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Chemically Modified Derivatives of the Activator Compound Cloxyquin Exert Inhibitory Effect on TRESK (K_{2P}18.1) Background Potassium Channel [S]

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ABSTRACT

Cloxyquin has been reported as a specific activator of TRESK [TWIK-related spinal cord K^+ channel (also known as K_{2P}18.1)] background potassium channel. In this study, we have synthetized chemically modified analogs of cloxyquin and tested their effects on TRESK and other K_{2P} channels. The currents of murine K_{2P} channels, expressed heterologously in *Xenopus* oocytes, were measured by two-electrode voltage clamp, whereas the native background K^+ conductance of mouse dorsal root ganglion (DRG) neurons was examined by the whole-cell patch-clamp method. Some of the analogs retained the activator character of the parent compound, but, more interestingly, other derivatives inhibited mouse TRESK current. The inhibitor analogs (A2764 and A2793) exerted state-dependent effects. The degree of inhibition by 100 μM A2764 (77.8% ± 3.5%, n = 6) was larger in the activated state of TRESK (i.e., after calcineurin-dependent stimulation) than in the resting state of the channel (42.8% ± 11.5% inhibition, n = 7). The selectivity of the inhibitor compounds was tested on several K_{2P} channels. A2793 inhibited TWIK-related acid-sensitive K^+ channel (TASK)-1 (100 μM, 53.4% ± 13, 5%, n = 5), while A2764 was more selective for TRESK, it only moderately influenced TREK-1 and TWIK-related alkaline pH-activated K^+ channel. The effect of A2764 was also examined on the background K^+ currents of DRG neurons. A subpopulation of DRG neurons, prepared from wild-type animals, expressed background K^+ currents sensitive to A2764, whereas the inhibitor did not affect the currents in the DRG neurons of TRESK-deficient mice. Accordingly, A2764 may prove to be useful for the identification of TRESK current in native cells, and for the investigation of the role of the channel in nociception and migraine.

SIGNIFICANCE STATEMENT

TRESK background potassium channel is a potential pharmacological target in migraine and neuropathic pain. In this study, we have identified a selective inhibitor of TRESK, A2764. This compound can inhibit TRESK in native cells, leading to cell depolarization and increased excitability. This new inhibitor may be of use to probe the role of TRESK channel in migraine and nociception.

Introduction

Background (leak) potassium currents are responsible for the generation of the resting membrane potential and also play an important role in the adjustment of cellular excitability. The molecular correlates of the background potassium currents are the two-pore domain potassium channels (K_{2P}). To date, 15 K_{2P} subunits have been identified in mammalian cells. These channels are regulated by a large variety of physicochemical factors, intracellular signaling pathways, and drugs (for review, see Enyedi and Czirják (2010).

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ABBREVIATIONS: cRNA, complementary RNA; DRG, dorsal root ganglion; KO, knockout; K_{2P}, two-pore domain potassium channel; mTRESK, mouse tandem of pore domains in a weak inward rectifying K^+ channel–related spinal cord K^+ channel; RR, ruthenium red; TALEN, transcription activator–like effector nuclease; TALK, TWIK-related alkaline pH-activated K^+ channel; TASK, TWIK-related acid-sensitive K^+ channel; THIK, tandem pore domain halothane-inhibited K^+ channel; TREK, TWIK-related K^+ channel; TRAAK, TWIK-related arachidonic acid-stimulated K^+ channel; TRESK, TWIK-related spinal cord K^+ channel; TWIK, tandem of pore domains in a weak inward rectifying K^+ channel.

TRESK [TWIK-related spinal cord K^+ channel (also known as $K_{2P}18.1$)] was first cloned from human spinal cord (Sano et al., 2003). TRESK is activated by the cytoplasmic Ca^{2+} signal, via the calcium/calmodulin-dependent phosphatase calcineurin (Czirják et al., 2004). TRESK is abundantly expressed in the primary sensory neurons of the dorsal root (DRG) (Yoo et al., 2009) and trigeminal ganglion (Bautista et al., 2008; Yamamoto et al., 2009). Single-channel studies indicated that TRESK provides a major component of the background potassium current in rat DRG neurons (Kang and Kim, 2006). It is a plausible hypothesis that TRESK modulates the activity of sensory neurons and the absence of TRESK current results in an increased excitability, leading to disorders in nociception. A decrease of TRESK expression has been reported in a model of neuropathic pain (Tulleuda et al., 2011). In further support of this hypothesis, a mutation of TRESK leading to a nonfunctional channel fragment with dominant-negative effect has been recognized in a cohort of patients suffering from migraine (Lafrenière et al., 2010).

To understand the role of TRESK in physiological and pathophysiological nociception, appropriate pharmacological tools are necessary for selective modulation of the channel. However, pharmacological targeting of TRESK and other K_{2P} channels is a difficult task (for a recent review on the pharmacology of TRESK, see Enyedi and Czirják, 2015).

