Two-pore-domain potassium channels (K$_{2p}$) are the major determinants of the background potassium conductance. They play a crucial role in setting the resting membrane potential and regulating cellular excitability. These channels form homodimers; however, a few examples of heterodimerization have also been reported. The K$_{2p}$ channel subunits TRESK and TREK-2 provide the predominant background potassium current in the primary sensory neurons of the dorsal root and trigeminal ganglia. A recent study has shown that a TRESK mutation causes migraine because it leads to the formation of a dominant negative truncated TRESK fragment. Surprisingly, this fragment can also interact with TREK-2. In this study, we determined the biophysical and pharmacological properties of the TRESK/TREK-2 heterodimer using a covalently linked TRESK/TREK-2 tandem construct to ensure the assembly of the different subunits. The tandem channel has an intermediate single-channel conductance compared with the TRESK and TREK-2 homodimers. Similar conductance values were recorded when TRESK and TREK-2 were coexpressed, demonstrating that the two subunits can spontaneously form functional heterodimers. The TRESK component confers calcineurin-dependent regulation to the heterodimer and gives rise to a pharmacological profile similar to the TRESK homodimer, whereas the presence of the TREK-2 subunit renders the channel sensitive to the selective TREK-2 activator T2A3. In trigeminal primary sensory neurons, we detected single-channel activity with biophysical and pharmacological properties similar to the TRESK/TREK-2 tandem, indicating that WT TRESK and TREK-2 subunits coassemble to form functional heterodimeric channels also in native cells.

Two-pore-domain K$^+$ channels (K$_{2p}$) are the molecular correlates of the leak potassium current. They play a major role in determining the resting membrane potential and regulating cellular excitability (1, 2). In mammals, the K$_{2p}$ family consists of 15 different members, which are grouped into six subfamilies based on sequence similarity. The TREK (TWIK-related K$^+$ channel) subfamily consists of three members: TREK-1, TREK-2, and TRAAK (TWIK-related arachidonic acid activated K$^+$ channel). TREK-2 is a thermo- and mechanosensitive channel regulated by various intracellular signaling pathways. TRESK and TREK-2 are the main determinants of the background potassium current in the neurons of the dorsal root ganglion (9, 10). TRESK and TREK-2 contribute to the regulation of nociceptive responses and are considered as potential therapeutic targets for the treatment of pain (11).

Heteromerization of pore-forming subunits is a widespread mechanism leading to increased functional diversity in both the voltage-gated (K$_v$) and inwardly rectifying (K$_i$) K$^+$ channel subfamilies and often generates channels with unique properties (12–20). K$_{2p}$ channels dimerize to form functional channels. Well-established examples of heterodimerization involve subunits from the same subfamily (16–20). Although some examples of heterodimerization between subunits from different K$_{2p}$ subfamilies have also been reported (21, 22), these results await independent confirmation.

A frameshift mutation (F139WFsX24), which leads to the expression of a nonfunctional TRESK subunit and causes neuronal hyperexcitability, has been identified in a family suffering from migraine (23, 24). Since then, another missense TRESK mutation (C110R) has been identified (25). Unexpectedly, the C110R mutant TRESK did not influence neuronal excitability, despite having a dominant negative effect on TRESK in heterologous expression systems (26). Recently, it was found that TRESK can interact with TREK channels in heterologous expression systems (27). Unexpectedly, the frameshift mutation creates an alternative translation initiation site in the TRESK mRNA, which results in the translation of two nonfunctional TRESK fragments (MT1 and MT2). The pathogenic TRESK frameshift mutation has a dominant negative effect on both TRESK and TREK channels. The N-terminal fragment (MT1) exerts a dominant negative effect on TRESK, whereas the MT2 fragment inhibits TREK channels. The expression of the C-terminal fragment (MT2) was sufficient to induce hyperexcitability.
in trigeminal ganglion neurons and mechanical allodynia in rat models of migraine (27).

In this paper, we used whole-cell and single-channel electrophysiology to investigate the heterodimerization of WT and different constitutively active or silenced mutant versions of TRESK and TREK-2 subunits. We report that the TRESK/TREK-2 heterodimer has distinctive biophysical, pharmacological, and regulatory properties compared with TRESK and TREK-2 homodimers. Based on these unique features of the heterodimeric channel, we show that WT TRESK and TREK-2 subunits also coassemble to form functional heterodimers in trigeminal ganglion neurons.

Results

**T2A3 is a selective activator of TREK-2**

Mouse TREK-2 channel was expressed in *Xenopus* oocytes. Background K\(^+\) currents were estimated at $-100 \text{ mV}$ in 80 mM K\(^+\) after subtraction of the nonspecific leak current measured in 2 mM K\(^+\). TREK-2 current was activated by T2A3 in a concentration-dependent manner (Fig. 1A). Application of 10 \muM T2A3 activated TREK-2 current 15.7 ± 7.2-fold ($n = 7$ oocytes), whereas 30 \muM T2A3 increased the current 43.2 ± 25.8-fold ($n = 7$ oocytes). The concentration–response relationship is summarized in Fig. 1B.

T2A3 was reported to have no effect on the currents of the closely related TREK-1 channel (28). To determine the specificity of the effect of T2A3 on TREK-2 in the whole K\(_{\text{r}}\)p subfamily, we applied 10 and 30 \muM T2A3 on several mouse K\(_{\text{r}}\)p channels: TASK-1, TASK-2, TASK-3, TREK-1, TREK-2, TRAAK, TASK-1, TASK-2, TASK-3, TALK-1, THIK-1, and TRESK. Among all examined K\(_{\text{r}}\)p channels, the stimulatory effect of T2A3 was the most robust in the case of TREK-2. TASK-2 was also stimulated by the compound (1.6 ± 0.4-fold at 10 \muM and 3.1 ± 1.8-fold at 30 \muM, $n = 5$ oocytes); however, the degree of stimulation was significantly smaller than that of TREK-2. All other K\(_{\text{r}}\)p channels were unaffected by the compound. For the summary of the results of our selectivity screening, see Fig. 1 (C and D).

