift the pK_a value of E306 to 6.0.

In the whole-cell configuration, the protonophore FCCP, which produces intracellular acidification (Hyllienmark and Brismar, 1996; Murai *et al.*, 1997), strongly activates TREK-1, while it fails to modulate the activity of both E306A and Δ 100/E306A, as predicted if E306 is the intracellular proton sensor.

Protonation of E306 tunes TREK-1 mechano-gating

E306 is located in a charge cluster critically required for mechano-gating (Patel *et al.*, 1998; Maingret *et al.*,

Fig. 8. (A) Helical wheel representation of the proximal C-terminal domain of TREK-1 (from G293 to F310). The residues sensitive to the alanine substitution, S300, T303, E306 and E309, are indicated in black. (B) Functional model of TREK-1 gating. TREK-1 flickers between two closed states and one open state. When E306 is protonated the channel is mechanically coupled to the bilayer (closed state 2). In this conformation, mild mechanical stimulation or chemical stimulation by polyunsaturated fatty acids such as AA reversibly induces TREK-1 opening. At acidic intracellular pH, TREK-1 opens at atmospheric pressure because of high mechano-coupling to the membrane. The E306A mutation mimics intracellular acidification and opens TREK-1 at atmospheric pressure. In contrast, at alkaline intracellular pH, when E306 is deprotonated, the channel becomes mechanically uncoupled (closed state 1) and is more resistant to membrane stretch as well as lipid stimulation. Similarly, phosphorylation of the PKA site S333 or partial truncation of the C-terminal domain ($\Delta 100$) locks TREK-1 in the mechanically uncoupled closed state 1.

1999b). The identification of a titrable residue, E306, in this region is of importance to understand TREK-1 gating. Protonation of E306 leads to TREK-1 WT opening at atmospheric pressure and, furthermore, normalizes the pressure sensitivity of a loss-of-function truncated mutant ($\Delta 100$). The protonation of E306 thus appears to directly control TREK-1 mechano-sensitivity. The activating mechanical force may be directly transmitted to TREK-1 via either the lipid bilayer or the tethered elements including the cytoskeleton (Patel *et al.*, 1998, 2001; Maingret *et al.*, 1999a,b). The protonation of E306 may induce a conformational change of TREK-1, such as a tilt of a helix, which could alter the mechanical coupling with the membrane and thus enhance channel mechano-sensitivity (bilayer hypothesis) (Sachs and Morris, 1998; Hamill and Martinac, 2001). Since disruption of the cytoskeleton sensitizes mechano-gated 2P domain K^+ channels to stretch, it has been proposed that the cytoskeleton may tonically repress channel mechano-gating (Patel *et al.*, 1998; Maingret *et al.*, 1999a). Protonation of E306 may reduce the binding to the

cytoskeleton and thus sensitize TREK-1 to stretch (tethered hypothesis) (Sachs and Morris, 1998; Hamill and Martinac, 2001). However, the effect of intracellular pH is observed in the excised inside-out patch configuration, in the absence of intracellular ATP, when the cytoskeleton is already destabilized (Maingret *et al.*, 1999a). It seems unlikely, therefore, that the effect of intracellular acidosis may be related to the modulation of the interaction with the cytoskeleton. Structural information, as well as identification of possible interacting proteins, will be required to determine how protonation of E306 may regulate the mechanical coupling of TREK-1.

Functional interaction between the proton sensor E306 and the PKA phosphorylation site S333

The release of serotonin by facilitating interneurons produces a cAMP/PKA-dependent inhibition of the *Aplysia* S-type K^+ channel (Siegelbaum *et al.*, 1982; Belardetti and Siegelbaum, 1988). This mechanism is responsible for short-term pre-synaptic facilitation and underlies a simple form of learning (Hawkins *et al.*, 1993). Similarly, activation of serotonin receptors inhibits TREK-1 opening via the cAMP/PKA pathway (Patel *et al.*, 1998). The PKA phosphorylation site S333 in the cytosolic C-terminal region is responsible for this negative regulation (Patel *et al.*, 1998). The down-modulation of TREK-1 by phosphorylation of Ser333 requires E306, while the up-modulation of TREK-1 by intracellular acidification or E306A mutation is independent of Ser333 (Ser333 is absent in $\Delta 100$). E306 and S333 may either interact directly within the three-dimensional structure of TREK-1, or/and the conformational change induced by the E306A mutation may prevent the phosphorylated S333 residue from inducing channel inhibition.

