Properties of single two-pore domain TREK-2 channels expressed in mammalian cells

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TREK-2 (K_{2P}10.1), a member of the two-pore domain K^+ (K_{2P}) channel family, provides the background K^+ conductance in many cell types, and is a target of neurotransmitters that act on receptors coupled to Gs and Gq. We report here that TREK-2 exhibits small (TREK-2S) and large (TREK-2L) conductance phenotypes when expressed in mammalian cell lines (COS-7, HEK293, HeLa) and in *Xenopus* oocytes. TREK-2S phenotype shows a noisy open state with a mean conductance of 54 pS (+40 mV). TREK-2L phenotype shows a full open state (202 pS) with several short-lived sub-conductance levels. Both phenotypes were strongly activated by arachidonic acid, membrane stretch (−40 mmHg) and intracellular acidification (pH 6.4). Phosphorylation of TREK-2 produced by treatment of cells with activators of protein kinases A and C, and okadaic acid (a serine/threonine phosphatase inhibitor) decreased the current contributed by TREK-2S and TREK-2L, and caused partial switching of conductance levels from those of TREK-2S and TREK-2L to more intermediate values. Under this condition, TREK-2 exhibited six conducting levels and one closed level. TREK-2 mutants in which putative protein kinases A and C phosphorylation sites were mutated to alanines (S326A, S359A, S326A/S359A) displayed mostly TREK-2S and TREK-2L phenotypes. However, S326D and S359D mutants (as well as the double mutants) that mimic the phosphorylated state showed all six conducting levels and low channel activity. The S326A and S359A mutants did not significantly affect the intrinsic voltage dependence of TREK-2 in Mg^{2+}-free solution. Phenotypes resembling TREK-2S and TREK-2L were also observed in cerebellar granule neurons that express TREK-2 mRNA. These results show that TREK-2 exhibits two primary modes of gating that give rise to two channel phenotypes under dephosphorylated conditions, and that its phosphorylation shifts the gating mode to include intermediate conducting levels. This represents a novel mechanism by which receptor agonists modulate the function of a K^+ channel to alter cell excitability.

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TREK-2 (K_{2P}10.1) is a member of the two-pore domain K^+ channel family and provides part of the background K^+ current in a number of cell types, including cerebellar granule neurons, dorsal root ganglion neurons and cortical astrocytes (Bang *et al.* 2000; Gnatenco *et al.* 2002; Gu *et al.* 2002; Han *et al.* 2002, 2003; Kang & Kim, 2006). Thus, TREK-2 helps to stabilize the resting membrane potential and oppose depolarization. Physical and chemical stimuli such as membrane stretch, unsaturated free fatty acids, intracellular acidification and volatile anaesthetics activate TREK-2, thereby causing hyperpolarization (Patel *et al.* 1999, 2001; Kim, 2003). In contrast, neurotransmitters that act on Gs- and Gq-coupled receptors inhibit TREK-2, thereby causing depolarization (Chemin *et al.* 2003; Kang *et al.* 2006). TREK-1 is a close relative of TREK-2, and exhibits similar sensitivity to the activators and receptor agonists. Thus, TREKs are likely to contribute to diverse physiological functions by regulating the excitability of the cell in response to various biological stimuli.

The modulation of TREK-2 by chemical and physical stimuli has been studied in depth. However, the properties of the single TREK-2 channels including its single channel

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conductance are still not well defined, presumably due to very high open channel fluctuations. Although the general increasing and decreasing actions of various modulators of TREK-2 are known, how they affect the single channel kinetics of TREK-2 is also not very clear. When TREK-2 was first cloned and expressed in Cos-7 cells, single channel recording showed two channel phenotypes, one of which was more active than the other. The conductance and the current–voltage relationship of the less active phenotype were close to that of the large Ca^{2+}-activated K^+ channel (BK). Thus, it was thought that the less active phenotype was an endogenous BK channel expressed in Cos-7 cells, and the more active phenotype was therefore assumed to represent TREK-2 (Bang *et al.* 2000). In subsequent studies, a range of values for the single channel conductance of TREK-2 was reported. At a depolarized potential (+40 mV), the conductance values measured in symmetrical 150 mM KCl ranged from 52 pS to 100 pS (Bang *et al.* 2000; Lesage *et al.* 2000; Han *et al.* 2003; Kim, 2003). Such large differences in single channel conductance values under similar ionic conditions suggest that TREK-2 may exhibit different gating modes under various conditions. It is well known that many ion channels exhibit sub-conductance states that may represent partially conducting states, in addition to the main open and closed states. However, the different conducting phenotypes of TREK-2 do not fit the description of the typically short-lived sub-conductance levels.

This study further investigates the presence of different gating modes of TREK-2 and associated phenotypes. Here we report that TREK-2 exhibits two main channel phenotypes under dephosphorylated (basal) conditions, and that phosphorylation causes switching of the conductance levels from two main phenotypes that have high open probability to those with intermediate conducting phenotypes with low open probability. Thus, agonist-induced inhibition of TREK-2 that occurs via phosphorylation is associated with switching of the channel phenotypes from those with low and high conductance levels to those with intermediate levels. Such changes in conductance levels represent a previously unrecognized mechanism that receptor agonists use to regulate the function of a background K^+ channel to regulate neuronal excitability.

Methods

Transfection in cultured cells

Rat TREK-2 and TREK-1 cDNAs were cloned in this laboratory (Bang *et al.* 2000). The coding regions of *TREK-2* and *TREK-1* were subcloned into pcDNA3.1 vector (Invitrogen). mSlo1 and its β subunit (β1) were gifts from Dr Toshi Hoshi (Univ. Pennsylvania). Cos-7, HEK293 and HeLa cells were seeded at a density of 2×10^5 cells per 35 mm dish 24 h prior to transfection in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cells were co-transfected with plasmids containing DNA for an ion channel and GFP in pcDNA3.1 using LipofectAMINE 2000 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 and a GFP filter (emission wavelength, 510 nm). Cells were used 1–2 days after transfection.

Cerebellar granule neuron culture

All animals were used in accordance with the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. NIH85-23). The use of all rats in this study was approved by the Animal Care and Use Committee of Rosalind Franklin University. The cerebellum was isolated from rapidly decapitated P6–P8 rat pups, washed with oxygenated physiological buffer solution at 4°C. The cerebellar cortex was cut into thin sections and incubated for 15 min in a solution containing papain (12 units ml^{-1}; Worthington, Lakewood, NJ, USA), albumin (0.2 mg ml^{-1}) and DL-cysteine (0.2 mg ml^{-1}). After digestion, the tissue was washed twice with PBS solution and resuspended in a solution containing Dnase I (1000 Kunitz ml^{-1}: Worthington). After gentle trituration of the solution using a fire-polished glass pipette, the suspended cells were gently passed through a 3 cc/25G syringe. The suspension was layered on top of sterilized fetal bovine serum and centrifuged at 100 g for 10 min. The pellet was resuspended in plating medium that contained NeuroBasal Media supplemented with B-27 (10 μl per ml of culture medium; Life Technologies, Rockville, MD, USA), glutamic acid (2.5 mM), glutamine (20 mM), gentamicin (50 mg ml^{-1}) and fungizone (2.5 mg ml^{-1}). The cells were plated on glass coverslips coated with poly-L-lysine at a density of 1×10^5 cells cm^{-2}. Cells were kept for 5 days at 37°C in a humidified incubator gassed with 95% air–5% CO_2 mixture.