**A pore mutant TRESK subunit exerts dominant negative effect on both TRESK and TREK-2 current**

A recent study (27) has shown that the migraine-causing TRESK frameshift mutation leads to the generation of nonfunctional TRESK fragments, which have dominant negative effects on both TRESK and TREK current. To examine the interaction between TREK-2 and the full-length TRESK subunit, we generated a loss-of-function, dominant negative (DN) TRESK subunit by mutating the second glycine residue of the first pore domain to glutamate (TRESK G131E, “TRESK\(^{\text{DN}}\)”). Similar mutations were previously used to generate dominant negative subunits in other K\(_{\text{r}}\)p channels (17, 18, 29). In the present study, we first verified that TRESK\(^{\text{DN}}\) exerted a dominant negative effect when coexpressed with WT TRESK. In these experiments, the target channel (WT TRESK) and the dominant negative mutant were expressed at a ratio of 1:3 while keeping the total amount of DNA fixed at 2 \muG. Coexpression of TRESK and the control pCD8 plasmid resulted in a large conductance (44.7 ± 23.1 pA/pF at 0 mV, $n = 9$ cells; Fig. 2A). The coexpression of TRESK and TRESK\(^{\text{DN}}\)-pCD8 efficiently decreased the current (11.5 ± 5.6 pA/pF at 0 mV, $n = 9$ cells; Fig. 2A). The effect of TRESK\(^{\text{DN}}\)-pCD8 expression on TRESK current is summarized in Fig. 2 (B and C). The decrease in current was statistically significant ($p = 0.003$).

In the next set of experiments, the effect of TRESK\(^{\text{DN}}\) on the TREK-2 current was examined. When TREK-2 and the control pCD8 plasmid were expressed, a large whole-cell conductance was recorded (44.6 ± 30.4 pA/pF at 0 mV, $n = 9$ cells; Fig. 2D). Coexpression of TREK-2 and TRESK\(^{\text{DN}}\)-pCD8 lead to greatly decreased current (5.2 ± 2.4 pA/pF at 0 mV, $n = 8$ cells; Fig. 2D) compared with the control plasmid. The current recorded in these cells is similar to the endogenous currents recorded in HEK293T cells that have been transfected with control pCD8 plasmid (5.9 ± 1.7 pA/pF at 0 mV, $n = 10$ cells; Fig. S1). The effect of TRESK\(^{\text{DN}}\)-pCD8 expression on TREK-2 current is summarized in Fig. 2 (E and F). The decrease in current was statistically significant ($p = 8 \times 10^{-5}$), showing that TRESK\(^{\text{DN}}\) has a dominant negative effect on TREK-2.

The inhibitory effect of TRESK\(^{\text{DN}}\) on both TRESK and TREK-2 current could be a consequence of decreased expression of the WT channel due to increased competition for the translational and processing machinery of the cell. To rule out this possibility, we examined the effect of TRESK\(^{\text{DN}}\) coexpression on the current amplitude of a distantly related potassium channel, K\(_{\text{r}}\)1.3. When K\(_{\text{r}}\)1.3 and the control pCD8 plasmids were expressed, a large voltage-sensitive conductance was recorded (62.4 ± 36.6 pA/pF at 0 mV, $n = 9$ cells; Fig. 2G). Coexpression of K\(_{\text{r}}\)1.3 and TRESK\(^{\text{DN}}\)-pCD8 lead to whole-cell currents with a similar amplitude (69.1 ± 37.8 pA/pF at 0 mV, $n = 9$ cells; Fig. 2G). TRESK\(^{\text{DN}}\)-pCD8 expression had no effect on K\(_{\text{r}}\)1.3 current ($p = 0.86$), as summarized in Fig. 2 (H and I).

Previous work from both our and other laboratories has shown that assembly of K\(_{\text{r}}\)p channel heterodimers can be forced by using a “tandem” construct in which the two different subunits are covalently linked (16–18). To study the properties of the TRESK/TREK-2 heterodimer, we assembled a TRESK/TREK tandem channel by joining the N terminus of the mouse TREK-2 subunit to the C terminus of the TRESK channel using a 2-amino acid linker. When the tandem construct was expressed in HEK293T cells, we were able to record a potassium conductance (62.6 ± 41.2 pA/pF at 0 mV, $n = 9$ cells; Fig. 2J). When we introduced the G131E mutation into the tandem channel, the current was negligible (7.5 ± 3.3 pA/pF at 0 mV, $n = 9$ cells; Fig. 2J), similar to the endogenous currents recorded in HEK293T cells that have been only transfected with pCD8 plasmid without channel DNA (5.9 ± 1.7 pA/pF at 0 mV, $n = 10$ cells; Fig. S1). The decrease in current caused by the mutation was statistically significant ($p = 4 \times 10^{-5}$). The current of the tandem constructs is summarized in Fig. 2 (K and L). Similar results were observed when these constructs were expressed in *Xenopus* oocytes (data not shown). Accordingly, the tandem construct can be considered as an appropriate model for heterodimerization of TRESK and TREK-2 and was used to determine the pharmacological and biophysical properties of the TRESK/TREK-2 heterodimer.
The TRESK/TREK-2 tandem channel has a unique pharmacological profile

The TRESK channel is activated via dephosphorylation by the calcium-dependent protein phosphatase, calcineurin. When the oocytes expressing the TRESK/TREK-2 tandem construct were stimulated with ionomycin (0.5 mM), the activation of the K\(^+\) current was observed (8.7 ± 3.2-fold, n = 7 oocytes; Fig. 3A). The degree of activation by ionomycin was similar to previously reported values for TRESK and the TRESK/TREK-1 tandem channel (5, 27). We have recently reported that an analog (A2764) of the selective TRESK activator cloxyquin inhibits TRESK (9). The application of A2764 (100 μM) to oocytes expressing the tandem channel inhibited the TRESK/TREK-2 current (24 ± 11% inhibition, n = 6 oocytes; Fig. 3B). When the current was activated by ionomycin before the application of A2764, the inhibition was significantly more pronounced (70 ± 7%, n = 6 oocytes; Fig. 3C; p = 8 × 10\(^{-6}\)). The effect of T2A3 on the TRESK/TREK-2 tandem was examined as described in Fig. 1. Application of T2A3 (10 μM) increased the current of the tandem channel 8.2 ± 6.2-fold (n = 7 oocytes; Fig. 3D), whereas a higher concentration of T2A3 (30 μM) evoked an even more substantial, 47.1 ± 22.3-fold activation.

