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A mammalian two pore domain mechano-gated S-like K^+ channel

Amanda J.Patel, Eric Honoré, François Maingret, Florian Lesage, Michel Fink, Fabrice Duprat and Michel Lazdunski[1]

Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

[1]Corresponding author
e-mail: ipmc@ipmc.cnrs.fr

A.J.Patel and E.Honoré contributed equally to this work

Aplysia **S-type K^+ channels of sensory neurons play a dominant role in presynaptic facilitation and behavioural sensitization. They are closed by serotonin via cAMP-dependent phosphorylation, whereas they are opened by arachidonic acid, volatile general anaesthetics and mechanical stimulation. We have identified a cloned mammalian two P domain K^+ channel sharing the properties of the S channel. In addition, the recombinant channel is opened by lipid bilayer amphipathic crenators, while it is closed by cup-formers. The cytoplasmic C-terminus contains a charged region critical for chemical and mechanical activation, as well as a phosphorylation site required for cAMP inhibition.**

Keywords: arachidonic acid/K^+ channel/serotonin/stretch/volatile general anaesthetics

Introduction

TWIK-1 (tandem of P domains in a weak inward rectifier K^+ channel) was cloned initially in human kidney and encodes a K^+ channel with four transmembrane segments and two pore regions called P1 and P2 (Lesage *et al.*, 1996). The overall structure of TWIK-1 is very different from that of other pore-forming K^+ channel subunits which have a hydrophobic core containing six transmembrane segments and one P domain for the *Shaker* channels, and two transmembrane segments and one P domain for the inward rectifier K^+ channels (IRK). Another important feature of TWIK-1 is the presence of an extended domain between M1 and P1 (~60–70 residues) that is absent in *Shaker* and IRK proteins. TREK-1, another member of the novel class of K^+ channels with two P domains (related to TWIK-1), was cloned from a mouse brain library (Fink *et al.*, 1996). Despite a similar overall structure to TWIK-1 (four transmembrane segments and two P regions), the sequence identity with TWIK-1 is only 25%. TREK-1 has a wide tissue distribution and is present at high levels in several brain regions including the olfactory bulb, the hippocampus and the cerebellum (Fink *et al.*, 1996). TASK (TWIK-1-related acid-sensitive K^+ channel) is the third TWIK-related channel that was identified in mouse and human (Duprat *et al.*, 1997). Human TASK cDNA encodes a 395 amino acid polypeptide sharing 25 and 28% sequence identity with TWIK-1 and TREK-1, respectively. TWIK-1 encodes a weak IRK channel, while both TREK-1 and TASK encode background outward rectifier K^+ channels (Fink *et al.*, 1996; Lesage *et al.*, 1996; Duprat *et al.*, 1997).

Behavioural sensitization is a simple form of learning that follows the presentation of a novel stimulus. In *Aplysia*, sensitization of the gill-withdrawal reflex with a noxious stimulus to the tail is caused by presynaptic facilitation of transmission between the sensory and the motor neurons of the reflex pathway (Castellucci and Kandel, 1976; Klein and Kandel, 1980; Klein *et al.*, 1982). Serotonin is released by facilitating interneurons and inhibits a resting K^+ conductance (S-type K^+ channels) in presynaptic terminals via cAMP-dependent phosphorylation (Klein and Kandel, 1980; Klein *et al.*, 1982; Siegelbaum *et al.*, 1982; Shuster *et al.*, 1985). Inhibition of the S channels enhances action potential duration allowing more calcium to flow into the terminal, thus increasing neurotransmitter release (Klein and Kandel, 1980; Blumenfeld *et al.*, 1990). In contrast, the FMRFamide neuropeptide released by inhibitory interneurons opens the S channels via the arachidonic acid (AA) pathway, decreases action potential duration, reduces transmitter release from the sensory neuron terminals and leads to presynaptic and behavioural inhibition (Belardetti *et al.*, 1987; Buttner *et al.*, 1989; Blumenfeld *et al.*, 1990). The molluscan S channel is a resting, outwardly rectifying, weak voltage-dependent, time-, and calcium-independent, 45–55 pS (physiological conditions) K^+ channel. The S-type channel is resistant to external Ba^{2+}, tetraethylammonium (TEA^+) and 4-aminopyridine (4-AP) but sensitive to quinidine (Shuster and Siegelbaum, 1987; Sigurdson and Morris, 1989; Vandorpe and Morris, 1992; Small and Morris, 1995; Winegar *et al.*, 1996). It is opened by volatile general anesthetics such as chloroform (Franks and Lieb, 1988; Winegar *et al.*, 1996). A last major feature which characterizes the molluscan S channel is its stretch sensitivity (Sigurdson and Morris, 1989; Vandorpe and Morris, 1992).

Mechanosensitive ion channels transduce mechanical stimuli into electrical responses in specialized sensory cells and are proposed to play a more general role in controlling cellular volume, shape and growth (for reviews see Roberts *et al.*, 1988; Sachs, 1988; Pickard and Ding, 1992; Sackin, 1995; Hamill and McBride, 1997; Sukharev *et al.*, 1997). Moreover, mechanosensitivity of membrane receptors, including the *N*-methyl-D-aspartate (NMDA) receptor, has been reported in cultured mouse central neurons (Paoletti and Ascher, 1994). Here, we show that TREK-1 encodes a mammalian mechano-activated K^+ channel which shares most of the properties of the *Aplysia* S-type K^+ channel.