Expression of TREK-2 in *Xenopus* oocytes

TREK-2 cDNA was subcloned into pGEMHE vector for expression in oocytes. The plasmid was linearized with *Xho*I, and cRNA was transcribed *in vitro* using T7 RNA polymerase (Ambion). Adult female *Xenopus laevis* frogs were anaesthetized with 0.2% tricaine and a partial ovarectomy performed once on each frog, a procedure that was approved by the Animal Care and Use committee of Rosalind Franklin University. The oocytes were defolliculated by treating them with 0.4% collagenase IA (Sigma). Stage V and VI oocytes were selected and pressure injected using a Nanoject

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variable microinjection apparatus (model no. 3-000-203, Drummond Scientific) with cRNA (~20 ng, 50 nl). Oocytes were incubated for 2 days at 19°C in modified Barth's solution (pH 7.4) containing (in mM) 88 NaCL, 1 KCL, 2.4 $NaHCO_3$, 15 Hepes, 0.3 $CaNO_3{}^*4H_2O$, 0.41 $CaCL_2{}^*6H_2O$, 0.82 $MgSO_4{}^*H_2O$, 550 mg/L pyruvate, and 50 μg ml-1 gentamycin, 100 μg ml-1 penicillin and 100 μg ml-1 streptomycin. The vitelline layer was removed using forceps before patches were formed on the membrane.

TREK-2 mutants

Mutations were done using QuikChange site directed mutagenesis kit (Stratagene) to generate serine-to-alanine and serine-to-asparate mutations in pcDNA3.1. Both strands of mutated TREK-2 DNA fragments were sequenced for confirmation.

Electrophysiological studies

Electrophysiological recording was performed using a patch clamp amplifier (Axopatch 200, Axon Instruments). Single channel current was filtered at 3 kHz using an 8-pole Bessel filter (−3 dB; Frequency Devices) and transferred to the computer and stored for analysis with the pCLAMP program (version 9). 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 amplitude histograms 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 min of current recording. The single channel current tracings shown in the figures were filtered at 1 kHz. In experiments using cell-attached and excised patches, pipette and bath solutions contained (mM): 150 KCl, 1 $MgCl_2$, 5 EGTA and 10 Hepes (pH 7.3). Okadaic acid and all other chemicals and enzymes were purchased from Sigma Chemical Co. For statistics, Student's t test was used with $P < 0.05$ as the criterion for significance. Data are represented as mean ± standard deviation (S.D.) unless specified otherwise.

Results

TREK-2 cDNA produces two channel phenotypes under basal condition: TREK-2S and TREK-2L

TREK-2 was expressed in Cos-7 cells, and channels were recorded from inside-out patches with bath and pipette solutions containing 150 mM KCl. Interestingly, two channel phenotypes could be recorded at both positive (+40 mV) and negative (−40 mV) membrane potentials in almost all patches ($n > 50$). Figure 1*A* shows two types of inward single channel currents from an inside-out patch held at −40 mV. The channel openings with the small and large amplitudes were designated TREK-2S and TREK-2L, respectively, and indicated by dotted lines S and L (Fig. 1). In this patch, simultaneous opening of TREK-2S and TREK-2L was also present, as indicated by the dotted line S+L. The K^+ selectivity of the two channels was confirmed by 53–56 mV negative shifts in reversal potential following a 10-fold reduction in $[K^+]_o$ (150 mM to 15 mM), and the absence of inward current when extracellular K^+ was replaced by Na^+. Replacing the extracellular Cl^- with glutamate did not significantly affect the reversal potential (3 ± 3 mV; $n = 3$) for both phenotypes.

The two channel phenotypes can be recognized more clearly when the current is recorded at +40 mV and outward. Single channel currents recorded from a patch containing both channel phenotypes at −40 mV and +40 mV are shown in Fig. 1*B*. At +40 mV, the difference in conductance levels between the two channel types is even greater than that at −40 mV. Although both TREK-2S and TREK-2L were present in most patches, a few patches showed only one phenotype, allowing a clear distinction between the two phenotypes (Fig. 1*C* and *D*). When both TREK-2S and TREK-2L were present in the patch, TREK-2S was generally more active, as judged by a higher channel activity (NP_o) of TREK-2S (0.32 ± 0.12) than TREK-2L (0.06 ± 0.02) determined from six patches showing one level of opening. TREK-2S always exhibited a high level of noise in the open state at both positive and negative membrane potentials, compared with that of TREK-2L. The current amplitudes of the channel openings at different membrane potentials were determined from the amplitude histograms, assuming one open level for TREK-2S and TREK-2L. These amplitude levels were then used to plot the current–voltage relationships of TREK-2 (Fig. 1*E*). The single channel conductance values of TREK-2S and TREK-2L were 52 ± 3 and 201 ± 9 pS, respectively, at +40 mV ($n = 4$). Patches from control Cos-7 cells transfected with *GFP* alone did not show TREK-2S- or TREK-2L-like K^+ channels ($n > 22$), suggesting that both phenotypes are formed by *TREK-2*.

To test whether TREK-2 forms two phenotypes (TREK-2S and TREK-2L) in other cell types, TREK-2 was also expressed in HEK293, HeLa and *Xenopus* oocytes. In HEK293 and HeLa cells, both phenotypes were clearly observed in cell-attached patches ($n > 12$ each). In mock (GFP or no DNA)-transfected cells, no K^+ channels with single channel kinetics similar to TREK-2S and TREK-2L were ever observed ($n = 12$ for each cell type). The appearance of two channel phenotypes in different cell lines that normally do not express any TREK-2-like channels makes it unlikely that an endogenous channel was induced. Human TREK-2 (98% amino acid identity with rat TREK-2) expressed in Cos-7 and HeLa cells also exhibited the same two phenotypes. In *Xenopus* oocytes, injection of TREK-2 cRNA also produced two phenotypes identical to TREK-2S and TREK-2L in all

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seven cell-attached patches formed after removing the vitelline layer. Therefore, the possibility that the transcriptional process produces two mRNA species and thus two channel phenotypes could be ruled out.

TREK-2L is not the high-conductance Ca^{2+}-activated K^+ channel

The high single channel conductance of TREK-2L is close to that of the large-conductance Ca^{2+}-activated K^+ (K_{Ca}) channel reported in earlier studies (Farley & Rudy, 1988; Latorre *et al.* 1989). K_{Ca} channel is also activated by membrane stretch and free fatty acids (Ordway *et al.* 1991, 1995; Kirber *et al.* 1992). These properties are also exhibited by TREK-2 (Kim, 2003). Therefore, it was possible that TREK-2L was an endogenous K_{Ca} channel whose expression was somehow induced by the transfection of *TREK-2* in Cos-7 cells. To directly compare the single channel and pharmacological properties of TREK-2L and the K_{Ca} channel, mouse Slo1 (mSlo1) and its β subunit (β1) that together form the K_{Ca} channel was expressed in Cos-7 cells, and the single channels recorded from inside-out patches in bath and pipette solutions containing 150 mM KCl. The single channel openings recorded at two membrane potentials for TREK-2 and mSlo1/β1 are shown in Fig. 2*A*. The conductance of mSlo1 co-expressed with its β1 subunit was 252 ± 12 pS at +40 mV and 268 ± 11 pS at −40 mV ($n = 3$; Fig. 2*A*). These values are just slightly higher than those of TREK-2L (202 ± 8 pS at +40 mV and 220 ± 10 pS at −40 mV; Fig. 2*A*). To further distinguish between TREK-2L and K_{Ca} channel, the effect of iberiotoxin (Candia *et al.* 1992), a potent inhibitor of the K_{Ca} channel, was tested. When applied to the extracellular side of outside-out patches, iberiotoxin (100 nM) showed no significant effect on TREK-2L ($n = 4$, $P > 0.05$), but markedly inhibited mSlo1/β1 ($n = 4$; Fig. 2*B*). Applying 10 μM Ca^{2+} to the bath solution to inside-out patches also showed no significant effect on TREK-2L ($n = 5$; $P > 0.05$), but strongly activated mSlo1/β1 ($n = 4$; Fig. 2*C*). Therefore,