In the next set of experiments, we applied T2A3 to previously activated tandem channels. In the experiment shown on Fig. 3E, channels were first activated by ionomycin (9.8 ± 3.7-fold, n = 8 oocytes). Application of 10 μM T2A3 stimulated the current a further 2.6 ± 0.6-fold (n = 8), leading to a final activation of 25.6 ± 8.6-fold, compared with the basal current. In a separate set of experiments, oocytes expressing the tandem channel were stimulated with the selective TRESK activator cloxyquin (100 μM), resulting in a 4.8 ± 1.1-fold activation of the current (n = 6 oocytes), as seen on Fig. 3F. Subsequent application of T2A3 further increased the current (2.5 ± 0.6-fold, n = 6). The final activation compared with the unstimulated current was 12.1 ± 1.2-fold.

Phosphorylation state of TRESK channel influences the open probability of the channel

The calcium/calcineurin-dependent regulation of the TRESK channel has been well-characterized in whole-cell recordings...
Functional heterodimerization of TRESK and TREK-2

A  TRESK  TRESK\textsuperscript{DN}

B  Current (pA/pF) vs Voltage (mV)

C  \( \star \)

D  TREK-2  TREK-2\textsuperscript{DN}

E  Current (pA/pF) vs Voltage (mV)

F  \( \star \)

G  K\textsubscript{V}1.3  K\textsubscript{V}1.3\textsuperscript{DN}

H  Current (pA/pF) vs Voltage (mV)

I  \( \star \)

J  TRESK/TREK-2  TREK-2/TRESK\textsuperscript{DN}

K  Current (pA/pF) vs Voltage (mV)

L  \( \star \)
performed in *Xenopus* oocytes and mammalian cells (5, 30–32). To date, the effects of the phosphorylation-dependent regulation have not been examined at the single-channel level. TRESK channels were expressed in HEK293T cells, and the currents were recorded in symmetrical high-K⁺ solutions in excised inside-out patches at −60, 0, and +60 mV. To obtain channels in the dephosphorylated state, cells were treated with 0.5 μM ionomycin prior to patch excision (Fig. 4A). We observed the unique asymmetric single-channel gating of TRESK (5) (i.e. at depolarized potentials, the channel produces long square wave-like openings, whereas at hyperpolarized potentials, the current emerged in bursts of short openings). For ease of analysis, we determined the single-channel conductance and open probability (Pₒ) at +60 mV. The single-channel conductance of the channel was 15.1 ± 2.8 pS (n = 6 patches), which is similar to previously reported values. The open probability of the dephosphorylated channel was 0.24 ± 0.08 (n = 6 patches). When channels were maintained in the phosphorylated state before recording by overnight treatment with the calcineurin inhibitor cyclosporine A (1 μM), the channel activity was much lower (Fig. 4B). The single-channel conductance under these conditions was 13.8 ± 0.7 pS (n = 6 patches), which was not significantly different from the conductance of the ionomycin-pretreated channels (p = 0.27). In contrast, the open probability of the cyclosporine-pretreated channels was significantly lower (Pₒ = 0.008 ± 0.007, n = 6 patches) than the Pₒ of the ionomycin-pretreated group (p = 9 × 10⁻⁴). For the summary of our results, see Fig. 4 (C and D).

**Figure 2.** Dominant negative action of the pore mutant TRESK subunit on both TRESK and TREK-2 currents. Channels (mouse TRESK, mouse TREK-2, and human Kv1.3) were coexpressed with a nonfunctional TRESK subunit, TRESK-G131E (TRESKON-pCD8), or pCD8 at a ratio of 1:3 in HEK293T cells. The WT TRESK/TREK-2 tandem and TREK-2/TRESK-G131E tandem were coexpressed with pCD8. Currents were measured in the whole-cell configuration every 4 s at the end of 1-s voltage steps ranging from −100 to +50 mV. The holding potential was −80 mV. Currents were normalized to cell capacitance. A, D, and G, representative current traces from cells expressing TRESK (A), TREK-2 (D), or human Kv1.3 in combination with either pCD8 (left traces) or TRESKON-pCD8 (right). Zero current levels are marked with a dash on the left side of the recordings. B, E, and H, current–voltage relationship of the different channels with the coexpression of pCD8 (black circles) or TRESKON-pCD8 (white circles) is summarized as mean ± S.D. (error bars). The number of cells in each experimental group is shown in parentheses. C, F, and I, current density measured at 0 mV of cells coexpressing either pCD8 (black circles) or TRESKON-pCD8 (white circles) is plotted as a scatter plot. The average values for each group are plotted as a column. The difference between the two groups was statistically significant (Mann-Whitney test). J, representative current traces from cells expressing the WT TRESK/TREK-2 tandem (left) or the TREK-2/TRESK-G131E tandem (right). Zero current levels are marked with a dash on the left side of the recordings. K, current–voltage relationship of the WT TRESK/TREK-2 tandem (black circles) or the TRESK-2/TRESK-G131E tandem (white circles) is summarized as mean ± S.D. (error bars). The number of cells in each experimental group is shown in parentheses. L, current density measured at 0 mV of cells coexpressing either the WT TRESK/TREK-2 tandem (black circles) or the TREK-2/TRESK-G131E tandem (white circles) is plotted as a scatter plot. The average values for each group are plotted as a column. The difference between the two groups was statistically significant (Student’s t test).
Phosphorylation state of TRESK/TREK-2 channel influences the open probability of the channel