The antiamoebic drug cloxyquin was identified as an activator of TRESK (Wright et al., 2013). We have recently reported that cloxyquin is a selective activator of TRESK in the K_{2P} family. The stimulatory effect of cloxyquin was independent of the Ca^{2+}/calcineurin pathway, suggesting that cloxyquin is a direct activator of the channel. We have also shown that cloxyquin activates TRESK in isolated mouse DRG neurons (Lengyel et al., 2017). In the present study, we have produced 28 chemically modified analogs of cloxyquin and examined their effects on TRESK channels. Some of these derivatives activated TRESK with similar efficiency and potency as the parent compound. On the other hand, we obtained several compounds suitable for the selective inhibition of TRESK current in an activation state–dependent manner.

Materials and Methods

Plasmids, Complementary RNA Synthesis. The cloning of mouse TWIK-related acid-sensitive K^+ channel (TASK)-1/2/3, TWIK-related K^+ channel (TREK)-2, TWIK-related alkaline pH-activated K^+ channel (TALK)-1, tandem pore domain halothane-inhibited K^+ channel (THIK)-1, and TRESK has been described previously (Czirják et al., 2004; Czirják and Enyedi, 2006). The plasmids coding mouse TREK-1 and TRAAK channels were provided by Professor M. Lazdunski and Dr. F. Lesage (Institut de Pharmacologie Moleculaire et Cellulaire -CNRS -UPR 411, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France). The plasmids were linearized and used for in vitro complementary RNA (cRNA) synthesis using the mMESSAGE mMACHINE T7 in vitro transcription kit (Ambion, Austin, TX). The structural integrity of the RNA was checked on denaturing agarose gels.

Animal Husbandry, Preparation, and Microinjection of *Xenopus* Oocytes. Generation of a TRESK Knockout Mouse Line: Isolation of DRG Neurons. *Xenopus laevis* oocytes were prepared as previously described (Czirják and Enyedi, 2002). 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, Sarasota, FL). *X. laevis* frogs were housed in 50-l 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.

FVB/Ant (FVB.129P2-Pde6b$^+$Tyrc-ch/Ant) mice were obtained from the Institute of Experimental Medicine of the Hungarian Academy of Sciences (Budapest, Hungary). TRESK knockout (KO) animals were generated by the transcription activator–like effector nuclease (TALEN) technique using plasmids ordered from Addgene (TALE Toolbox Kit number 1000000019, deposited by Feng Zhang) (Broad Institute of MIT and Harvard). Mouse TRESK (mTRESK)-specific TALEN recognition sites were designed for the genomic sequence of the first exon (corresponding to the N-terminal intracellular domain of the channel) using the 5′-TN^{19} N$^{14-20}$ N^{19}A-3′ formula, with the following sequences: left TALEN recognition site, 5′tgaggagccacctgaggcca; right TALEN recognition site, 5′ccctgggaaggccagggga; and an 18 base pair (5′ggagatgctgtcctgagg) FokI nuclease dimerization and cutting sequence in between. mTRESK recognizing TALEN plasmids were assembled according to the protocol of Sanjana et al. (2012). TALEN mRNAs were produced using the Ambion mMESSAGE mMACHINE T7 in vitro transcription kit (Ambion). TALEN mRNAs were microinjected at a concentration of 20–20 ng/μl into the pronuclei of fertilized eggs of FVB/Ant mice. Pups were analyzed with Surveyor assay plus sequencing. In 12 mice, among the 61 born pups the TRESK (KCNK18) gene was changed, and a founder bearing a 33 base pair deletion and also a mutation introducing a stop codon was chosen to establish a colony.

Adult female wild-type and TRESK KO mice (2–3 months of age) were used for the patch-clamp experiments in this study. The animals were maintained on a 12-hour light/dark cycle with free access to food and water in a specific pathogen-free animal facility. Mice were killed humanely by CO_2 exposure (CO_2 was applied until death of the animals). DRGs were dissected from the thoracic and lumbar levels of the spinal cord and collected in sterile PBS (137 mM NaCl, 2.7 mM KCl, and 10 mM NaH_2PO_4, pH adjusted to 7.4 with NaOH) at 4°C. Ganglia were incubated in PBS containing 2 mg/ml collagenase enzyme (type I; Worthington Biochemical Corporation, Lakewood, NJ) for 30 minutes with gentle shaking at 37°C. For further details regarding the isolation and culturing of the cells, see Braun et al. (2015). All experimental procedures using animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted by the National Institutes of Health, local state laws, and institutional regulations. All animal experiments were approved by the Animal Care and Ethics Committee of Semmelweis University (approval ID: XIV-I-001/2154-4/2012).