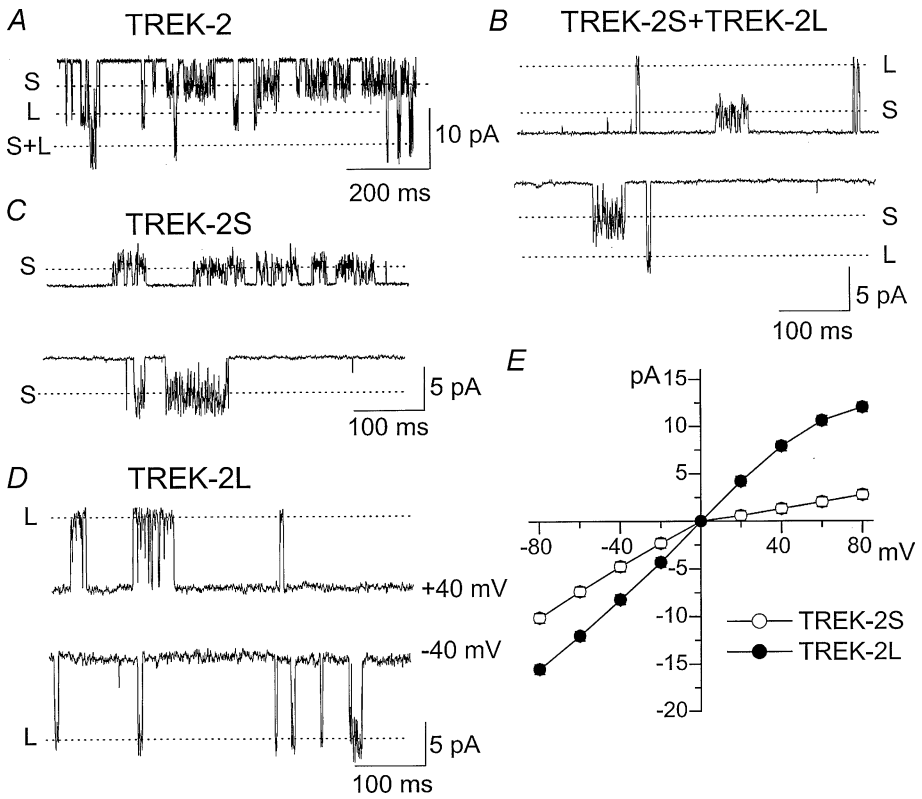

Figure 1. TREK-2 forms two channel phenotypes: TREK-2S and TREK-2L
A, inside-out patches were formed on Cos-7 cells expressing rat TREK-2. Cell membrane potential was held at −40 mV (pipette potential, +40 mV) to record inward current. Dotted lines indicate the open state for small (S) and large (L) conductance channels. S+L represents the state when both types of channels are open. Pipette and bath solutions contained 150 mM KCl. *B*, current recordings show channel openings at +40 mV (top) and −40 mV (bottom) in a patch that showed the two types of channels more clearly. *C*, current recordings from a patch that contained only one TREK-2S channel at +40 and −40 mV. *D*, current recordings from a patch that contained only one TREK-2L channel at +40 and −40 mV. *E*, the current–voltage relationships of TREK-2S and TREK-2L. Each point is the mean ± S.D. of 4 determinations.

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the evidence is clear that TREK-2L is not the K_{Ca} channel, despite their similar single channel conductance and behaviour.

TREK-2S and TREK-2L have similar sensitivity to activators and inhibitors

Arachidonic acid (10 μM), intracellular acidic solution (pH_i 6.0) and negative pressure applied to the patch are potent activators of TREK-2S (Patel & Honore, 2001; Kim, 2005). Whether TREK-2L is also sensitive to these activators has not been demonstrated previously. To test this, Cos-7 cells were transfected with *TREK-2*, and inside-out patches containing low levels of TREK-2S and TREK-2L activity were exposed to the three activators. Figure 3*A* illustrates an example of how the experiments were done. An inside-out patch containing both TREK-2S and TREK-2L was formed, and then the desired stimulus applied to the patch. Figure 3*B–D* shows expanded current tracings from such experiments before and after the application of a stimulus. All three activators caused similar increases in both TREK-2S and TREK-2L activity (Fig. 3*E*), showing that TREK-2L is also sensitive to the three stimuli. Applying Ba^{2+} (1 mM) to the bath solution containing inside-out patches reduced the outward currents of both TREK-2S and TREK-2L to a similar extent ($62 \pm 8\%$ *versus* $68 \pm 6\%$ inhibition; $n = 4$). Tetraethylammmonium (1 mM) had no effect on both TREK-2S and TREK-2L ($P > 0.05$; $n = 3$). These results provide additional evidence that both TREK-2S and TREK-2L are formed by *TREK-2*.

TREK-2S phenotype shows rapid transitions among three conducting levels

Expanded current tracings of TREK-2S recorded at +40 mV from two patches are shown in Fig. 4*A* and *B*. Although the open state appears noisy, two and three levels of opening can be identified in the top (Fig. 4*A*) and middle tracings (Fig. 4*B*), respectively. When the dotted lines are used to set the amplitude of conducting levels, amplitude histograms determined from these current tracings showed clear two and three open levels, respectively, as if there were two or three channels in the patch. Based on this analysis, the smallest conductance level of TREK-2S was determined to be 34 ± 2 pS at +40 mV ($n = 5$). Thus, the rapid transitions between the two or three levels give rise to the TREK-2S phenotype. TREK-2S showing three conducting levels (Fig. 4*B*) appeared more frequently (71 of 96 patches) than that showing only two levels (Fig. 4*A*). Do TREK-2S levels represent opening of two or three TREK-2 channels, or do they represent sub-conductance states of a single TREK-2 channel? So far, we have not been able to obtain a patch that contained only a single level of opening (34 pS), despite our efforts using pipettes with very small diameter tips and low expression levels. This is suggestive of the likelihood that TREK-2S represents sub-conductance levels of a single channel, rather than multiple channels present in the patch membrane.