In two-electrode voltage-clamp experiments, we have shown that similarly to the TRESK homodimer, the TRESK/TREK-2 tandem channel can also be activated by ionomycin application. The calcineurin-dependent regulation of the TRESK/TREK-2 single channels were examined by a similar approach as used for the homodimeric TRESK channel. When HEK293T cells expressing the TRESK/TREK-2 tandem were pretreated with 0.5 mM ionomycin to facilitate channel dephosphorylation prior to patch excision, channels with high activity were observed in inside-out excised patches ($P_o = 0.142 \pm 0.096$ at $-60\text{ mV}$, $P_o = 0.146 \pm 0.063$ at $+60\text{ mV}$, $n = 5$ patches; Fig. 5A). In contrast, when the cells were treated with the calcineurin inhibitor cyclosporin A ($1\mu M$, overnight treatment), the channel activity was much lower ($P_o = 0.003 \pm 0.003$ at $-60\text{ mV}$ and $P_o = 0.003 \pm 0.001$ at $+60\text{ mV}$, $n = 6$ patches; Fig. 5B). The open probability of the dephosphorylated TRESK/TREK-2 tandem was significantly higher than that of the phosphorylated channel at both negative and positive potentials ($p = 0.031$ and $p = 0.007$ at $-60$ and $+60\text{ mV}$, respectively). The open probabilities were not influenced by the membrane potential. The data about the open probability of the tandem channel is summarized in Fig. 5C. The single-channel conductance of the channel was not affected significantly by either pretreatment (ionomycin: $-60\text{ mV}$, $47.3 \pm 18.1$ pS; $+60\text{ mV}$, $39.7 \pm 11.5$ pS; cyclosporin A: $-60\text{ mV}$, $32.2 \pm 9.0$ pS; $+60\text{ mV}$, $40.3 \pm 15.3$ pS). Furthermore, the single-channel conductance was not influenced by the membrane potential under either condition. The data for the single-channel conductance of the tandem channel are shown in Fig. 5D.

The mutations mimicking the different phosphorylation states influence the open probability of the TRESK channel

We have previously reported that mutations of the calcineurin target serine residues in the intracellular loop of TRESK may result in increased (S264A and S276A mutants) or decreased (S264E and S276E mutants) basal activity (5). However, the single-channel properties responsible for the difference of the whole-cell currents of these mutants mimicking the phosphorylated or dephosphorylated state have not yet been examined. We have expressed single- and double-point mutant TRESK channels (S276A; S264A,S276A; S276E; and S264E, S276E) in HEK293T cells and performed excised-patch re-

Figure 4. Phosphorylation state of TRESK channel regulates the open probability of the channel. Mouse TRESK channels were expressed in HEK293T cells. Experiments were done on excised inside-out patches. Currents were measured at $+60$, $0$, and $-60\text{ mV}$ in symmetrical $140\text{ mM} \text{KCl}$ solutions. The current level corresponding to the closed state of the channel is marked with a dash on the left side of the recordings. A, representative recording of a dephosphorylated TRESK channel. To obtain channels in the dephosphorylated state, HEK293T cells expressing mouse TRESK were pretreated with $0.5 \mu M$ ionomycin ($\text{Iono}$) before patch excision. B, representative recording of a phosphorylated TRESK channel. To obtain channels in the phosphorylated state, HEK293T cells expressing mouse TRESK were pretreated overnight with $1 \mu M$ cyclosporine A ($\text{CsA}$). C, open probability of dephosphorylated ($\text{Iono}$) and phosphorylated ($\text{CsA}$) TRESK channels determined at $+60\text{ mV}$ is displayed as a scatter plot. The average values for each group are plotted as a column. The difference between the two groups was statistically significant (Student’s t test). D, single-channel conductance of dephosphorylated ($\text{Iono}$) and phosphorylated ($\text{CsA}$) TRESK channels determined at $+60\text{ mV}$ is displayed as a scatter plot. The average values for each group are plotted as a column. The difference between the two groups was not statistically significant (Student’s t test).
cordings in the inside-out configuration (for representative recordings, see Fig. 6 (A–D)). Single-channel conductance and open probabilities were determined at +60 mV. Mimicking the dephosphorylated state of TRESK by the substitution of the main target residue of calcineurin, serine 276, with alanine (S276A) produced a channel with high open probability ($P_o = 0.440 \pm 0.012$, $n = 5$ patches). Mutation of the other main calcineurin target residue, Ser-264, to alanine did not increase the open probability further (S264A,S276A: $P_o = 0.37 \pm 0.09$, $n = 6$ patches). The replacement of serine 276 residue with glutamate (S276E, mimicking the phosphorylated state of the channel) led to a channel with low basal activity ($P_o = 0.05 \pm 0.04$, $n = 6$ patches). The additional mutation of residue Ser-264 to glutamate (S264E,S276E mutant) decreased further the open probability ($P_o = 0.005 \pm 0.002$, $n = 6$ patches). In contrast to the drastic changes in open probability, the single-channel conductance has not been significantly altered by the different mutations (S264A, S276A:12.9 ± 2.3 pS; S276A: 11.5 ± 1.9 pS; S276E: 12.2 ± 2.3 pS; S264E,S276E: 12.8 ± 1.3 pS). For the summary of the results regarding the TRESK mutants, see Fig. 6 (E and F).

**The mutations mimicking the different phosphorylation states of TRESK influence the open probability of the TRESK/TREK-2 tandem channel**

To determine the role of the calcineurin target residues in the regulation of the tandem channel, we produced mutant TRESK/TREK-2 tandem constructs containing the serine point mutations, which were characterized in the previous set of experiments. The mutant tandem channels were expressed in HEK293T cells, and recordings were performed on excised patches in the inside-out configuration at −60 and +60 mV, as shown in Fig. 7 (A–D). The mutations had a similar effect on the open probability as in the case of the TRESK homodimer. The mutation of the Ser-276 residue had drastic effects on the open probability; the S276A mutation caused high activity ($P_o = 0.125 \pm 0.028$ at −60 mV and $P_o = 0.113 \pm 0.019$ at +60 mV, $n = 6$ patches), whereas the S276E mutation resulted in a channel with significantly lower activity ($P_o = 0.006 \pm 0.003$ at −60 mV and $P_o = 0.004 \pm 0.004$ at +60 mV, $n = 6$ patches). The mutation of residue Ser-264 had no further effect on the open probability (S264E,S276E: $P_o = 0.003 \pm 0.003$ at −60 mV and $P_o = 0.005 \pm 0.003$ at +60 mV, $n = 6$ patches; S264A,S276A, 12414 J. Biol. Chem. (2020) 295(35) 12408–12425
$P_o = 0.108 \pm 0.035$ at $-60$ mV and $P_o = 0.096 \pm 0.065$ at $+60$ mV, $n = 5$ patches). Statistical significance was determined with one-way ANOVA followed by Tukey’s post hoc test (the data are summarized in Fig. 7E). We observed that the single-channel conductance values of the glutamate mutants were smaller than that of the alanine mutants; however, this trend was not statistically significant. For the summary of the single-channel conductances of the TRESK/TREK-2 tandem mutant constructs, see Fig. 7F.