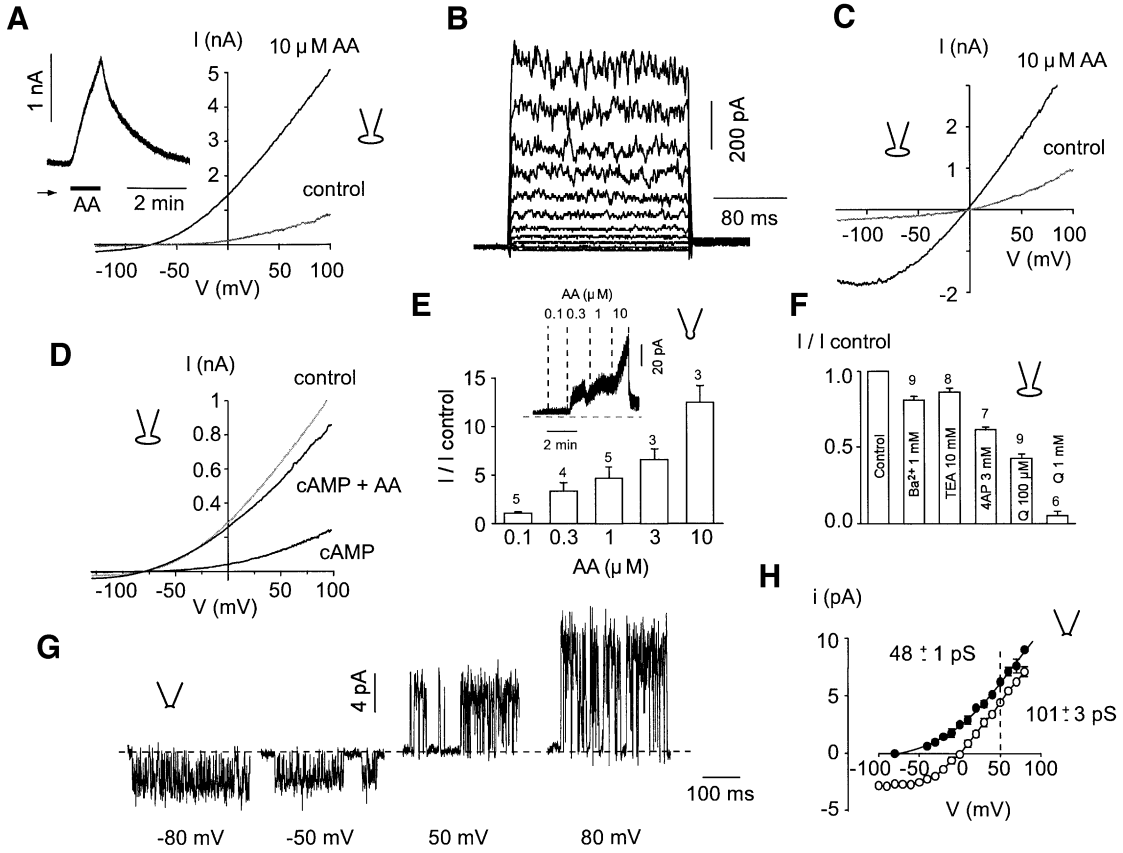

Fig. 1. TREK-1 and the *Aplysia* S channels share the same biophysical, pharmacological and regulation properties. (**A**) Effects of 10 μM AA on the whole-cell I–V curve of a TREK-1-transfected COS cell. The I–V curve was constructed with a voltage ramp of 600 ms in duration starting from a holding potential of –80 mV. The cell was bathed with the standard external solution containing 5 mM K^+. The inset shows a 1 min application of 10 μM AA on the whole-cell TREK-1 current measured at 0 mV [a different cell from that shown in (A)]. The arrow indicates the zero current. (**B**) The AA-induced current was obtained by digitally subtracting current traces in the presence and absence of 10 μM AA. The holding potential was –80 mV and the voltage steps were incremented by 20 mV from –100 to 100 mV [a different cell from that shown in (A)]. (**C**) I–V curve of the cell illustrated in (A) bathed in the presence of 155 mM K^+. (**D**) Effects of 500 μM CPT-cAMP on both the basal and the AA- (10 μM) stimulated TREK-1 current. (**E**) Dose–effect curve of AA on outside-out patches from TREK-1-transfected cells. The patches were maintained at 0 mV. The inset shows an example of such an experiment. The histogram bars represent the ratio of the mean current amplitude in the presence and absence of increasing concentrations of AA. The numbers of patches tested are indicated. (**F**) Pharmacological properties of TREK-1. The histogram bars represent the ratio of the current amplitude in the presence and absence of blocker. The numbers of cells tested are indicated by numbers above the bars. (**G**) Single-channel recording of TREK-1 in a symmetrical gradient (155 mM K^+). The membrane voltages are indicated. (**H**) Single-channel I–V curves of TREK-1 in physiological (5 mM K^+) or symmetrical K^+ (155 mM K^+) gradients. The single-channel conductance at 50 mV was 48 ± 1 pS and 101 ± 3 pS in the presence of 5 and 155 mM K^+ in the external medium, respectively. Six patches were analysed in each K^+ condition.