Two-Electrode Voltage-Clamp and Patch-Clamp Measurements. Two-electrode voltage-clamp experiments were performed 1–3 days after the microinjection of cRNA into *Xenopus* oocytes, as previously described (Czirják et al., 2004). For each channel type, the oocytes contributing to the n number (the exact n number is indicated in the text or on 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-millisecond-long voltage steps to −100 mV applied every 4 seconds. The low-potassium recording solution contained the following (in mM): NaCl 95.4, KCl 2, $CaCl_2$ 1.8, and HEPES 5, at pH 7.5, adjusted by NaOH. The high-potassium solution contained 80 mM K^+ (78 mM NaCl in the low-potassium solution was replaced with KCl). For the measurement of TREK-1, TREK-2, and TRAAK currents, the high-potassium solution contained 40 mM K^+. 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 (Molecular Devices, Sunnyvale, CA).

Whole-cell patch-clamp experiments in the voltage-clamp configuration were performed as described previously (Lengyel et al., 2016). The resting membrane potential was recorded in the current-clamp mode with no current injection (I = 0 mode). The rheobase was determined by injecting depolarizing current (in 100 pA increments,

up to 1500 pA) for 1 second every 4 seconds. Isolated DRG neurons were used for experiments 1 to 2 days after isolation. For the current-clamp study of DRG neurons, only cells with a membrane potential between −45 and −70 mV were accepted, as described in previous studies (Petruska et al., 2000). Because the focus of this study was to examine the effects of our new cloxyquin analogs on the electrophysiological parameters of isolated DRG neurons, no other selection criteria were used. The cutoff frequency of the eight-pole Bessel filter was adjusted to 200 Hz, and data were acquired at 1 kHz. The pipette solution contained (in mM): 140 KCl, 3 $MgCl_2$, 0.05 EGTA, 1 Na_2-ATP, 0.1 Na_2-GTP, and 10 HEPES. The low-potassium solution contained (in mM): 140 NaCl, 3.6 KCl, 0.5 $MgCl_2$, 2 $CaCl_2$, 11 glucose, and 10 HEPES. The high-potassium solution contained 30 mM KCl (26.4 mM NaCl of the low-potassium solution was replaced with KCl). The pH of the bath solutions was adjusted to 7.4 with NaOH. Experiments were performed at room temperature (21°C). Data were analyzed using pCLAMP 10.7 software (Molecular Devices).

Data and Statistical Analysis. Results are expressed as the mean ± S.D. Normalized concentration-response curves were fitted with the following modified Hill equation: $\alpha \times c^n/[\ c^n + K_{1/2}{}^n\ ] + 1$, where c is the concentration, $K_{1/2}$ is the concentration at which half-maximal stimulation occurs, n is the Hill coefficient, and α is the effect of the treatment. The 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 the 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 $P < 0.05$. The difference in sample sizes among different groups is a consequence of the differing number of cells suitable for experimentation; sample sizes were not modified after obtaining initial results. The determined P values are descriptive and not the result of testing prespecified hypotheses. Curve fitting was performed using SigmaPlot10 (Build 10.0.0.54; SyStat Software, San Jose, CA). Statistical calculations were performed using Statistica (version 13.2; Dell, Round Rock, TX).

Materials. Chemicals of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (Milwaukee, WI), and Merck (Whitehouse Station, NJ). Enzymes and kits for molecular biology were purchased from Ambion, Thermo Fisher Scientific (Waltham, MA), New England Biolabs (Beverly, MA), or Stratagene (La Jolla, CA). Cloxyquin and ruthenium red (RR) were purchased from Sigma-Aldrich. Ionomycin (calcium salt) was purchased from Enzo Life Sciences, Inc. (Farmington, NY), dissolved in DMSO as a 5 mM stock solution, and stored at −20°C. Synthesis and chemical analysis of the new compounds described in this study are detailed in the Supplemental Material.

Results

A2764 Is a State-Dependent Inhibitor of Mouse TRESK Channel. We have synthesized 28 compounds structurally similar to cloxyquin and tested their effects on mTRESK channels expressed in *X. laevis* oocytes using the two-electrode voltage-clamp technique. The background K^+ current was estimated at −100 mV in an extracellular solution containing high K^+ (80 mM) after the subtraction of the small nonspecific leak current measured in 2 mM K^+. Seven derivatives had a stimulatory effect on mTRESK activity in a manner similar to that of cloxyquin, 10 compounds did not influence the K^+ current, while 11 analogs inhibited the channel. Based on our pilot screening of the compounds, the most promising inhibitors (A2764 and A2793) and an activator (A2797) of mTRESK were chosen for in-depth analysis. For the chemical structure of the novel cloxyquin analogs, see Fig. 1 (for the chemical structures of the new compounds and details of synthesis and chemical analysis for A2764 and A2793, see the chemical structures of compounds and the Supplemental Material).