A binomial distribution test of channel gating of TREK-2S was done to determine whether subunits act independently or show cooperatively, as described for nicotinic and purinergic receptor channels (Keleshian

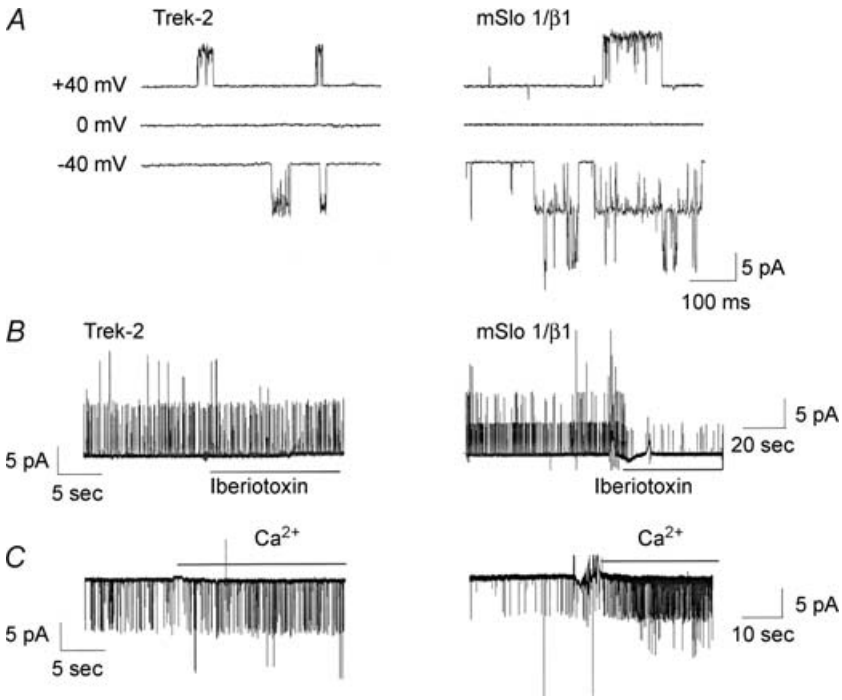

Figure 2. Effect of iberiotoxin and Ca^{2+} on TREK-2L and BK (mSlo1/β1)
A, single channel recordings of TREK-2L and BK expressed in Cos-7 cells at +40 mV and −40 mV. *B*, iberiotoxin (100 nM) added to the extracellular side of outside-out patch had no effect on TREK-2L but inhibited BK. Membrane potential was held at +40 mV for TREK-2 and +20 mV for Slo1. *C*, Ca^{2+} (10 μM) added to the bath solution in inside-out patches had little or no effect on TREK-2L, but greatly activated BK. Membrane potential was held at +40 mV for TREK-2 and +20 mV for Slo1.

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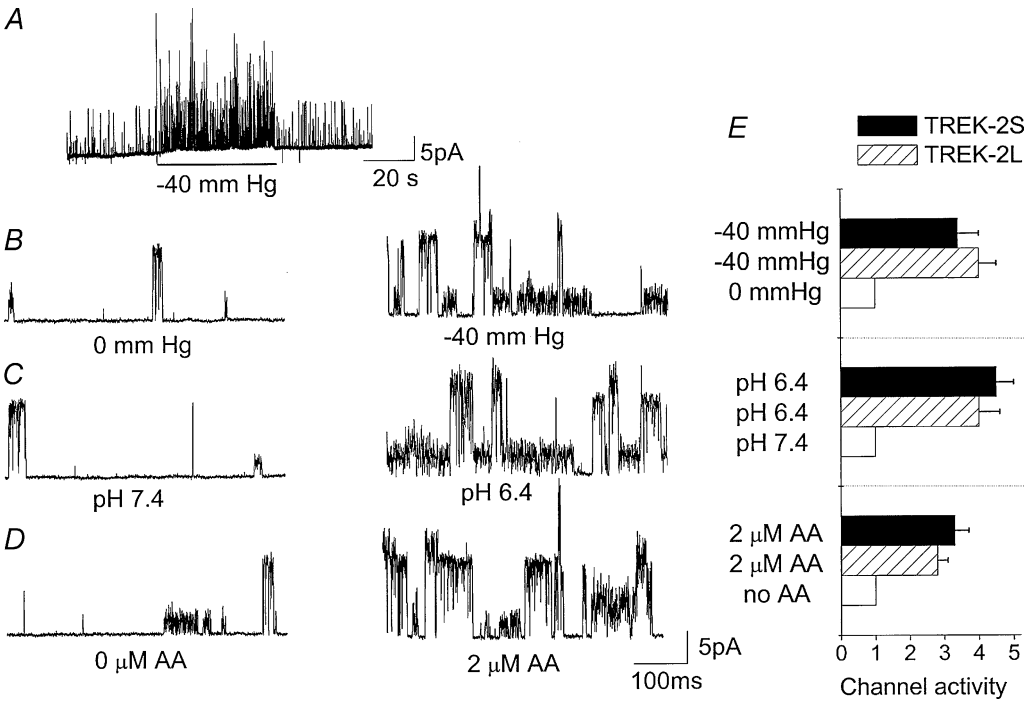

Figure 3. Activation of both TREK-2S and TREK-2L by membrane stretch, intracellular acidification and arachidonic acid in inside-out patches
Cell membrane potential was held at +40 mV. *A* and *B*, negative pressure (−40 mmHg) was applied to the patch membrane containing TREK-2. *C*, the pH of the bath solution was reduced from 7.4 to 6.4. *D*, arachidonic acid (2 μM) was applied to the bath solution. *E*, summary graphs showing the effect of the three activators on the relative channel activity of TREK-2S and TREK-2L. Each bar is the mean ± S.D. of 4 experiments.

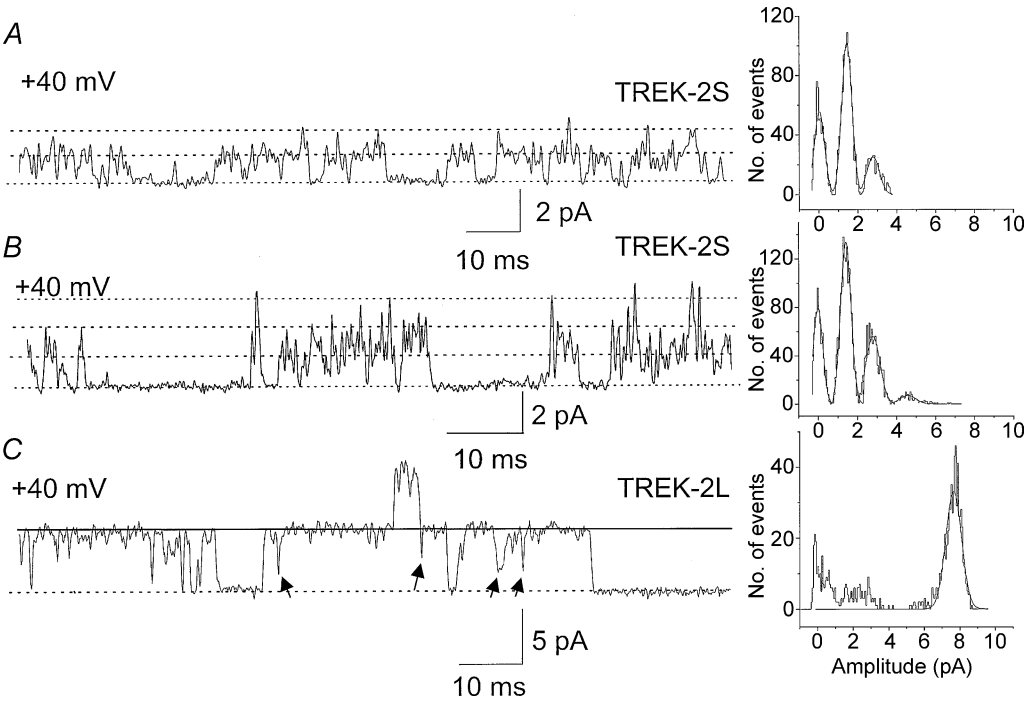

Figure 4. Different conductance levels of TREK-2S, and sub-conductance levels of TREK-2L
Cell membrane potential was held at +40 mV. *A*, this cell-attached patch contained the TREK-2S phenotype in which only two open levels were present, as indicated by dotted lines. Amplitude histogram is shown on the right. *B*, this patch contained the TREK-2S phenotype in which three open levels can be identified. *C*, a patch containing TREK-2L is shown. Arrows indicate the short-lived sub-conductance levels. Amplitude histogram showing the main peak is also shown