**TRESK, TREK-2, and the TRESK/TREK-2 tandem have distinct single-channel conductances**

If the single-channel conductance values of the TRESK and TREK-2 homodimers and the tandem channel are different, then this provides a feasible approach to confirm heterodimerization also when assembly is not ensured by covalent linkage of the two expressed subunits. We expressed TREK-2 channels in HEK293T cells and determined the single-channel conductance in excised inside-out membrane patches. In the previous experiments, we determined the single-channel conductances of both TRESK and the TRESK/TREK-2 tandem channel after ionomycin or cyclosporin A pretreatment. Because the pretreatments had no effect on the single-channel conductance (Figs. 4D and 5D), we pooled the data and compared the conductance values with TREK-2 (for representative recordings, see Fig. 8A). As expected on the basis of the literature (33), TREK-2 channels had large conductances ($117.9 \pm 36.1$ pS at $-60$ mV and $144.0 \pm 33.6$ pS at $+60$ mV, $n = 7$ patches), whereas TRESK has a relatively small
single-channel conductance (14.4 ± 6.1.9 pS at -60 mV and 14.4 ± 2.1 pS at +60 mV, n = 12 patches). The single-channel conductance of the TRESK/TREK-2 tandem channel is intermediate between the two homodimers (39.1 ± 15.3 pS at -60 mV and 40.0 ± 13.0 pS at +60 mV, n = 11 patches). The difference among the conductance of the tandem channel and the homodimers was statistically significant (Fig. 8B); therefore, in heterologous expression systems, the TRESK/TREK-2 heterodimer can be distinguished from both TRESK and TREK-2 based on its single-channel conductance.

**TRESK and TREK-2 form functional heterodimers in HEK293T cells**

To verify formation of TRESK/TREK-2 heterodimers on the single-channel level, TRESK and TREK-2 subunits were coexpressed in different ratios. Single-channel conductances were determined in excised inside-out membrane patches at -60 and +60 mV and plotted against the different DNA ratios. The results are summarized in Fig. 9A. As a comparison, we have also plotted the average ± S.D. single-channel conductance of the TRESK/TREK-2 tandem channel. The channels clearly formed three distinct groups based on their single-channel
conductance: TREK-2 homodimers with a large conductance (gray triangles, single-channel conductance >80 pS), small conductance channels (10–19 pS) corresponding to TRESK homodimers (white circles), and channels with an intermediate conductance value (23–70 pS) corresponding to the TRESK/TREK-2 heterodimers (black diamonds). Increasing the ratio of TRESK DNA while keeping the total amount of channel DNA constant (100 ng of channel DNA transfected/35 mm Petri dish) lead to an increase of heterodimer and TRESK homodimer formation (compare the column marked 3:1 with the columns marked 1:3 and 1:15 in Fig. 9A).

In a separate set of experiments to ensure an equimolar ratio of TRESK and TREK-2 subunits, we expressed the two subunits using a plasmid where the two subunits were linked with a self-cleaving peptide (TRESK-T2A-TREK2). Single-channel conductances were determined and plotted as in Fig. 9A and summarized in Fig. 9B. The same three distinct groups were observed as in the case when the channel subunits were expressed using separate plasmids. Accordingly, TRESK/TREK-2 heterodimer formation was confirmed on the single-channel level using two separate approaches for coexpression of TRESK and TREK-2.

A2764 and T2A3 modulate TRESK/TREK-2 activity in excised patches

Our previous two-electrode voltage-clamp experiments show that the TRESK/TREK-2 tandem channel has a unique pharmacological sensitivity compared with the parent subunits. To examine the effect of our channel modulators on the TRESK/TREK-2 heterodimer, we expressed the TRESK/TREK-2 tandem channel in HEK293T cells and applied A2764...
and T2A3 to excised inside-out membrane patches. As seen on the representative recording in Fig. 10A, application of A2764 (100 μM) to excised membrane patches (channels were preactivated by 0.5 μM ionomycin applied before patch excision) led to a reversible decrease in channel activity (87.3 ± 4.3% reduction, n = 6; Fig. 10B). The decrease in channel activity was a consequence of a decrease in open probability, because the unitary current of the channel was unaffected by the drug. In good agreement with our oocyte data, application of T2A3 (10 μM) led to a reversible increase in channel activity as seen on Fig. 10C (649.0 ± 306.8% compared with control, n = 6; Fig. 10D). Therefore, the unique pharmacological properties of the heterodimer are also present at the single-channel level and can be used to identify the TRESK/TREK-2 heterodimer.