The background K^+ current was inhibited by 42.8% ± 11.5% when A2764 (100 μM) was applied to the oocytes expressing mTRESK ($n = 7$ oocytes) (Fig. 2A). In addition to the inhibition of the basal current, we also examined the effect of A2764 on the current of activated mTRESK. Ionomycin was used to evoke the elevation of the cytoplasmic $[Ca^{2+}]$, which has been previously shown to activate the channel by dephosphorylation via the stimulation of the serine phosphatase calcineurin (Czirják et al., 2004). When mTRESK current was increased 7.1 ± 1.8-fold by the pretreatment with ionomycin (0.5 μM), the subsequent application of A2764 (100 μM) strongly inhibited the current (77.8% ± 3.5% inhibition, $n = 6$ oocytes) (Fig. 2B). The effect of A2764 on mTRESK activated by dephosphorylation was more pronounced than the inhibition of the basal current ($P < 0.05$, Mann-Whitney U test) (Fig. 2C). The concentration dependence of the channel inhibition was also shifted; while the IC_{50} value evoked by A2764 was 11.8 μM for the activated channel, the inhibitory potency of the drug was an order of magnitude lower for mTRESK in the resting state (Fig. 2D).

A2764 Is the Most Selective Modulator Among the Cloxyquin Analogs, Tested Among a Wide Range of K_{2P} Channels. To estimate the selectivity of the novel cloxyquin analogs within the K_{2P} subfamily, we tested them on several mouse K_{2P} channels (at least one member from each subfamily being functional under physiologic ionic conditions): TASK-1 ($K_{2P}3.1$), TASK-2 ($K_{2P}5.1$), TASK-3 ($K_{2P}9.1$), TALK-1 ($K_{2P}16.1$), TREK-1 ($K_{2P}2.1$), TREK-2 ($K_{2P}10.1$), TRAAK ($K_{2P}4.1$), and THIK-1 ($K_{2P}13.1$). The channels were expressed in *Xenopus* oocytes, and the effect of the compounds was measured by two-electrode voltage clamp (n = 5–7 oocytes per channel type). Among the three examined cloxyquin analogs, A2764 proved to

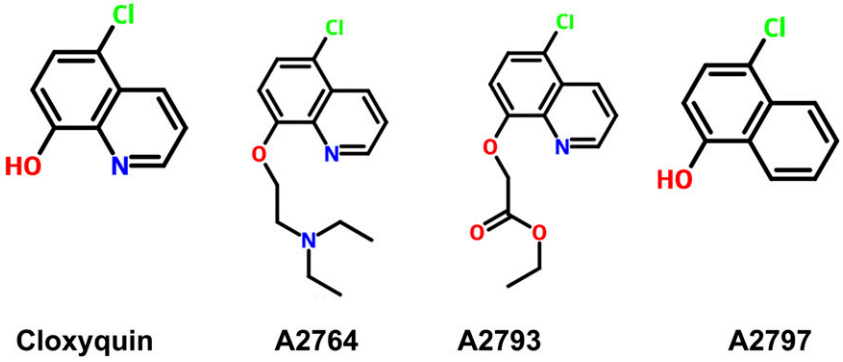

Fig. 1. Chemical structures of novel cloxyquin derivatives. Chemical structures of the new cloxyquin analogs reported in this study (A2764, A2793, and A2797). The structure of the parent compound is shown for comparison.