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et al. 2000; Ding & Sachs, 2002). If channel subunits act independently, the probability of k channels being in the open state simultaneously is given by the binomial distribution equation: $P(\mathrm{k}) = (N{:}\mathrm{k})\, P^{\mathrm{k}}(1-P)^{N-\mathrm{k}}$, where N is taken as 4 (total number of subunits), and P (0.3) was estimated from our current tracings. If subunits act independently, the ratio of experimentally obtained open probability (P_{obs}) and open probability calculated from binomial distribution (P_{bin}) should be 1.0 for each level. For TREK-2S, $P_{\mathrm{obs}}/P_{\mathrm{bin}}$ was 0.9 (0.38/0.41) for the first level, 1.4 (0.36/0.26) for the second level and 1.4 (0.09/0.076) for the third level. The deviation from unity indicates that subunits do not act independently, and that positive co-operativity is present.

TREK-2L phenotype shows short-lived sub-conductance levels

Figure 4*C* shows an expanded current tracing of TREK-2L recorded at +40 mV. When the channel current was analysed assuming that the peak current (continuous line in Fig. 4*C*) was due to opening of one channel, the amplitude histogram showed one main peak with a mean of ~8 pA, as expected. By visual inspection, two sub-levels could be identified for TREK-2L, as indicated by the arrows (Fig. 4*C*). The sublevels of TREK-2L were generally short-lived, typical of sub-levels of many ion channels. There were no indications that the fully open level arose because of summation of several channels.

In a few inside-out patches in which only TREK-2S was present, no spontaneous switching to the TREK-2L phenotype occurred during 30 min recordings ($n = 7$). Similarly, when only TREK-2L was observed in the patch on rare occasions, it never switched to the TREK-2S phenotype ($n = 4$). These results suggest that TREK-2S and TREK-2L are highly stable conformations of the same channel. Openings of both TREK-2S and TREK-2L, and summation of their open levels, were commonly observed, as if they were two separate channels.

Phosphorylation causes switching of open levels of TREK-2

Our experiments described in Fig. 3 show that various activators of TREK-2 simply increase the frequency of opening of TREK-2S and TREK-2L, and do not cause switching of phenotypes. We asked whether inhibition of TREK-2 by protein kinase A- and C-mediated phosphorylation is associated with switching of channel phenotypes (Lesage *et al.* 2000; Kang *et al.* 2006). Cells expressing TREK-2 were incubated with forskolin (10 μM) and isobutylmethylxanthine (IBMX; 0.1 mM) for ~60 min to activate PKA. Cell-attached patches from these cells showed mixed channel phenotypes. Close inspection of channel openings at expanded scales indicated that there were six conductance levels, as illustrated by lines drawn through open levels (Fig. 5*C* and *D*). To analyse the conductance levels of TREK-2 in more detail, amplitude histograms were obtained from recordings from cell-attached patches (held at +40 mV) obtained from untreated cells and cells treated with forskolin–IBMX. The number of open levels was set at six, based on our initial estimate of conducting levels. Patches containing mainly single openings of TREK-2S and TREK-2L phenotypes were used for analysis. Double openings of TREK-2S, and summed openings of TREK-2S and TREK-2L, were skipped during analysis. Patches that showed one each of TREK-2S and TREK-2L were commonly obtained using our transfection and recording protocols.

For TREK-2 recorded from control cells, amplitude histograms showed six peaks. The number of openings to the third and fourth conducting levels was relatively low, although it was not zero, indicating that some intermediate conductance levels were also present in control patches (Fig. 5*A*, right graph). The amplitudes of the first two and last two peaks were always high compared with those of the middle two peaks. Forskolin/IBMX treatment produced a shift in the distribution of six open levels such that openings to intermediate levels (level 3 and 4) were increased while openings to low and high levels were decreased (Fig. 5*B*). TREK-2 recorded from cells incubated with phorbol myristate acetate (PMA; 1 μM) for 30 min to activate PKC also showed increased intermediate conductance levels (Fig. 5*C*). Another intervention that should lead to phosphorylation of TREK-2 is inhibition of phosphatases. Cells were incubated with okadaic acid (1 μM, 2 h), a potent inhibitor of phosphatases PP1 and PP2A (Cohen *et al.* 1990), and TREK-2 recorded from cell-attached patches. Similar to forskolin–IBMX, okadaic acid treatment resulted in increased intermediate conducting levels and reduced low and high conducting levels.

Open probability (P_{o}) from each conductance level was determined by setting the amplitude limits for each level. Multiplying P_{o} and the mean single channel amplitude at +40 mV gives an estimate of the channel current at this depolarized potential (for example, $I = i_1 P_{\mathrm{o1}}$ for the first conductance level). The first three amplitude levels are contributed by TREK-2S and the last two are contributed by TREK-2L in the untreated, basal state. Channel current contributed by each conductance level is shown in Fig. 6. Channel current from all six levels were then added to obtain the total current in the patch ($I = i_1 N P_{\mathrm{o1}} + i_2 N P_{\mathrm{o2}} + \ldots + i_6 N P_{\mathrm{o6}}$ where N is taken as 1). When the total currents (I) were determined during the same recording time periods (30 s), the results showed that forskolin–IBMX, PMA and okadaic acid reduced the current to 32 ± 4%, 37 ± 6% and 28 ± 7% of control, respectively. Therefore, these results suggest that

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phosphorylating agents not only reduce channel activity, but also shift the distribution of conductance levels of TREK-2.

Whether the intermediate levels of TREK-2 are also sensitive to physical and chemical stimuli was tested in cells treated with okadaic acid. In inside-out patches containing TREK-2 and showing multiple channel sub-levels, negative pressure (−40 mmHg) or arachidonic acid (5 μM) was applied to the patch. Channel activities (NP_o) at different conducting levels before and after the two treatments were analysed for 10 s duration each, and plotted in Fig. 6*B*. Both arachidonic acid and application of negative pressure caused an increase in the activity of all sub-levels. These results show that all conformations of TREK-2 are sensitive to the two most potent activators of this channel.