**TRESK and TREK-2 subunits assemble into heterodimeric channels in trigeminal ganglion neurons**

The high expression of TREK-2 and TRESK subunits in the primary somatosensory neurons of the dorsal root and trigeminal ganglia has been demonstrated previously by various methods (9, 10, 34). This led us to investigate whether WT TRESK and TREK-2 subunits form functional heterodimers also in primary neurons. Based on our two-electrode voltage-clamp data from *Xenopus* oocytes, T2A3 activates any K2P potassium channel that contains at least one TREK-2 subunit. The sensitivity of the same channel conductance of the TRESK-TREK2 tandem is plotted for comparison (as mean ± S.D. (error bars)). Conductance values corresponding to different channels are plotted as follows: TREK-2 homodimers are plotted with gray triangles, TRESK homodimers are marked with white circles, and TRESK/TREK-2 heterodimers are plotted as black diamonds. B, the TRESK-T2A-TREK2 construct was expressed in HEK293T cells. Single-channel conductances were determined and plotted as in A.
(determined by measuring the reversal potential of the currents) was detected in 19 of 29 membrane patches. The effects of T2A3 (10 μM) and A2764 (30 μM) on channel activity were measured. In 19 membrane patches, 29 distinct potassium channels were identified. 16 channels were found to be resistant to both T2A3 and A2764; seven channels were activated by T2A3 but were not inhibited by A2764; these channels correspond to TREK-2 homodimers (Fig. 11A). We found six channels that were sensitive to both T2A3 and A2764 (Fig. 11B). This unique pharmacological profile indicates that these potassium channels found in trigeminal ganglion neurons are formed by the heterodimeric assembly of TRESK and TREK-2 subunits. Thus, the heterodimeric assembly of the WT TRESK and TREK-2 subunits was confirmed in native cells. The sensitivity of the channels to T2A3 and A2764 is summarized as a scatter plot in Fig. 11C. In addition to their unique pharmacological profiles, the two channel populations could also be distinguished from each other based on single-channel conductance (for a summary, see Fig. 11D).

**Discussion**

TREK and TRESK channels are highly expressed in the primary sensory neurons of the dorsal root and trigeminal ganglia. They play an important role in regulating the excitability of neurons involved in thermo- and nociception. Accordingly, they have been implicated in the pathophysiology of nociceptive disorders and migraine (11, 35, 36).

TREK-2 and TRESK represent the majority of the background (K2P) potassium channels in the sensory neurons of the dorsal root and trigeminal ganglia (10). However, addressing their relative contribution to the standing outward potassium current of these cells (which is the major determinant of their excitability) has been impeded by the lack of pharmacological tools that specifically target the different channel types. In the absence of specific pharmacological agents, our knowledge was mainly based on single-channel conductance data (10), studies on TRESK- and TREK-deficient mice (37, 38), or mRNA expression profiles (34).

Recently, specific pharmacological tools for modifying TRESK activity have been identified: the TRESK activator cloxyquin and its inhibitory analog, A2764 (9, 39, 40). We have also published results about the identification of a novel TREK-2 activator in a high-throughput screen of >70,000 compounds; T2A3 substantially activates TREK-2 but not the closely related TREK-1 channel (28). In this study, we further examined the specificity of T2A3 among a broad group of mouse K2P channels (at least one channel was tested from each subfamily). We found that T2A3 is highly specific for TREK-2; only two

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**Figure 10. The TRESK/TREK-2 tandem channel is sensitive to both T2A3 and A2764.** The TRESK/TREK-2 tandem construct was expressed in HEK293T cells. Experiments were done on excised inside-out patches. Currents were measured at –60 mV in symmetrical 140 mM KCl solutions. The current levels corresponding to the closed state and the different open levels of the channels in the patch are marked with dashes on the left side of the recordings. Channel activity (NPo) was determined before, during, and after application of the drugs. A, representative recording showing that 100 μM A2764 inhibits TRESK/TREK-2 tandem channel activity in excised inside-out patches. B, channel activity after application of A2764 is displayed as a scatter plot, whereas the average values are plotted as a column. C, representative recording showing that 30 μM T2A3 increases TRESK/TREK-2 tandem channel activity in excised inside-out patches. D, channel activity after application of T2A3 is displayed as a scatter plot, whereas the average values are plotted as a column.
channels of the alkaline-activated subfamily (TASK-2 and TALK-1) were moderately influenced by the drug. However, these channels are not expressed in sensory neurons (34). Based on these results, we propose that T2A3 can be used to identify TREK-2 in native sensory neurons.

A migraine-associated stop-mutation of TRESK was recently shown to result in two channel fragments with a C-terminal fragment exerting a dominant negative effect on TREK-1 and TREK-2 (27). In this study, a tandem of TREK-1/TRESK was constructed and used for the verification of potential heterodimerization of the two different channel subunits. However, TREK-1/TRESK heterodimer assembly from individual subunits was not demonstrated (27).

In the present experiments, first we covalently linked TRESK and TREK-2 subunits to force heterodimerization. Our data indicated that the tandem construct only forms intramolecular heterodimer channels: introducing a dominant negative pore mutation into the TRESK component abolished the current entirely, excluding the potential intermolecular association of the functional TREK-2 subunits as homodimers. The tandem...
Functional heterodimerization of TRESK and TREK-2

The phosphorylation-dependent regulation of TRESK has been studied in great detail under whole-cell conditions in Xenopus oocytes and mammalian cells (5, 30, 32). However, there were no data available regarding the effects of the phosphorylation state on the single-channel properties of the TRESK channel. We found that in excised membrane patches, the phosphorylation state of the channel influences TRESK channel activity by modifying the open probability ($P_o$). Dephosphorylated TRESK had a high $P_o$, whereas $P_o$ was an order of magnitude lower if TRESK was phosphorylated. Similar differences in $P_o$ were observed when known calcineurin target residues were mutated to mimic different phosphorylation states. The single-channel conductance (14 pS) was not influenced by the phosphorylation state of the channel or the point mutations and was identical to previously reported values for TRESK channels under the conditions when their activity was not manipulated (5, 10).

To determine the effect of the calcineurin-dependent regulation on the single-channel properties of the heterodimer, we expressed the TRESK/TREK-2 tandem in HEK293T cells and performed single-channel recordings as in the case of the TRESK homodimer. Pharmacological manipulation of the phosphorylation state or mutation of the calcineurin target residues had similar effects on the open probability of the TRESK/TREK-2 tandem as they had on TRESK, while leaving unaffected the single-channel conductance, which was around 40 pS (significantly different from the TRESK homodimer). Accordingly, activation of channels containing at least one TRESK subunit via calcineurin-mediated dephosphorylation or point mutations was mediated by an increase in open probability.

The single-channel conductance of TRESK is 14 pS, and that of the TRESK/TREK-2 heterodimer is around 40 pS, whereas TREK-2 is characterized by a large single-channel conductance (120 pS). Taking advantage of the significantly different conductance values, we were able to distinguish the different channel types when TRESK and TREK-2 subunits were coexpressed in HEK293T cells. Indeed, three unique conductance levels were observed: small conductance channels corresponding to TRESK, large conductance channels (TREK-2 homodimers), and channels with an intermediate conductance level similar to the TRESK/TREK-2 tandem. The presence of this intermediate conductance entity proves that TRESK and TREK-2 subunits can spontaneously form a functional hybrid pore, which is in good agreement with our results obtained in whole-cell experiments using the dominant negative TRESK subunit.