Results and discussion

The biophysical, pharmacological and regulation properties of TREK-1 were investigated in transiently transfected COS cells. The amplitude of the basal whole-cell TREK-1 current (31.4 ± 4.8 pA/pF at 100 mV, n = 31) is significantly higher than the control current in mock CD8-transfected cells (4.2 ± 0.5 pA/pF at 100 mV, n = 18). Figure 1A shows that the addition of 10 μM AA in the external medium strongly potentiates (530 ± 78%, n = 23) the whole-cell TREK-1 current whereas ethanol (solvent of AA) has no effect (n = 8; not shown). The effects are completely reversible upon washing (Figure 1A, inset). In mock CD8-transfected cells, no current activation is observed in the presence of 10 μM AA (5 ± 7%, n =18). TASK, another member of the two P domain K^+ channel family (Duprat *et al.*, 1997), is weakly inhibited by 10 μM AA (–34 ± 7%, n = 10). TREK-1 activation is independent of internal Ca^{2+} (5 mM EGTA in the internal medium) and can be reproduced several times without rundown. The AA-induced current (I_{AA}) reverses at the predicted value for the K^+ equilibrium potential (–83 ± 2 mV, n = 13), has no apparent voltage activation threshold, is time-independent and is noisy at positive potentials (Figure 1B). When external Na^+ is substituted with K^+, I_{AA} remains outwardly rectifying and the reversal potential (–6 ± 2 mV, n = 15) corresponds to the equilibrium potential for K^+ in symmetrical conditions (Figure 1C). Addition of the cAMP permeant derivative CPT-cAMP reversibly inhibits the basal whole-cell TREK-1 current (–64 ± 5%, n = 9) (Figure 1D). Interestingly, when TREK-1 is co-expressed with the 5-HT_4 receptor which increases intracellular cAMP (Claeysen *et al.*, 1997), serotonin application (100 μM) mimics the effects of cAMP addition (–72 ± 5%, n = 6; not shown). AA antagonizes and overrides the cAMP inhibition (n = 9; Figure 1D).

Activation of TREK-1 by AA is also observed in excised outside-out patches (Figure 1E). The activation is