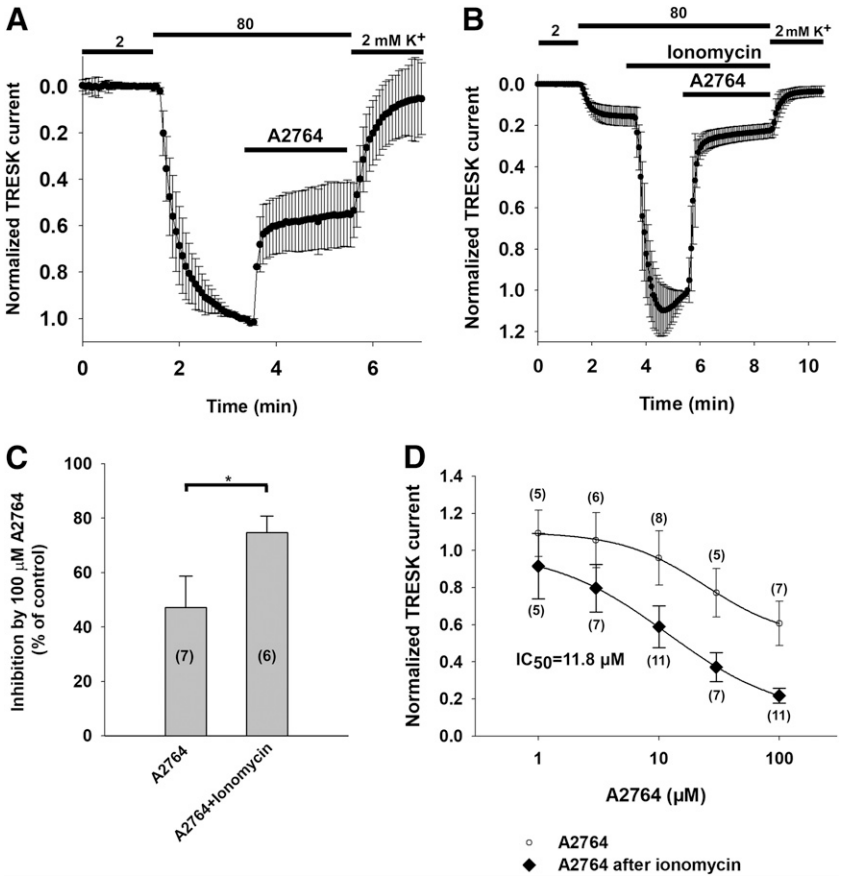

Fig. 2. A2764 is a state-dependent inhibitor of mTRESK. (A) Normalized currents of oocytes expressing mTRESK, measured by the two-electrode voltage-clamp technique. Currents were measured at the end of 300-millisecond voltage steps to −100 mV applied every 4 seconds from a holding potential of 0 mV. The extracellular K^+ concentration was increased from 2 to 80 mM, as indicated by the bars above the graph. Oocytes were challenged with A2764 (100 μM; see the horizontal bar). Currents were normalized to the value measured in 80 mM K^+ before the application of the drug. The current measured in 80 mM K^+ before the application of A2764 was 2.9 ± 1.5 μA. Data are plotted as the mean ± S.D. (B) The effect of A2764 on the activated mTRESK channels was determined under the same experimental conditions as in (A). The current measured in 80 mM K^+ before the application of ionomycin was 1.5 ± 0.8 μA. The TRESK current was activated by the application of 0.5 μM ionomycin (by 7.1 ± 1.8 fold), which was followed by the addition of A2764 (100 μM) to the bath solution (the timing of the changes of K^+ concentration and drug application are marked by horizontal bars above the graph). Currents were normalized to the value measured after ionomycin stimulation, before the application of A2764. (C) The data from (A and B) have been summarized as a column graph. Mean inhibitions of the resting and activated TRESK currents are plotted, and the number of oocytes in each group is given in parentheses. The difference in the degree of inhibition was statistically significant (*: $P < 0.05$, Mann-Whitney U test). (D) Concentration-response relationship of A2764 and mTRESK current. The inhibitory effects of different concentrations of A2764 (in a range from 1 to 100 μM) on basal (white circles) and activated (black diamonds) TRESK current are plotted. Each data point represents the average of 5–11 oocytes (the exact number is indicated in parentheses). The data points were fitted with a modified Hill equation (see *Materials and Methods*).

be the most selective agent; it inhibited the current of TRESK under resting conditions and in the activated state by 42.8% ± 11.5% (n = 7) and 77.8% ± 3.5% (n = 6), respectively (Fig. 3). TREK-1 and TALK-1 currents were reduced only by about 20%, while the other channels were even less affected. The difference in the degree of inhibition between TRESK (both in the resting and in the activated state) and all the examined K_{2P} channels was statistically significant.

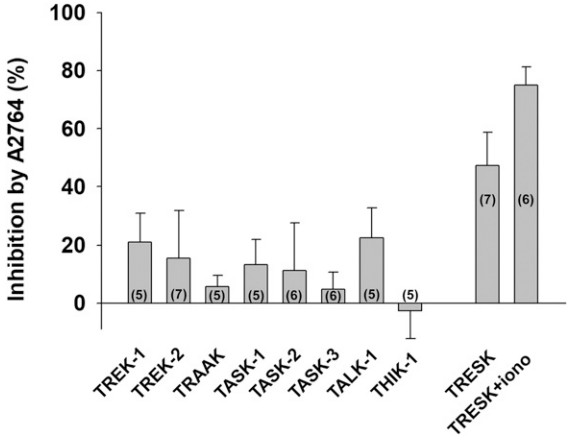

Fig. 3. A2764 is a selective inhibitor of mTRESK. Mouse K_{2P} channels were expressed in *Xenopus* oocytes. The effect of A2764 (100 μM) on the inward current in 80 mM extracellular K^+ (or 40 mM in the case of TREK-1, TREK-2, and TRAAK) was determined in 5–7 oocytes per channel type, as in Fig. 2A. As a comparison, the data points corresponding to the inhibition of basal or activated TRESK current from Fig. 2 are also plotted. The mean inhibition for each channel type is plotted as a column graph. The error bars represent the S.D. iono, ionomycin.

The efficiency of the other tested inhibitory analog (A2793) was higher than that of A2764. At 100 μM concentration, A2793 inhibited the unstimulated channel by 43.0% ± 8.9% (n = 5), whereas after ionomycin activation the reduction of the TRESK current was 85.5% ± 2.9% (n = 5) and was practically reduced to the nonactivated level (Fig. 4, A and B). The concentration-response relationship was also determined for both the activated and the basal mTRESK currents (Fig. 4D). The activated channel was inhibited with an IC_{50} of 6.8 μM, whereas the inhibitory potency of the compound was more than an order of magnitude smaller in the case of TRESK at basal activity. The specificity of A2793 showed a strikingly different pattern from A2764; it inhibited TASK-1 by 53.4% ± 13.5% (n = 5), while all the other channels were influenced by less than 20% (Fig. 4C). These properties may have advantage under experimental conditions when the presence of TASK-1 can be excluded. This was not the case in our studies on native DRG cells, accordingly, we used A2764 in our subsequent experiments.