The characteristic single-channel conductance of TRESK and TREK2 homodimers and their heterodimer allows clear distinction of the expressed channels when the presence of other $K_\text{Ca}$ channels can be excluded; however, in native cells, this is not the case. Therefore, we combined single-channel recordings with pharmacological tools. We tested the effect of T2A3 and A2764 on the single-channel activity of the TRESK/TREK-2 channel. Application of A2764 to the heterodimer, which was preactivated by ionomycin before patch excision, led to a decrease in open probability, while having no effect on the unitary current. The degree of $N_P$, reduction was similar to the inhibition of the ionomycin-activated TRESK/TREK-2 current in whole-cell recordings. Perfusion of the membrane patches with T2A3 increased open probability in a reversible manner as reported previously for TREK-2 (28). The increase of the open probability by T2A3 was comparable with the degree of TRESK/TREK-2 activation in whole-cell recordings.

In a recent study, overexpression of a nonfunctional truncated TRESK channel fragment led to neuronal hyperexcitability and mechanical allodynia by having a dominant negative effect on TREK-2 (27). Intrigued by these results, we investigated whether WT TRESK can assemble into a functional heterodimer with TREK-2 in trigeminal ganglion neurons. The effects of T2A3 and A2764 on channel activity were determined in inside-out membrane patches derived from freshly isolated trigeminal neurons. We found channels with large single-channel conductances that could be activated by T2A3 but were resistant to A2764. These channels correspond to TREK-2 homodimers. A subset of channels had intermediate conductances similar to the TRESK/TREK-2 tandem. These channels were...
activated by T2A3 (indicating the presence of a TREK-2 subunit in the dimer), whereas they were also efficiently inhibited by the TREK inhibitor A2764, indicating the contribution of TREK to the channel pore. In conclusion, the pharmacological and single-channel parameters of these channels correspond to the profile of the TREK/TREK-2 heterodimer. We did not find channels with properties similar to those of the TREK homodimer, which could be a consequence of significantly higher TREK-2 expression compared with TREK, in which case formation of TREK homodimers is unlikely compared with heterodimer formation.

The TREK/TREK-2 heterodimer has a significantly larger single-channel conductance than the TREK homodimer; however, it is also efficiently activated by calcineurin. The assembly of heterodimers instead of TREK homodimers leads to an increase in the background potassium conductance, resulting in decreased cellular excitability, without changing the sensitivity of the current to calcium-mobilizing agonists.

**Experimental procedures**

**Materials**

Chemicals of analytical grade were purchased from Sigma, Fluka (Milwaukee, WI, USA), or Merck (Whitehouse Station, NJ, USA). Enzymes and kits for molecular biology applications were purchased from Ambion (Austin, TX, USA), Thermo Scientific (Waltham, MA, USA), New England Biolabs (Beverly, MA, USA), and Stratagene (La Jolla, CA, USA). Ionomycin (calcium salt) was purchased from Enzo Life Sciences Inc. (Farmington, NY, USA), dissolved in DMSO as a 5 mM stock solution, and stored at −20°C. Cloxyquin was purchased from Sigma, dissolved in ethanol as a 100 mM stock solution, and stored at −20°C. Synthesis and characterization of A2764 was described previously (9). A2764 was dissolved in ethanol as a 100 mM stock solution and stored at −20°C. The TREK-2 activator T2A3 (C$_{14}$H$_{12}$ClNO$_2$; 2-[(4-chloro-3-methylphenyl)amino] benzoic acid) was dissolved in DMSO as a 30 mM stock solution and stored at −20°C (28).

**Molecular biology**

The cloning of mouse TASK-1/2/3, TREK-2, TALK-1, THIK-1, and TREK-1, and TREK-2 has been described previously (5, 41). Generation of the different TREK mutants has also been described in previous papers (5, 42). Introduction of point mutations into the coding sequence of the different channels was done via site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmids coding mouse TREK-1 and TRAAK were provided by Prof. M. Lzdunski and Dr. F. Lesage. The plasmid coding human Kv1.3 was a gift from Prof. G. Panyi. For the concatenation of the TREK and TREK-2 subunits (tandem constructs), the coding sequence of TREK (without the stop codon) and that of TREK-2 (without the start codon) were amplified from mTREK-pEXO and mTREK-2-pEXO plasmids by PCR, respectively.

The TAA stop codon of the TREK subunit and the ATG start codon of the TREK-2 subunit were replaced with a unique Munl restriction enzyme site. This introduced a two-amino acid (glutamate and leucine) linker between the two concatenated subunits after final ligation into the pXEN vector. TREK-2/TRESK tandems were assembled using a similar strategy. Tandem channels containing mutant TREK subunits were produced by using the same strategy; however, the WT template in the PCR was replaced with the DNA of the appropriate mutant subunit.

For the expression in mammalian cells, TREK-2 was subcloned into pcDNA3.1 vector and TREK channels (WT or mutant) were subcloned into pRES-CD8 vector as described previously (16, 42). TREK/TREK-2 tandem constructs (containing WT or mutant TREK subunit) were subcloned into pRES-CD8 vector. For equimolar expression of TREK and TREK-2 subunits, the two subunits were linked with a self-cleaving viral peptide, T2A, which was previously shown to ensure equimolar expression of the linked proteins (43, 44). All constructs were verified by automated sequencing.

**cRNA synthesis**

For expression in Xenopus oocytes, plasmids coding different constructs were linearized and used as a template for *in vitro* cRNA synthesis performed with the mMESSAGE mMACHINE T7 *in vitro* transcription kit (Ambion, Austin, TX). The structural integrity of the RNA was checked on denaturing agarose gels, and the quantity of RNA was determined by spectrophotometry.