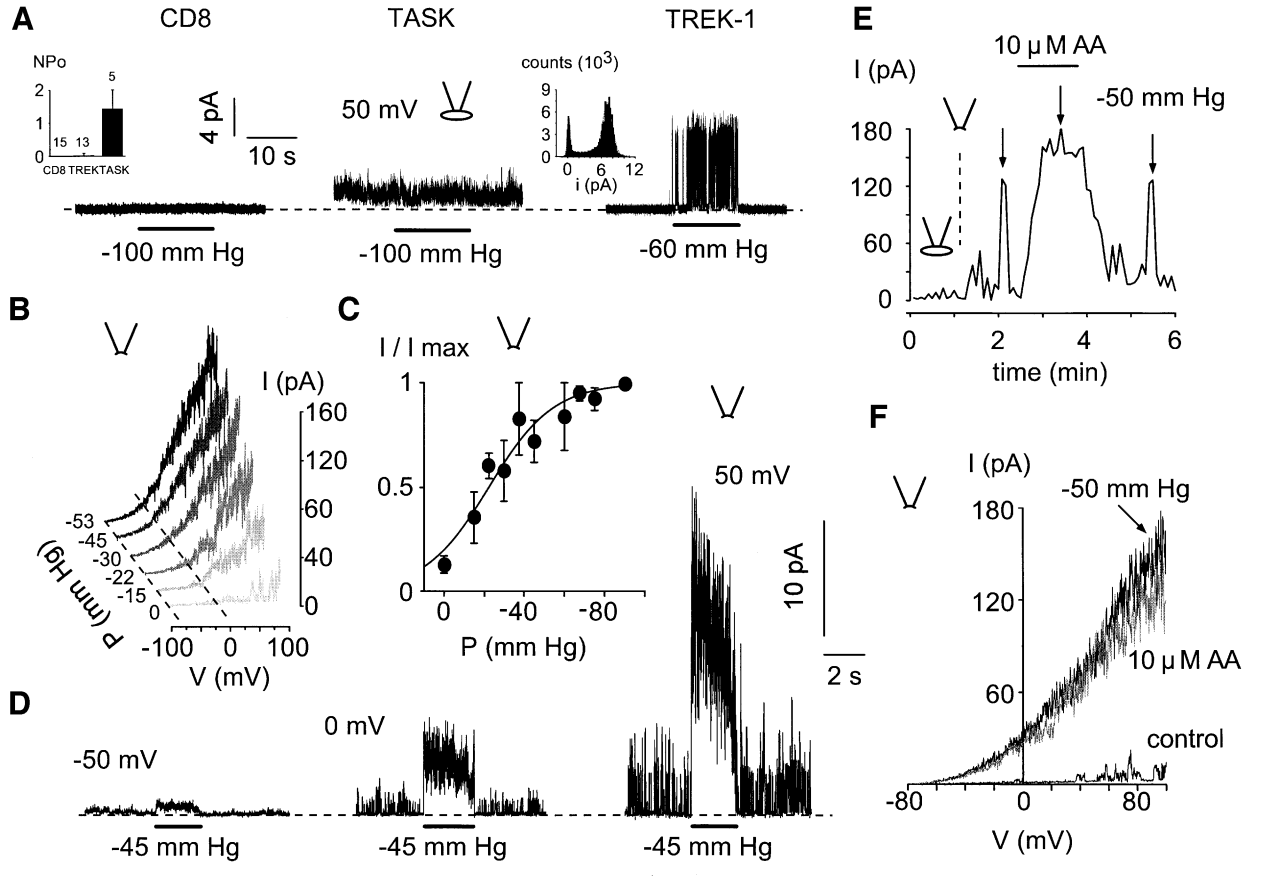

Fig. 2. TREK-1 is a stretch-activated K^+ channel. (**A**) The right panel shows the reversible activation of TREK-1 by membrane stretch in a cell-attached patch held at 50 mV. The right inset shows the amplitude histogram (80 bins). The left and middle panels demonstrate the lack of effect of membrane stretch on CD8 and TASK channel activities recorded in cell-attached patches. The left inset shows mean resting channel activities (NxPo) in cell-attached patches in mock- (CD8), TREK-1- and TASK-transfected cells. In all experiments illustrated in (A), patches were held at 50 mV. (**B**) Effects of increasing stretch stimulations (in mmHg) on the I–V curve of an inside-out patch from a cell expressing TREK-1. The I–V curves were constructed using voltage ramps from a holding potential of –80 mV (800 ms in duration). (**C**) Dose–response curve for TREK-1 activation by membrane stretch in inside-out patches (n = 6). The experimental points representing the normalized ratio of the mean current amplitude (I/I_{max}) were fitted with a Boltzmann relationship (half-activation: –23 mmHg). (**D**) Effects of membrane voltage (as indicated) on stretch-induced TREK-1 activation in an inside-out patch. (**E**) Combined effects of stretch and AA on TREK-1 channel activity recorded at 100 mV in an inside-out patch. The patch was stimulated with a voltage ramp (800 ms in duration) protocol [as illustrated in (F)] every 5 s. AA (10 µM) was added as indicated by a horizontal bar. During the time course of the experiment, the patch was stimulated by a –50 mmHg stretch (indicated by arrows). (**F**) I–V curves corresponding to the experiment illustrated in (E).

dose-dependent with a threshold concentration of 100 nM. Oleate (C18Δ9) (100 µM) and docosahexaenoate (C20Δ4,7,10,13,16,19) (10 µM), two other polyunsaturated fatty acids, mimic the effect of AA (n = 4; not shown). Derivatives of AA with an alcohol or a methyl ester substituted in the carboxylic function are inactive (n = 3). Saturated fatty acids such as palmitate (C16) and arachidate (C20) have no effect on TREK-1 (n = 3). Finally, TREK-1, unlike the *Aplysia* S channel, is insensitive to 12-hydroperoxyeicosatetranoic acid (12-HPETE) (0.5 µM) (n = 9; not shown). Another member of the two P family, the TRAAK channel, is also activated by AA but is not affected by variations in intracellular cAMP and therefore does not correspond to an S-type channel (Fink *et al.*, 1998).

Figure 1F shows that TREK-1 is resistant to high concentrations of Ba^{2+}, TEA^+ and 4-AP, but is blocked by quinidine. The single-channel properties of TREK-1 are illustrated in Figure 1G and H. The I–V curve of TREK-1 is outwardly rectifying in both physiological and symmetrical K^+ conditions. The single-channel conductance calculated at 50 mV is 48 ± 1 pS (n = 6) and 101 ± 3 pS (n = 6) in physiological and symmetrical K^+ conditions, respectively. Basal channel activity in inside-out patches is characterized by a flickery bursting behaviour with spontaneous transitions between high and low steady-state open probability (mean basal NPo: 0.44 ± 0.22; n = 13). In symmetrical K^+ conditions, the NPo ratio at +50 mV and –50 mV shows wide variability (mean NPo ratio: 11.1 ± 3.9; n = 13). These results demonstrate that both at the macroscopic and the microscopic levels, TREK-1 and the S channels share similar biophysical, pharmacological and regulation properties. However, the single S-type channel shows Goldman–Hodgkin–Katz-type rectification in symmetrical K^+ conditions while TREK-1 displays strong outward rectification.

Recent reports demonstrate that the *Aplysia* S channel is mechano-activated (Sigurdson and Morris, 1989; Vandorpe and Morris, 1992). Interestingly, TREK-1 channel activity, which is low in the cell-attached configuration (Figure 2A, left panel inset), can also be elicited by membrane stretch (in the absence of AA) (Figure 2A, right panel

and inset). Stretch has no effect on either control mock CD8- (n = 15) or TASK-transfected cells (n = 5) (Figure 2A, left and middle panel). Figure 2B and C shows that TREK-1 opening in inside-out patches is graded similarly by stretch, with a half-maximal activation at –23 mmHg stretch (n = 6). Figure 2D shows that stretch-induced TREK-1 channel activity can be elicited at both negative and positive potentials. In the same inside-out patch, TREK-1 can be opened by both membrane stretch and AA (Figure 2E and F). Lipid-free bovine serum albumin (BSA; 10 μM) (binding AA) added to either side of the membrane of inside-out patches has no effect on TREK-1 stretch activation, ruling out the possible involvement of a mechano-activated phospholipase releasing AA (n = 5; not shown). In the whole-cell configuration, a laminar shear stress (Olesen *et al.*, 1988) induced by increasing superfusion flux rate from 0.1 to 0.8 ml/min reversibly enhances TREK-1 current amplitude (40 ± 4%, n = 14) (to be shown later). To ensure that mechano-gating of TREK-1 is not dependent on specific endogenous COS cell components, we also expressed TREK-1 in *Xenopus* oocytes. TREK-1 cDNA was injected into the nuclei of oocytes and channel activity was recorded in the cell-attached patch configuration (Figure 3). *Xenopus* oocytes express endogenous cationic stretch-activated channels which can be differentiated easily from the exogenous TREK-1 channels due to their single-channel conductance, gating and reversal potentials (Figure 3A, inset). The endogenous stretch-activated cationic channels reverse at 0 mV, have a single-channel conductance of 15–20 pS and are characterized by long-lasting openings. In the oocyte, TREK-1 is opened similarly in a graded manner by membrane stretch (Figure 3A). Large outward currents are measured at 0 mV where no endogenous cationic channel current is observed (Figure 3B and C). Negative pressure is significantly more efficient than positive pressure for TREK-1 activation ($I_{-50\ mmHg}/I_{+50\ mmHg}$: 3.3 ± 0.3, n = 6). Figure 3D–F shows that the oocyte basal macroscopic TREK-1 current is also modulated by the cellular volume. When the osmolarity of the frog saline solution is increased by addition of mannitol (417 mOsm), basal TREK-1 current amplitude is significantly decreased compared with both mock CD8- and TASK-injected oocytes. Conversely, a reduction in osmolarity to 97 mOsm increases TREK-1 current amplitude by 28 ± 4% (n = 4; not shown). The effects of osmolarity are complete and reversible within 10 min (Figure 3E). TREK-1 basal current reverses at the predicted equilibrium potential for K^+ (–80 mV) and the amplitude is decreased at all membrane voltages by increasing osmolarity with sucrose (instead of mannitol) (Figure 3F). Taken together, these data demonstrate that mechanosensitivity is an intrinsic property of TREK-1.