Effect of mutations at putative phosphorylation sites on TREK-2 phenotypes

In TREKs, two serine residues close to the proximal carboxyl terminus are targets of phosphorylation by protein kinases A and C (Patel *et al.* 1998; Murbartian *et al.* 2005; Kang *et al.* 2006). To provide further evidence for the role of phosphorylation in the regulation of TREK-2 gating modes, serine residues were mutated to alanine or aspartate. Mutation of serine to alanine should keep the

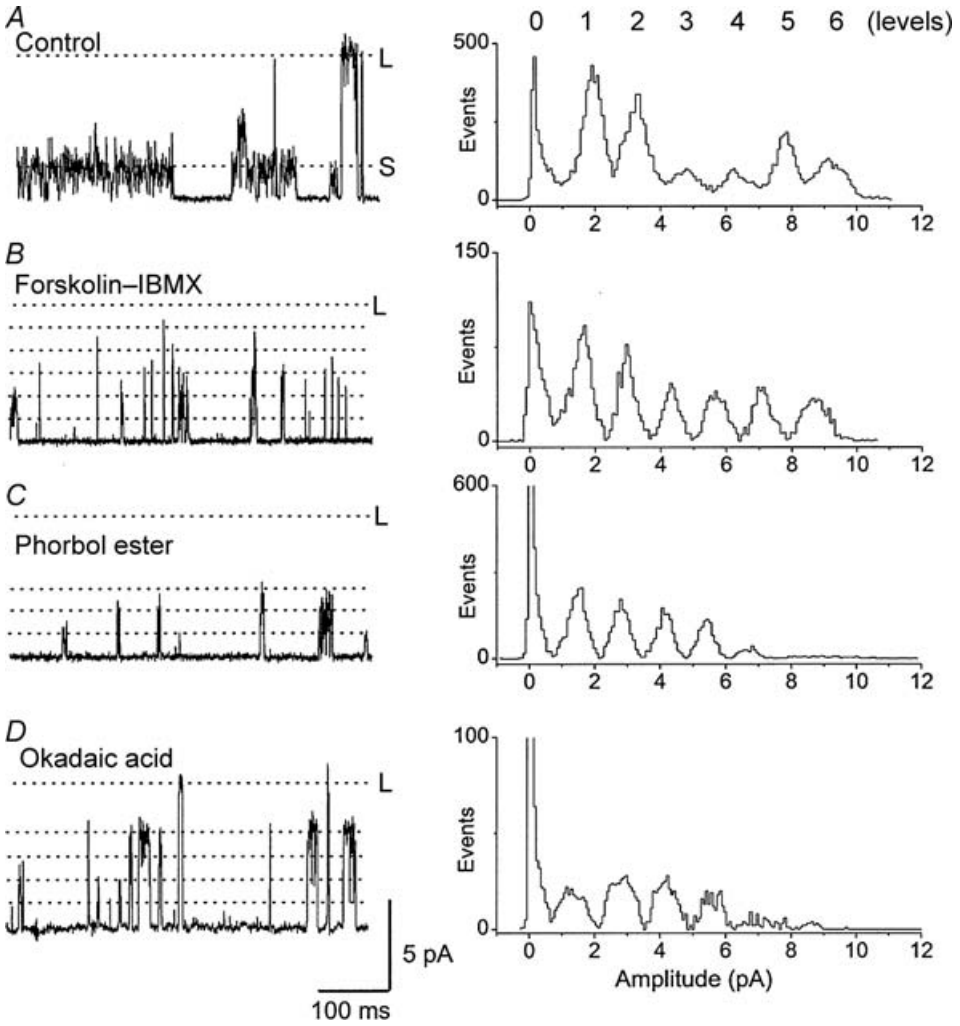

Figure 5. Changes in the distribution of TREK-2 conductance levels by phosphorylating agents
A, channel recording from cell-attached patches of Cos-7 cells expressing TREK-2. Cell membrane potential was held at +40 mV. Dotted lines indicate the conducting levels of TREK-2L and TREK-2S. On the right is shown an amplitude histogram obtained after setting six open levels. *B*, Cos-7 cells expressing TREK-2 were incubated with 10 μM forskolin and 0.1 mM IBMX for ∼60 min in culture medium, and single channels were recorded from cell-attached patches. Dotted lines were drawn assuming six open levels. *C*, cells were incubated with phorbol myristate acetate (PMA; 1 μM) for 30 min, and channels recorded from cell-attached patches. *D*, cells were incubated with 1 μM okadaic acid for ∼2 h, and channels recorded from cell-attached patches.

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channel in the dephosphorylated state, whereas mutation to aspartate should mimic the phosphorylated state. In cells expressing the TREK-2 mutant in which serine was mutated to alanine (S326A and S359A), TREK-2S and TREK-2L were the two main phenotypes, and intermediate levels (3rd and 4th) were very low (Fig. 7). When channels were recorded from the S326D and S359D mutants, intermediate levels were increased, similar to that observed after treatment with okadaic acid. In the S326A/S359A double mutant, the gating mode of TREK-2 was similar to those of single S326A and S359A mutants, showing mainly TREK-2S and TREK-2L phenotypes. The frequency of opening of channels to the intermediate conductance levels (3rd and 4th) increased relative to those of TREK-2S and TREK-2L in the S326D/S359D double mutant. Thus, phosphorylation at only a single site was sufficient to produce a shift in the gating mode of TREK-2. The relative channel activities of those mutants that mimic the phosphorylated state are much lower than those that mimic the dephosphorylated state (Fig. 7*B*). These results provide additional evidence that phosphorylation at the serine residues alter the relative distribution of TREK-2 conducting sub-levels.

In the wild type TREK-2 and TREK-2 (S/A) mutants, the ratio of channel activity at +40 to that at −40 mV ranged from 1.52 to 1.97 (Fig. 7*C*), showing a small voltage dependence in symmetrical 150 mM KCl. In TREK-2 (S/D) mutants, this ratio increased to an average value of 3.66, showing a small increase in voltage dependence. In Mg^{2+}-free solution, the voltage dependence was not significantly different among TREK-2 wild type S/A and S/D mutants (Fig. 7*C*). The ratio of channel activity of TREK-2S and TREK-2L at +40 mV to that at −40 mV was also determined in Mg^{2+}-free solution. They were 2.10 ± 0.94 (TREK-2S/wild type), 3.22 ± 1.06 (TREK-2L/wild type), 2.45 ± 0.76 (TREK-2S/S326A), 2.78 ± 0.72 (TREK-2L/S326A), 2.32 ± 0.76 (TREK-2S/S359A) and 2.84 ± 0.86 (TREK-2L/S359A). None of the values observed with the mutants were significantly different from those obtained with the TREK-2 wild type ($n = 5$ each, $P > 0.05$). Thus, the mutations that affect the phosphorylated state of TREK-2 have little or no effect on the intrinsic voltage dependence of the channel. These effects of phosphorylation on the voltage dependence of TREK-2 are smaller than those reported for TREK-1 (Bockenhauer *et al.* 2001), as previously argued by Maingret *et al.* (2002).