The chemical structure of the activator analog (A2797) differed in the aromatic ring from cloxyquin. Its potency was not favorable compared with cloxyquin, and it activated TRESK to a similar degree (437.6% ± 139.8% of control, n = 9 oocytes) as the parent compound. Unlike cloxyquin, it activated other K_{2P} channels (TREK-2, TASK-2, and TRAAK) in addition to TRESK (details of these data are available in Supplemental Fig. 1).

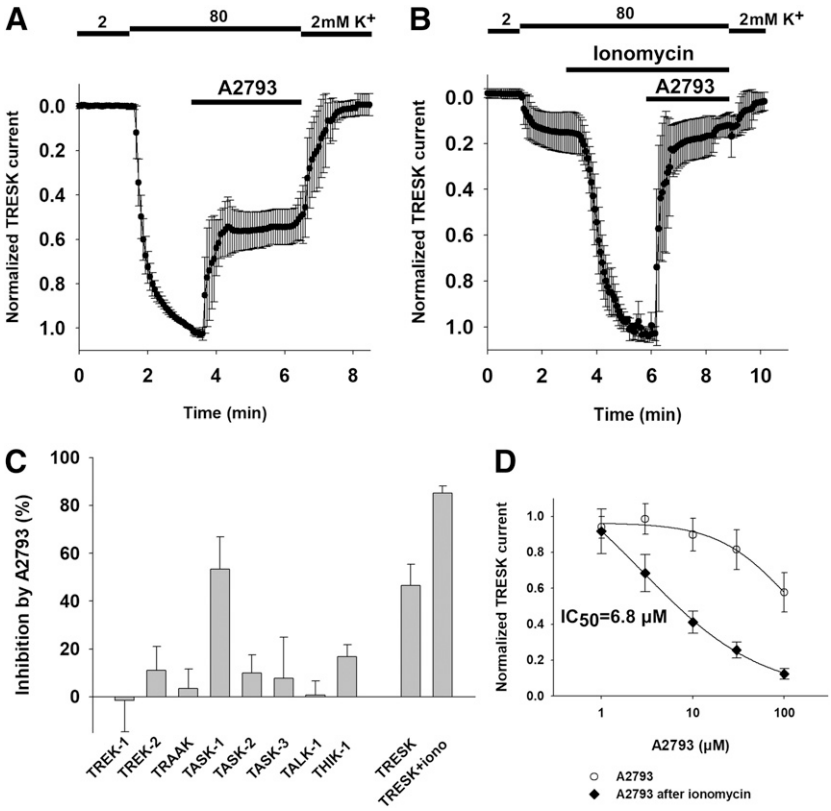

Fig. 4. A2793 is an inhibitor of mTRESK and mTASK-1. (A) mTRESK was expressed in *Xenopus* oocytes. The oocytes were challenged with A2793 (100 μM). The current measured in 80 mM K^+ before application of A2793 was 2.6 $\pm$ 0.6 μA. Inhibition by A2793 was determined and presented as described in Fig. 2A. (B) The effect of A2793 on the activated mTRESK current was determined under the same experimental conditions detailed in Fig. 2B. The current measured in 80 mM K^+ before the application of ionomycin was 1.7 $\pm$ 0.7 μA. Ionomycin activated the current by 6.7 $\pm$ 2.0 fold. (C) To determine the selectivity of A2793, mouse K_{2P} channels were expressed in *Xenopus* oocytes. The effect of A2793 on the inward current in 80 mM extracellular K^+ (or 40 mM in the case of TREK-1, TREK-2, and TRAAK) was determined in five oocytes per channel type, as in Fig. 3. As a comparison, the data points corresponding to the inhibition of TRESK current from (A and B) are also plotted. Data are plotted as the mean + S.D. (D) Concentration-response relationship of A2793 and mTRESK current. The inhibitory effect of different concentrations of A2793 (in a range from 1 to 100 μM) on basal (white circles) and activated (black diamonds) TRESK current are plotted. Each data point represents the average of five oocytes. The data points were fitted with a modified Hill equation (see *Materials and Methods*). iono, ionomycin.

A2764 Inhibits the Background K^+ Currents in DRG Neurons. To examine the TRESK current in native cells by the application of A2764, we isolated DRG neurons and performed whole-cell patch-clamp recordings. Since the functional state of TRESK in cell culture may vary depending on inhibitory kinases and the calcium homeostasis of the neuron, we uniformly induced calcineurin-dependent TRESK dephosphorylation by adding 0.5 μM ionomycin to our recording solutions before the application of A2764. In addition to the inhibitory effect of A2764 (100 μM), the sensitivity of the currents to RR (30 μM) was also examined. It is well established that TREK-2, TRAAK, and TASK-3 K_{2P} channels are inhibited by RR, and TREK-2 and TRAAK were previously reported to be expressed in large subpopulations of DRG neurons.