The *Aplysia* S-type channel was shown to be opened by the volatile general anaesthetic chloroform (Franks and Lieb, 1988; Winegar *et al.*, 1996). We also observed that chloroform reversibly opens TREK-1 in COS cells (Figure 4A and B). The I–V curve of the chloroform-activated current is identical to the AA-sensitive current (Figure 1A–C). Under symmetrical K^+ conditions, the I–V curve remains outwardly rectifying. Chloroform does not activate any current in either mock CD8- (–14 ± 7%, n = 5) or TASK-transfected cells (–16 ± 12%, n = 7). The

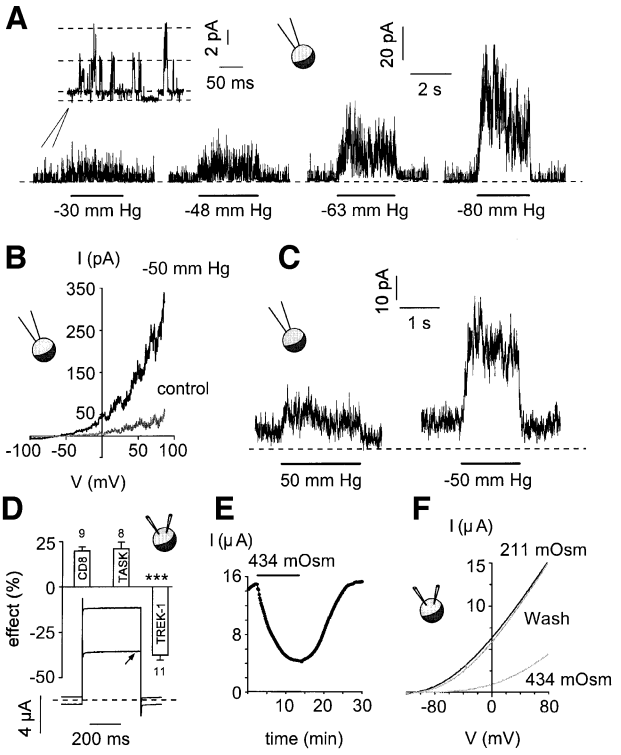

Fig. 3. TREK-1 is opened by membrane stretch, as well as cell swelling, in *Xenopus* oocytes. (**A**) TREK-1 activation was graded with membrane stretch in a cell-attached patch from a TREK-1 cDNA-injected oocyte. The patch was held at 50 mV and stretch amplitudes are indicated. The inset shows channel openings with a faster time scale, as indicated. In this patch, a small conductance endogenous channel was also present. (**B**) I–V curves in control and during a –50 mmHg stretch in a cell-attached patch from a TREK-1-expressing oocyte. The patch was held at a holding potential of –80 mV and was depolarized with a voltage ramp of 600 ms in duration every 5 s. (**C**) Negative pressure (right panel) was more efficient than positive pressure (left panel) in activating TREK-1 in a cell-attached patch held at 0 mV. (**D**) Comparison of the effects of a hypertonic solution (417 mOsm with addition of mannitol) on CD8-, TASK- and TREK-1-expressing *Xenopus* oocytes. Currents were measured during a voltage jump from –80 to 80 mV (shown in inset; the arrow indicates the hyperosmotic condition). (**E**) Kinetics of the effects of a 434 mOsm hypertonic solution (saccharose instead of mannitol). Each current was measured at 80 mV during voltage ramps from –80 mV to 80 mV. (**F**) Reversible effects of a hypertonic solution (434 mOsm with saccharose) on the I–V curve of TREK-1 constructed with a voltage ramp protocol (600 ms in duration).

concentrations of chloroform required to open TREK-1 are in the millimolar clinical range (Figure 4D).