Expression of TREK-2S and TREK-2L in cerebellar granule neurons

Cerebellar granule neurons express a high level of TREK-2 mRNA. To test whether different gating modes of TREK-2 observed in Cos-7 cells are also found in native cells expressing TREK-2, cerebellar granule (CG) neurons were cultured from neonatal rat. In an earlier study in CG neurons, we have identified and characterized TREK-2S but not TREK-2L (Han *et al.* 2002). In inside-out patches formed on the cell body, a K^+ channel with kinetic properties nearly identical to TREK-2S (mean conductance, 54 ± 5 pS; $n = 3$) was observed frequently (Fig. 8*A*). Patches that contained both TREK-2S- and TREK-2L-like channels (205 ± 12 pS; +40 mV; $n = 3$) were observed in 4 of 18 patches and one of them is shown in Fig. 8*A*, along with its amplitude histogram. Both TREK-2S- and TREK-2L-like channels in CG neurons were easily activated by low pH (pH 6.4) and membrane stretch (−40 mmHg), further confirming their identity as TREK-2 (Fig. 8*B*). Addition of 10 μM Ca^{2+} to the cytoplasmic side of inside-out patches did not affect channel activity of TREK-2S and TREK-2L ($P > 0.05$; $n = 4$), indicating that TREK-2L was not the large-conductance Ca^{2+}-activated channel. In three patches, arachidonic acid (10 μM) also activated both TREK-2S and TREK-2L-like channels in CG neurons. For reasons yet unknown, most patches (~80%) showed only TREK-2S in these neurons, suggesting that additional factors are involved in the expression of different TREK-2

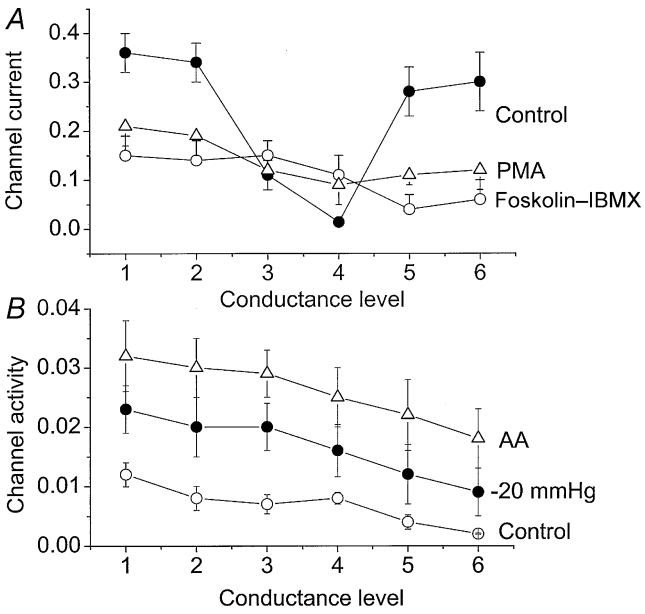

Figure 6. Relative channel current of TREK-2 in control and drug-treated cells

A, open probability of the channel at each conductance level was determined, and then multiplied by the mean single channel amplitude at +40 mV to estimate the channel current. Phosphorylating agents caused a shift in the distribution of channel openings from low and high conductance levels to more intermediate levels. Each point is the mean ± S.D. of 6 determinations. *B*, Cos-7 cells expressing TREK-2 were treated with 1 μM okadaic acid (control), and channel activity determined for each conducting level before and after application of two activators, as indicated in the graph. Both activators caused significant increases in the channel activity at all conducting levels. Each point is the mean ± S.D. of 4 determinations.

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phenotypes in the native system. Since CG neurons express several other types of K^+ channels (such as TASK-1, TASK-3, type 4 channel, etc.) with conductance and open times close to those of okadaic acid-treated TREK-2, it was not possible to clearly determine whether phosphorylation also caused a shift in the conductance levels of TREK-2 in these neurons.

Discussion

The main finding of this study is that TREK-2 exhibits multiple conductance levels and phenotypes under different conditions. Under basal, unstimulated conditions, the TREK-2 gene forms small- (TREK-2S) and large-conductance (TREK-2L) channel phenotypes when transfected into mammalian cell lines and oocytes. Under phosphorylating conditions, TREK-2 not only is reduced in channel activity, but also undergoes major switching of the conductance levels from low and high to intermediate levels. The occurrence of two stable gating modes of TREK-2 under basal conditions is an unusual phenomenon among ion channels, as most ion channels show two main states, open and closed, corresponding to two stable conformations of the ion permeation pathway, with only one conducting phenotype. The unusual behaviour of TREK-2 is difficult to explain at the mechanistic level, but may be related to different conformations of the conducting pore region of the channel protein that is somehow modulated by the phosphorylated state of the channel. Switching of conductance levels by phosphorylation is an interesting way of modulating cell excitability.

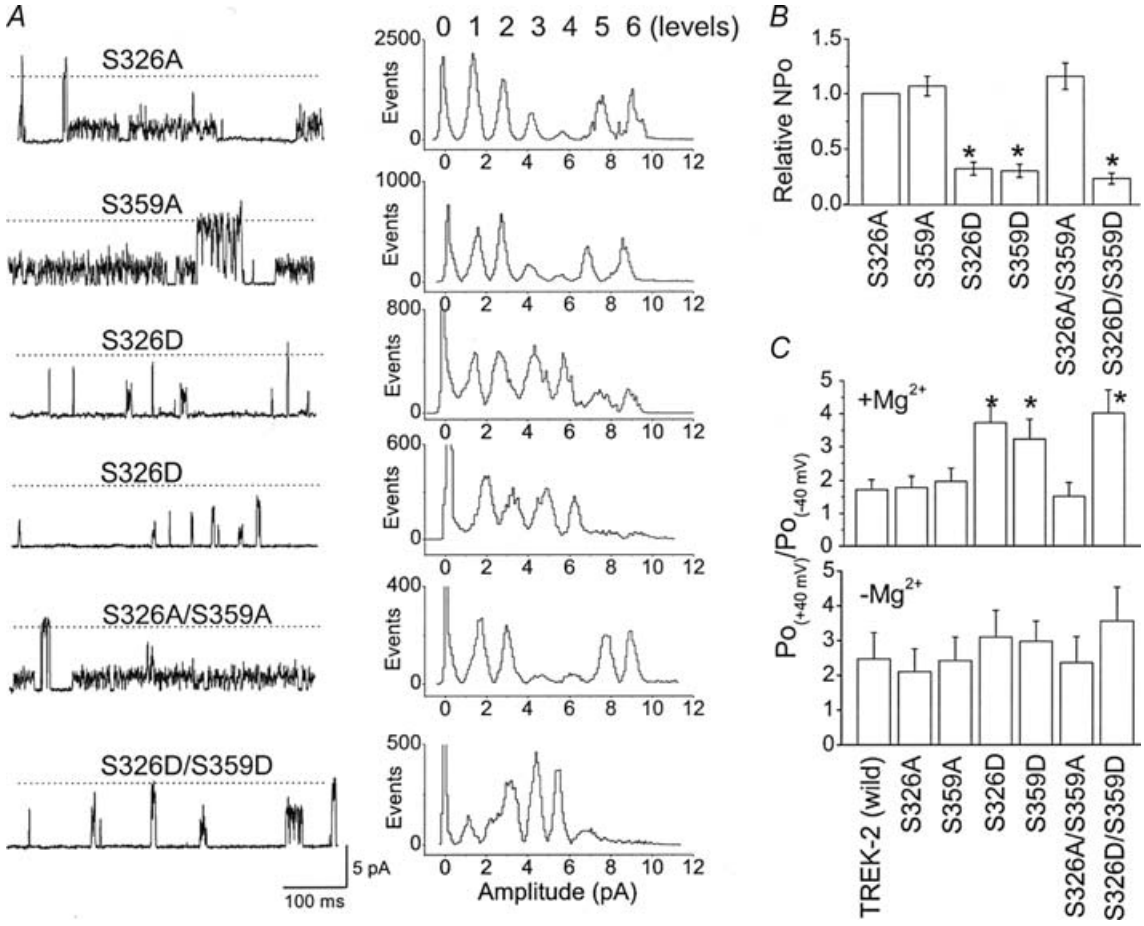

Figure 7. Single channel phenotypes and amplitude histograms of TREK-2 mutants
A, channels were recorded from cell-attached patches expressing one of the TREK-2 mutants indicated. Cell membrane potential was +40 mV. Amplitude histograms were obtained from each current tracing, and one example typical of those from five determinations (for each mutant) is shown in the right panel. Analysis was done after setting six open levels as in Fig. 6. *B*, relative channel activities of mutants are plotted. Each bar is the mean ± S.D. of 5 determinations. *Significant difference from the S326A mutant. *C*, channel activities at +40 mV and −40 mV were determined in Mg^{2+}-containing and Mg^{2+}-free solutions, and the ratios (NP_o at +40 mV/NP_o at −40 mV) are plotted for TREK-2 wild type and mutants. Each bar is the mean ± S.D. of 5 determinations. *Significant difference from TREK-2 wild type ($P < 0.05$).