We applied the two inhibitors (A2764 and RR) into the bath solution successively. A recording from a representative, A2764-sensitive DRG neuron is shown in Fig. 5A. The sensitivity of the background current of the examined individual cells to A2764 and RR is presented on a scatter plot; 6 of the examined 10 cells showed substantial A2764 sensitivity (above 40%), whereas in the other four cells the degree of inhibition was below 20% (Fig. 5B). At the same time, we observed a significant negative correlation ($r = -0.9499$, $P < 10^{-4}$, Pearson's correlation coefficient) between the amplitudes of A2764-sensitive and RR-sensitive current components (Fig. 5B). The current remaining after the elimination of the A2764-sensitive component was substantially reduced by RR (the residual current was 16% $\pm$ 9.2%), suggesting that the A2764-sensitive (TRESK) and RR-inhibited (most probably TREK-2 and TRAAK) currents were mainly responsible for the background K^+ conductance in the majority of DRG neurons of the examined unselected population.

To validate the assumption that the A2764-sensitive current component of these native cells was indeed TRESK current, we also performed whole-cell patch-clamp experiments in the DRG neurons from TRESK KO mice under identical conditions. As shown on a representative recording in Fig. 5C, the application of A2764 (100 μM) had no effect, but RR substantially inhibited the background K^+ current in this neuron. As apparent on the scatter plot in Fig. 5D, A2764 (100 μM) did not influence the background K^+ current of the DRG neurons derived from the TRESK KO animals (the average effect on the K^+ current was marginal, 6.5% $\pm$ 15.8% inhibition, $n = 8$). This was significantly different from the substantial inhibition of the current in the wild-type cells (43.0% $\pm$ 29.7%, $n = 10$; $P < 0.05$, Mann-Whitney U test). This result indicates that the A2764-sensitive component corresponds to TRESK current, and A2764 is suitable for the investigation of the channel in native cells.

A2764 Increases the Excitability of DRG Neurons. The effect of A2764 on the resting membrane potential and excitability of wild-type and TRESK KO DRG neurons was determined by using the current-clamp technique. In the case of the wild-type DRG neurons, the application of A2764 (100 μM, in low-K^+ recording solution) depolarized the membrane potential by 6.6 $\pm$ 5.1 mV, from -57.3 ± 8.5 to -50.7 ± 12.5 mV ($P < 0.05$, paired t test; $n = 15$) (for representative recording, see Fig. 6A, middle panel). In TRESK KO DRG neurons, the application of A2764 did not change the resting membrane potential (-55.8 ± 7.3 vs. -55.0 ± 7.6 mV, before and after A2764, respectively, $n = 13$) (for representative recording, see Fig. 6B, middle panel). The effect of A2764 on the membrane potential was significantly different between the wild-type and the KO animals ($P < 0.05$,