In order to define the cellular and molecular elements responsible for TREK-1 mechanosensitivity, we first altered the integrity of the cytoskeleton. In inside-out patches, internal application of the cytoskeleton-disrupting agents colchicine (500 μM) and cytochalasin D (1 μg/ml) does not alter TREK-1 stretch activation (n = 4; not shown). We then tried to modify the bilayer properties. Amphipathic molecules previously have been shown to cause erythrocyte membranes to either crenate or to form cups (Sheetz and Singer, 1974). These changes in cell shape are thought to be due to the preferential insertion of crenators into the external leaflet while cup-formers insert into the internal leaflet of the bilayer (Sheetz and Singer, 1974) (Figure 4, right panel). Moreover, amphipaths have drastic effects on the *Escherichia coli*

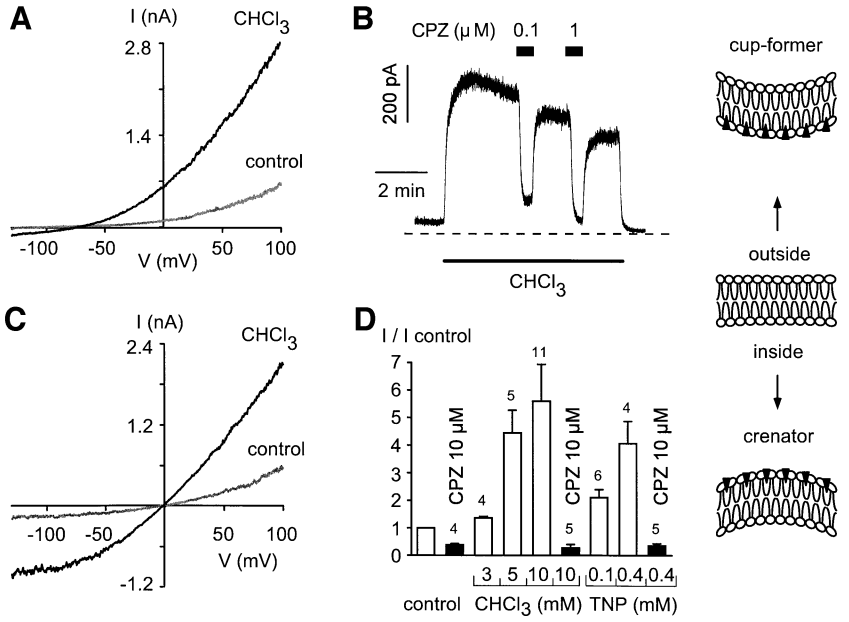

Fig. 4. Anionic amphipaths and chloroform open, while cationic amphipaths close TREK-1. (**A**) Effects of chloroform (5 mM) on the whole-cell I–V curve from a TREK-1-transfected COS cell. The I–V curve was constructed with a voltage ramp of 600 ms in duration starting from a holding potential of –80 mV. The cell was bathed with the standard external solution containing 5 mM K^+. (**B**) Activation of TREK-1 by chloroform (5 mM) in the whole-cell configuration at 0 mV. Chlorpromazine (CPZ 0.1 μM and 1 μM) was added as indicated. (**C**) I–V curve of the cell illustrated in (A) bathed in the presence of 155 mM K^+. (**D**) Histogram showing the effects of increasing concentrations of chloroform and various crenators and cup-formers on the whole-cell TREK-1 current. The currents were measured during voltage ramps at 100 mV and the histogram bars represent the ratio of the current in the presence and absence of the drugs. Numbers indicate the number of cells tested. The cartoon on the right hand side of the figure illustrates the bilayer couple model of Sheetz and Singer (1974). Anionic and neutral amphipaths preferentially insert into the outer half of the bilayer and produce crenation. Cationic amphipaths insert into the negatively charged inner half of the bilayer and lead to cup formation.

mechanosensitive cationic channels (Martinac *et al.*, 1990). In the present study, we found that the anionic crenator trinitrophenol (TNP) and the neutral crenator lysolecithin are able to mimic the activatory effects of AA and chloroform (Figure 4D) (100 ± 9% with 1 μM lysolecithin, $n = 3$; not shown). In contrast, we found that cationic amphipathic cup-formers such as chlorpromazine (CPZ) and tetracaine (–79 ± 2% at 100 μM tetracaine, $n = 4$; not shown) inhibit TREK-1 channel activity. As illustrated in Figure 4C and D, both the basal and the stimulated TREK-1 channel activities (by chloroform or TNP) are blocked by low concentrations of CPZ. These data show that the opening of TREK-1 can be modulated differentially by various amphipaths acting on the bilayer membrane. These results suggest that the expansion of the outer half of the bilayer (Sheetz and Singer, 1974) may be responsible for TREK-1 opening. The higher efficiency of negative versus positive pressure in cell-attached experiments seems to favour this hypothesis (Figure 3C). These data further imply that mechanical force may be transmitted directly to the channel via the lipid bilayer as seems to be indicated by the lack of effect of cytoskeleton-disrupting agents on inside-out patches (see above).

At this point, it remained to identify the TREK-1 region(s) responsible for activation by AA, mechanical stimulation and inhibition by cAMP. The cytoplasmic N- and C-termini were focused upon since they are the least conserved regions between the two P domain channels TREK-1 and TASK (Fink *et al.*, 1996; Duprat *et al.*, 1997). Truncation of the N-terminus of TREK-1 up to and including Met42 does not affect either the basal or the AA-stimulated TREK-1 current ($n = 5$; not shown). Similarly, truncation of the C-terminus up to Thr322 (mutant D1) does not have any significant effect (Figure 5A–C). However, further deletion of the C-terminal region up to Arg311 (mutant D2) results in a large decrease in the amplitude of the basal current and a reduction by 2.5-fold of the AA stimulation (Figure 5A–C). Truncation at Gly308 (mutant D3) does not affect channel activity further, but truncation at Val298 (mutant D4) completely suppresses both basal and AA-induced TREK-1 current (Figure 5A–C). We rescued basal channel activity by fusing the C-terminal portion of TASK (Duprat *et al.*, 1997) to the D4 mutant (chimera C2) (Figure 5A and B). However, chimera C2 remains insensitive to both AA and mechanical stimulation by laminar shear stress (Figure 5C and D). Chimera C1 confirms the results obtained with deletion D1, in that it is activated by AA, and chimera C3 behaves similarly to chimera C2 (Figure 5B and C). These results indicate that the channel region located between Val298 and Thr322 is crucial for chemical and mechanical activation and that both stimulations may act via a common molecular site. To try to answer the question of whether this region is sufficient by itself to provide chemical and mechanical sensitivities, we replaced the C-terminal region of TASK (not stimulated by AA and mechanical stimulation) with that of TREK-1 (chimera C4 with TASK ending at Val242) (Figure 5A–C). While chimera C4 has a basal activity comparable with that of TREK-1 (Figure 5B), AA (Figure 5C) and shear stress do not have any significant effect ($n = 4$; not shown). This result demonstrates that the C-terminal region of TREK-1