A functional model for TREK-1

Kinetic analysis of single-channel recordings with a hidden Markov algorithm (QuB program suite) indicates that there are one open and two closed states (Qin *et al.*, 2000). The briefer closure (closed state 2) corresponds to the flickery transition within open bursts, while the longer closure (closed state 1) is related to the time separating bursts of activity (Figure 8B). It was recently demonstrated that PKA phosphorylation selectively increases the duration of the longer close time (closed state 1) (Bockenhauer *et al.*, 2001). On the contrary, the E306A mutation, as well as intracellular acidosis, stretch and AA, decrease the interval between bursts (Figures 2 and 8B). We previously proposed that anionic amphipaths, including AA, open TREK-1 because of a membrane crenation effect (Patel *et al.*, 1998, 2001). Lipid activation would thus be directly related to mechano-gating (Figure 8B). The present report suggests that mechanical coupling of TREK-1 is dynamically regulated by protonation of E306 (Figure 8B). When E306 is deprotonated at intracellular alkaline pH, mechanical coupling is loose and high pressure is required to open the channel (closed state 1). Partial truncation of the C-terminal region ($\Delta 100$) or PKA phosphorylation of S333 lock the channel in the closed state 1 and thus similarly reduce mechano- and lipid-sensitivity (Figures 5 and 7). Upon protonation of E306, the mechano-coupling of TREK-1 would be increased and the channel would become more sensitive to stretch (closed state 2)

(Figures 5 and 8B). Ultimately, at very low intracellular acidic pH, the mechanical coupling would be high enough to allow channel opening at atmospheric pressure (Figure 3). The E306A mutation mimics the effect of a maximal protonation and leads to TREK-1 WT channel opening at atmospheric pressure and rescues the mechano-sensitivity of the $\Delta 100$ mutant. The E306A mutation locks TREK-1 in the mechanically coupled closed state 2 and thus favours channel opening (Figure 8B).

The identification of E306 as an intracellular proton sensor that dynamically tunes TREK-1 mechano-gating, is important to understand how TREK-1 will open during either physiological or disease states.

Materials and methods

Electrophysiological recording

COS cell culture, transfection and electrophysiology have been described extensively elsewhere (Fink *et al.*, 1996, 1998; Lesage *et al.*, 1996; Duprat *et al.*, 1997). Briefly, COS cells were seeded at a density of 20 000 cells per 35 mm dish 24 h prior to transfection. Cells were then transfected by the DEAE-dextran protocol (0.1 μ g of DNA was routinely transfected). Mouse TREK-1 was amplified by PCR and subcloned into the pCI.IRES-CD8 vector (Fink *et al.*, 1998). Transfected cells were visualized 28–72 h after transfection using the anti-CD8 antibody-coated beads method. For whole-cell and excised patch experiments, the internal solution was 155 mM KCl, 3 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES pH 7.2 with KOH. In some experiments, HEPES was substituted by Mes and the pH was lowered to 5.0. Other experiments included internal Cd^{2+} at a free concentration of 100 μ M (0.5 mM EGTA). The external medium contained 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 3 mM $MgCl_2$ and 10 mM HEPES pH 7.4 with NaOH (physiological K^+ gradient). The K^+ -rich solution contained 155 mM KCl instead of NaCl (symmetrical K^+ gradient). In some experiments, extracellular divalent cations were omitted and 5 mM EGTA and 1 mM EDTA were added. The HCO_3^- -rich solution contained 90 mM $NaHCO_3$ instead of NaCl and pH was equilibrated by bubbling CO_2 . Superfusion of the bicarbonate-rich solution lowers intracellular pH by 1 unit (Fakler *et al.*, 1996). Similarly, addition of 3 μ M FCCP to the HEPES extracellular solution lowers intracellular pH by 0.46 ± 0.07 pH unit ($n = 12$) (K.J. Buckler, personal communication). However, a possible direct effect of FCCP at the plasma membrane cannot be ruled out entirely. Cells were continuously superfused with a microperfusion system during the time course of the experiments (0.1 ml/min) performed at room temperature. A RK300 patch-clamp amplifier (Biologic, Grenoble, France) was used for whole-cell as well as single-channel recordings. Ionic currents were monitored and recorded continuously using a DAT recorder (Biologic). Subsequently, data were replayed and sampled using pClamp software. Data analysis was performed using clampfit (pClamp) for whole-cell recordings as well as Biopatch (Biologic) software for single-channel recordings. Kinetic analysis of single-channel recordings was performed with a hidden Markov algorithm (QuB program suite) (Qin *et al.*, 2000). Membrane capacitance was measured during a 10 mV hyperpolarizing step from a holding potential of -80 mV. Membrane stretch (negative pressure is expressed in mm Hg) was applied as described previously (1 mm Hg = 7.5×10^{-3} Pa) (Maingret *et al.*, 1999b). Student's *t*-test was used for statistical analysis ($P < 0.001$ indicated by an asterisk).

Construction of mutants

PCR was used to generate the mutant channels. All PCRs were performed using the Advantage-GC cDNA polymerase mix (Clontech) according to the manufacturer's protocol. PCR products were cloned into pCI.IRES-CD8, a derivative of pCI (Promega). The clones obtained in this manner were sequenced in their entirety using an automatic sequencer (Applied Biosystems).

Reagents

All chemical reagents were obtained from Sigma. AA and FCCP were dissolved as a stock solution at a concentration of 100 and 50 mM in ethanol, respectively. Rotenone was dissolved at 100 mM in DMSO. CPT-cAMP and cyanide were prepared daily in the saline solution at a concentration of 0.5 and 10 mM, respectively.

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