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Appearance of two TREK-2 phenotypes

In earlier studies, only TREK-2S was thought to be the K^+ channel encoded by the TREK-2 gene (Bang *et al.* 2000; Gnatenco *et al.* 2002; Han *et al.* 2002; Kang *et al.* 2004). We now show that the TREK-2 gene encodes both TREK-2S and TREK-2L. What would cause the channel to adopt a conformation that exhibits either TREK-2S or TREK-2L? Injection of TREK-2 cRNA into oocytes also produced TREK-2S or TREK-2L phenotypes, eliminating the possibility that two different TREK-2 mRNA species was produced or RNA editing occurred. None of the physical and chemical stimuli tested so far (pressure, arachidonic acid, intracellular acidosis, glutathione, NADH, hydrogen peroxide, ATP, GTP, PIP_2) was able to cause interconversion between the two phenotypes. TREK-1 expressed in HEK293 cells has also been reported to display both low and high conductance channels (Xian Tao *et al.* 2006). Thus, the expression of two or more phenotypes with different conducting levels may be a property of the TREK subfamily of K_{2P} channels, but the molecular mechanisms that produce different phenotypes are yet to be uncovered.

Phosphorylation of TREK-2 reveals six sub-conducting levels

Studies on phosphorylation provided the initial evidence suggesting that TREK-2S and TREK-2L represent different conductance levels of the same channel. Intermediate conductance levels appeared much more frequently when cells were treated with phosphorylating agents. Increased intermediate levels were also observed in the S326D and S359D mutants that mimic the phosphorylated state. The effects of phosphorylation on TREK-2 activity and gating modes suggest that agonists that act via PKA and PKC should also produce similar effects. We have not been able to directly test this on membrane patches because of the difficulty of recording clear outward single channel currents under physiological conditions that is necessary for the agonists to work. Nevertheless, previous whole-cell studies showing that agonists inhibit TREK-2 via phosphorylation, together with the results of our studies using various phosphorylating agents, strongly suggest that receptor agonists would have the same effect on TREK-2 gating. Thus, one mechanism by which receptor agonists increase cell excitability is to switch the conformations that provide highly active channel phenotypes (TREK-2S and TREK-2L) to those with low activity phenotypes (intermediate conductance levels). Depending on the cell type and the basal phosphorylating capacity of the cell, TREK-2 is likely to exhibit different phenotypes. This should be kept in mind when studying TREK-2 in the native system. In unstimulated cerebellar granule neurons, we recorded mainly TREK-2S, but a few patches also showed TREK-2L together with TREK-2S. The reason for the low rate of functional expression of TREK-2L in the native system is not known, but may involve additional cellular factors that regulate channel conformation.

Sub-conductance levels that probably represent partially open conformations of the channel can be observed for many channels, but they are usually very brief and thus are relatively unstable and transient conformations. What could account for the different gating modes of TREK-2? Ion channel gating involves complex conformational

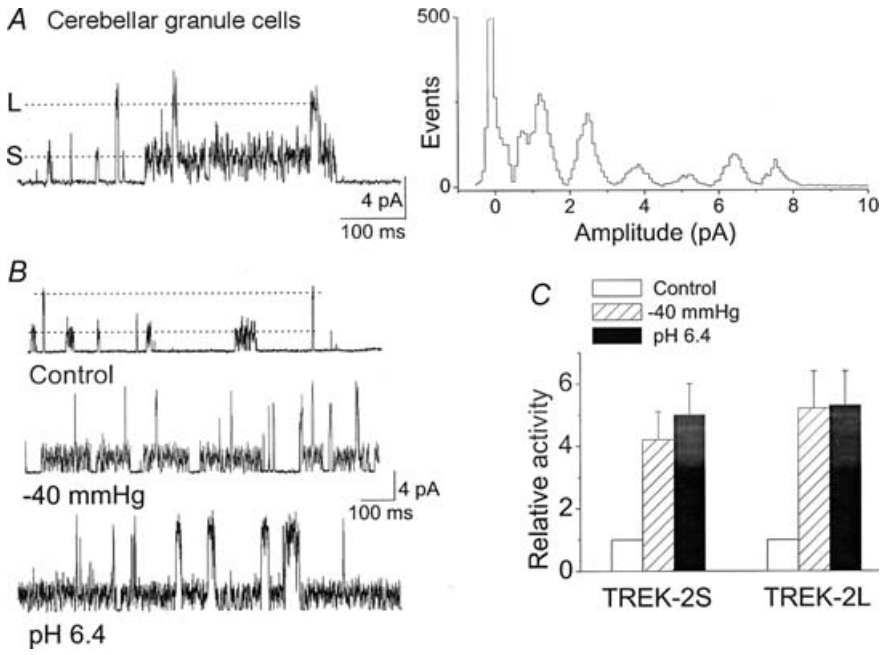

Figure 8. TREK-2S and TREK-2L expressed in cerebellar granule neurons
A, single channels were recorded from inside-out patches formed on cerebellar granule neurons held at +40 mV. A typical amplitude histogram is shown on the right. *B*, single channels were recorded from inside-out patch before and after application of negative pressure and low pH solution. *C*, a graph showing the effect of negative pressure and low pH on TREK-2 activity. Each bar represents the mean ± S.D. of 5 determinations. Both low and high conductance K^+ channels with phenotypes similar to TREK-2S and TREK-2L were analysed.

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changes in many regions of the channel molecule. The function of the pore regions that include the selectivity filter of the channel is probably ultimately responsible for generating the 'phenotype' of a channel as well as its sub-conductance levels (Chapman & VanDongen, 2005) This idea is supported by the recent work of Perozo and colleagues showing that different types of gating can occur as a result of microscopic rearrangements at the selectivity filter (Cordero-Morales *et al.* 2006). Evidence has also been presented to suggest that the sub-conductance levels may represent distinct heteromeric pore conformations in which some but not all of the subunits are in a conformation that supports ion permeation (Chapman & VanDongen, 2005). Therefore, one could imagine a model in which two different pore conformations that are relatively stable give rise to the two TREK-2 phenotypes. Within each phenotype, additional heteromeric conformations could account for the rapid open level transitions observed with TREK-2S and TREK-2L. For TREK-2S, the opening of sub-levels does not fit the binomial distribution expected from subunits acting independently, and may be explained better by a positive co-operative interaction among subunits. Future studies using pore and chimeric mutants should provide more information on how the two stable phenotypes arise. Recently, an alternative translation initiation was reported to produce a truncated $K_{2P}2.1$ with increased Na^+ permeability (Thomas *et al.* 2007). Therefore, the possibility that TREK-2S and TREK-2L are products of the same TREK-2 mRNA from alternative translation initiation sites will need to be tested.

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