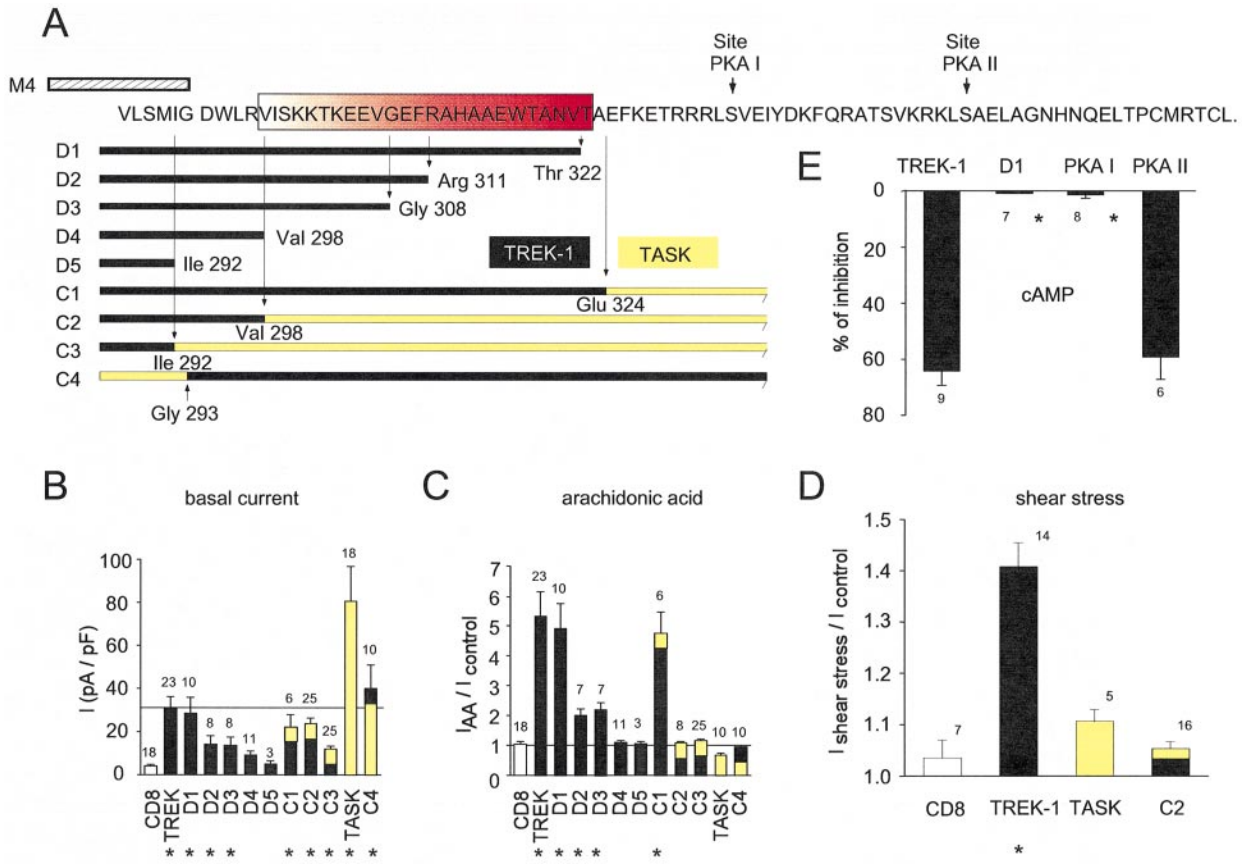

Fig. 5. The cytoplasmic C-terminal region of TREK-1 is critical for up-regulation by AA, laminar shear stress and down-regulation by cAMP. (**A**) Cartoon showing the truncated C-terminal mutants and chimeras. The sequence of the cytoplasmic C-terminus of TREK-1 starting at Gly293 is indicated. (**B**) Basal current density (whole-cell recording) measured at 100 mV in cells transfected with various TREK-1 and TASK constructs. Asterisks below the histograms indicate the significance of the difference ($P < 0.001$) between the mutants and the CD8 mock condition. (**C**) AA stimulation measured as the ratio of the current amplitude in the presence and the absence of 10 μM AA (whole-cell recording). (**D**) Effects of shear stress on CD8, TASK, TREK and C2 currents (whole-cell recording). The histogram bars represent the ratio of the current measured before and after shear stress. (**E**) Effects of point mutations in PKA I and PKA II sites on the CPT-cAMP (500 μM) down-modulation of the basal TREK-1 channel activity (whole-cell recording). The asterisks indicate the significance ($P < 0.001$) of the difference between the mutant and TREK-1. The numbers of cells tested are indicated.

is necessary but not sufficient to provide mechanical and chemical sensitivities. TASK chimeras with larger TREK-1 C-terminal portions up to and including the fourth transmembrane domain were inactive (not shown). Interestingly, we identified a charge cluster (**R**VIS**KK**T**KEE**) in the C-terminal region (Arg297–Glu306 as indicated in red in Figure 5A) of TREK-1 which is critical for channel activation and resembles the one identified in the C-terminus of the mechanosensitive cationic large conductance *E.coli* MscL channel (**RKKEE**P) (Sukharev *et al.*, 1994, 1997). Similar molecular mechanisms involved in mechanosensitivity could therefore be conserved through evolution.