**Cell culture, transient transfection**

Cell culture dishes were purchased from Greiner Bio-One GmbH (Kremsmuenster, Austria). HEK293T cells were obtained from ATCC (Manassas, VA, USA). Cells were seeded at a density of 20,000–100,000 cells/35-mm dish 24–48 h prior to transfection in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (DMEM/FBS). Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) in serum-free DMEM according to the manufacturer’s instructions. DMEM and FBS were purchased from Lonza (Basel, Switzerland). For excised-patch experiments, cells were transfected with 10–100 ng of channel DNA (depending on channel type) and 900–990 ng of DNA coding CD8 (leading to a total of 1 μg of DNA per transfection) per 35-mm dish. For whole-cell experiments, cells were transfected with 0.5 μg of channel DNA and 1.5 μg of DNA coding CD8 or mutant TREK (leading to a total of 2 μg of DNA/transfection) per 35-mm dish. Cells were used for experiments 24 h after transfection. Transfected cells were identified using anti-CD8 Dynabeads (Thermo Fisher Scientific).

**Animals, isolation, and microinjection of Xenopus laevis oocytes and isolation of trigeminal ganglion neurons**

*Xenopus laevis* oocytes were prepared as described previously (19). For the expression of the different channels, the oocytes were injected with 57 pg to 4 ng of cRNA (depending on the channel type) 1 day after defolliculation. Injection was performed with a Nanoliter Injector (World Precision Instruments, Saratosa, FL, USA). *Xenopus laevis* frogs were housed in 50-liter tanks with continuous filtering and water circulation. Room temperature was 19°C. Frogs were anesthetized with 0.1% tricaine solution and killed by decerebration and pithing. Adult female WT mice (2–3 months in age) were used for the
patch-clamp experiments in this study. The animals were maintained on a 12-h light/dark cycle with free access to food and water in a specific pathogen–free animal facility. Mice were killed humanely by CO₂ exposure. Trigeminal ganglia were dissected from the cranium and collected in sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, pH adjusted to 7.4 with NaOH) at 4°C. Ganglia were incubated in PBS containing 2 mg/ml collagenase enzyme (type I; Worthington) for 30 min with gentle shaking at 37°C. Following the digestion, the tissue was gently triturated with a cut 1-ml pipette tip 10–15 times in DMEM/FBS and centrifuged at 200 × g for 5 min. Pelleted cells were resuspended in DMEM/FBS and plated on 35-mm cell culture dishes coated with poly-l-lysine. For further details regarding the isolation and culturing of the cells, see Ref. 45. All experimental procedures using animals were conducted in accordance with local state laws and institutional regulations. All animal experiments were approved by the Animal Care and Ethics Committee of Semmelweis University (approval ID: XIV-1-001/2154-4/2012).

Two-electrode voltage-clamp and patch-clamp experiments

Two-electrode voltage-clamp experiments were performed 1–3 days after the microinjection of cRNA into Xenopus oocytes, as described previously (19). For each channel type, the oocytes contributing to the n number (the exact n number is indicated in the text or in the figures) were derived from at least two, but usually three, separate frogs. The holding potential was 0 mV. Background potassium currents were measured at the end of 300-ms-long voltage steps to −100 mV applied every 4 s. Low-potassium recording solution contained 95.4 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 5 mM HEPES (pH 7.5, adjusted by NaOH). The high-potassium solution contained 80 mM K⁺ (78 mM NaCl of the low-potassium solution was replaced with KCl). Solutions were applied to the oocytes using a gravity-driven perfusion system. Experiments were performed at room temperature (21°C). Data were analyzed by pCLAMP 10 software.

For patch-clamp experiments, pipettes were pulled from thick-walled borosilicate glass (Standard Glass Capillaries, 4 inches, 1.2/0.68 OD/ID, Filament/Fire Polished (item no. 1B120F-4) from World Precision Instruments, Sarasota, FL, USA) by a P-87 puller (Sutter Instrument Co., Novato, CA, USA) and fire-polished. Pipettes were filled with pipette solution containing 140 mM KCl, 1 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.4 with KOH). The bath solution contained 140 mM KCl, 1 mM MgCl₂, and 10 mM HEPES (pH adjusted to 7.4 with KOH). T2A3 and A2764 were dissolved in the bath solution containing 140 mM KCl, 3 mM MgCl₂, 0.05 mM EGTA, 1 mM Na₂-ATP, 0.1 mM Na₂-GTP, and 10 mM HEPES.

Excised-patch recordings were low pass–filtered at 200 Hz with pCLAMP10 software before analysis. Currents were recorded at the membrane potentials of +60 mV, 0 mV, and −60 mV. Due to the filtering, events shorter than 1 ms were excluded from the analysis. The recordings were analyzed to obtain amplitude histograms and channel activity (NP_0, where N is the number of channels in the patch, and P_0 is the probability of a channel being open). Single-channel current amplitudes were determined from the amplitude histograms. NP_0 was determined from at least 30–60 s of current recording. In whole-cell experiments, currents were measured at the end of 1,000-ms voltage steps in a range from −100 to +50 mV. The holding potential was −80 mV. For analysis, current amplitudes were normalized to cell capacitance values.

Experimental design and statistical analyses

Results are expressed as mean ± S.D. Normality of the data was estimated using the Shapiro–Wilk test. If the Shapiro–Wilk test showed a significant difference between the examined groups, statistical significance was determined using the Mann–Whitney U test. Otherwise, statistical significance was determined with Student’s t test or Fisher’s ANOVA followed by Tukey’s post hoc test for multiple groups. Results were considered to be statistically significant at p < 0.05. Statistical calculations were done using Statistica (version 13.2, Dell Inc., Tulsa, OK, USA).

Data availability

All data generated in this study are found in the paper.

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Functional heterodimerization of TRESK and TREK-2

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Abbreviations—The abbreviations used are: K2P, two-pore-domain K+ channel(s); Kv, voltage-gated K+ channel(s); KvP, inwardly rectifying K+ channel(s); TREK, TWIK-related K+ channel; TRAAK, TWIK-related arachidonic acid–activated K+ channel; TRESK, TWIK-related spinal cord K+ channel; DN, dominant negative; pF, picofarad(s); pS, picosiemens(s); ANOVA, analysis of variance; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.

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