The C-terminal region of TREK-1 is also characterized by the presence of two protein kinase A (PKA) consensus phosphorylation sites (Figure 5A). The truncated mutant D1 lacks these two sites and is insensitive to both cAMP and serotonin application ($n = 5$), while it remains sensitive to AA (Figure 5C and E). These results suggest that phosphorylation of these sites possibly may be involved in the down-modulation of TREK-1 by cAMP. In order to identify the phosphorylation site involved in this cAMP regulation, we independently replaced both serines with alanines. Figure 5E demonstrates that only site I for PKA phosphorylation (Figure 5A) is involved in the cAMP down-modulation of TREK-1.

The present study demonstrates that the mammalian TREK-1 and the molluscan S channel share very similar biophysical, pharmacological and regulation properties. Additionally, similar background K^+ channels activated by AA have been described in mammalian hippocampal and pre-motor respiratory neurons (Premkumar *et al.*, 1990; Wagner and Dekin, 1993). Both the S-type and TREK-1 K^+ channels are opened by membrane stretch. Since most cellular organisms are able to respond and in some cases adapt to vibration, touch, gravity and osmolarity, and considering the ubiquitous distribution of TREK-1 (Fink *et al.*, 1996), we believe that it may also play important physiological and pathophysiological roles in non-neuronal tissues. Stretch-activated K^+ channels, sharing the properties of the *Aplysia* S channel, have been implicated in several physiological functions such as modulation of the resting membrane potential in cleaving embryos, cardiac electrogenesis, auditive mechano-transduction, macrophage activation, neurite elongation, and cell locomotion and cell volume regulation (for reviews on mechanosensitive ion channels see Roberts *et al.*, 1988; Sachs, 1988; Pickard and Ding, 1992; Sackin, 1995;

Hamill and McBride, 1997; Sukharev *et al.*, 1997). Considering that TREK-1 is also expressed in the heart, it is likely that it may also be involved in the control of cardiac cell electrogenesis (Fink *et al.*, 1996). Activation of TREK-1 by mechanical stimulation during contraction may contribute to repolarization of cardiomyocytes.

Identification of TREK-1 as the first mammalian recombinant mechano-gated S-type K^+ channel may contribute to a better understanding of the major physiological functions of this type of channel and may also have important implications for several human pathologies.

Materials and methods

COS cell transfection, culture and electrophysiology have been described extensively elsewhere (Fink *et al.*, 1996; Duprat *et al.*, 1997). A 1100 bp *Cla*I–*Mun*I TREK-1 fragment amplified by PCR was subcloned into the *Cla*I–*Eco*RI-digested pIRES-CD8 vector (Fink *et al.*, 1998). Transfected cells were visualized 48 h after transfection using the anti-CD8 antibody-coated beads method (Fink *et al.*, 1998). For whole-cell experiments, the internal solution was 150 mM KCl, 3 mM $MgCl_2$, 5 mM EGTA and 10 mM HEPES–KOH pH 7.2. The external medium contained 150 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES–NaOH pH 7.4. Cells were superfused continuously with a microperfusion system during the time course of the experiments (0.1 ml/min). For shear stress experiments, a fast perfusion system was used (0.8 ml/min). For cell-attached experiments, the external medium bathing the cells contained 155 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES–NaOH pH 7.4. The pipette medium contained either the standard external medium (5 mM K^+) or a K^+-rich solution (155 mM K^+).

Xenopus oocyte isolation, cDNA injection and electrophysiology have been described elsewhere (Fink *et al.*, 1996; Duprat *et al.*, 1997). Oocytes were kept in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM HEPES–NaOH pH 7.4). The osmolarity of ND96 measured with the freezing point procedure (Knauer automatic osmometer) was 203 mOsm and was hypotonic compared with the standard amphibian Ringer's solution. For osmotic experiments, ND96 was diluted by 50% (ND48) and osmolarity was adjusted with either mannitol or saccharose as indicated in the text. Pressure applied to the membrane patches was monitored with a Bioblock pressure meter. AA (Sigma) stock solutions were prepared in ethanol at the concentration of 0.1 M, flushed with N_2 and kept at –20°C. TNP was obtained from Aldrich and the other pharmacological agents from Sigma. The Student's *t*-test was used for statistical analysis ($P < 0.001$).

PCR was used to generate N-terminal deletions in TREK-1 (DDBJ/EMBL/GenBank accession No. U73488) by utilizing Met42 or by introducing a methionine just before Val47, whereas C-terminal deletions were generated by introducing a stop codon at the indicated amino acids (see Figure 5). Chimeras between TREK-1 and TASK (DDBJ/EMBL/GenBank accession No. AF006823) were generated as described elsewhere (Yon and Fried, 1989) at the indicated positions in Figure 5, as well as the point mutations in the PKA I and PKA II sites Ser334 and Ser352 to Ala, respectively. 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), to permit visual selection of cells which had been transfected using anti-CD8-coated beads. The clones obtained in this manner were sequenced in their entirety using an automatic sequencer (Applied Biosystems).

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