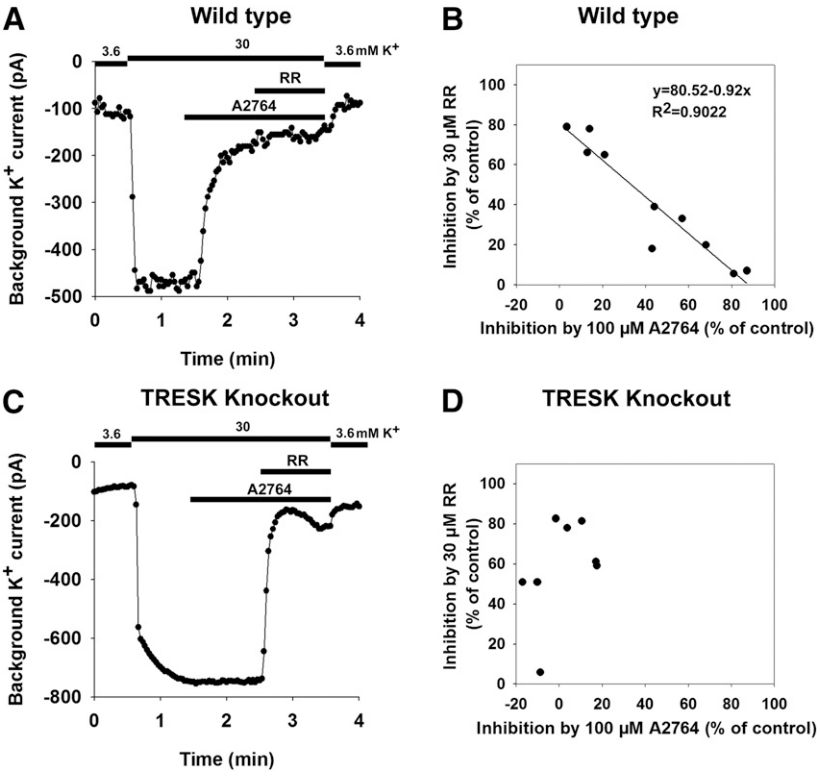

Fig. 5. A2764 inhibits the background K^+ current in DRG neurons. DRG neurons isolated from either wild-type or TRESK KO mice were used for whole-cell patch-clamp experiments. Inward currents were measured in 3.6 or 30 mM extracellular K^+ at the end of 200-millisecond voltage steps to −100 mV from a holding potential of −80 mV. Changes in extracellular K^+ concentration and the application of A2764 (100 μM) and RR (30 μM) are marked above the graphs. To convert TRESK channels into their active state, the bath solution contained 0.5 μM ionomycin. (A) A representative recording is shown from a wild-type, A2764-sensitive DRG neuron. (B) The effect of A2764 and RR on the background K^+ current was measured in 10 DRG neurons. The degrees of inhibition by A2764 and RR were plotted against each other. Linear regression was performed, and the regression line was plotted in addition to the data points (the corresponding equation and R value are also shown). (C) A representative recording is shown from a TRESK KO DRG neuron. (D) The effect of A2764 and RR on the background K^+ current was measured in eight DRG neurons isolated from TRESK KO mice. The degrees of inhibition by A2764 and RR were plotted against each other.

t test) (Fig. 7A), indicating that the depolarization by A2764 was mediated by the inhibition of TRESK.

We also investigated the effect of A2764 application on the excitability of DRG neurons. The rheobase of DRG neurons before and after the application of A2764 (100 μM) was determined by the injection of depolarizing current impulses of gradually increasing amplitude. The minimum amplitude of current injection necessary for the generation of action potential was reduced after the application of A2764 in most neurons (for representative recording, see Fig. 6A, left and right panels). The reduction of the rheobase was statistically significant (control rheobase: 500 ± 223 pA, $n = 6$; A2764 rheobase: 350 ± 160 pA, $n = 6$; $P < 0.05$, paired t test) (Fig. 7B, left panel). In sharp contrast, the rheobase of TRESK-deficient DRG neurons was not influenced by A2764 (for representative recording, see Fig. 6B, left and right panels). The rheobase was 320 ± 130 pA ($n = 5$) before and 360 ± 134 pA ($n = 5$) after, respectively, the application of the cloxyquin derivative (Fig. 7B, right panel).

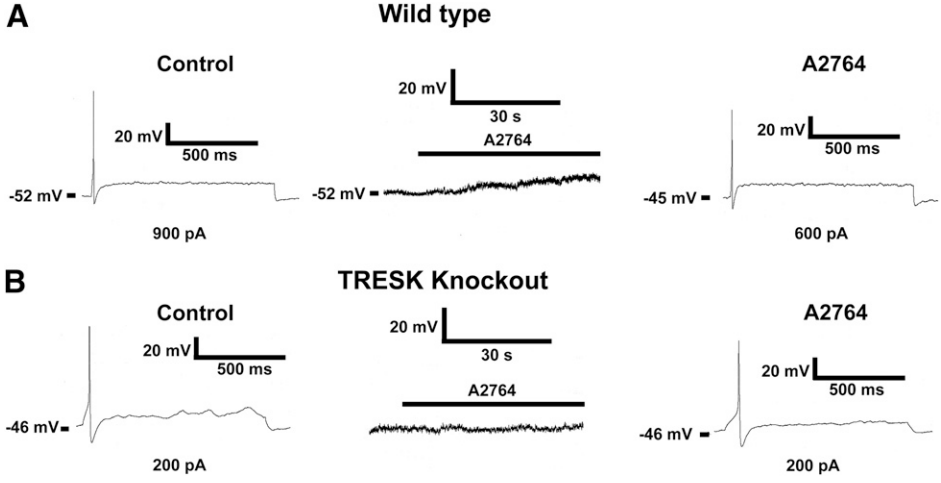

Fig. 6. A2764 increases the excitability of DRG neurons by inhibiting TRESK. DRG neurons isolated from either wild-type or TRESK KO mice were used for current-clamp experiments. The rheobase was determined by injecting depolarizing current impulses in 100 pA increments before and after the application of A2764 (100 μM). The effect of A2764 (100 μM) was determined without any current injection ($I = 0$ mode). (A) Representative recordings are shown from a wild-type DRG neuron. (Left and right panels) The first traces where current injection resulted in an action potential before and after the application of A2764 are plotted. (Middle panel) The application of A2764 leads to depolarization of the resting membrane potential. Application of the drug is marked by the horizontal line above the recording. (B) Representative recordings are shown from a TRESK KO DRG neuron. (Left and right panels) The first traces where current injection resulted in an action potential before and after the application of A2764 are plotted. (Middle panel) The application of A2764 has no effect on the resting membrane potential. Application of the drug is marked by the horizontal line above the recording.

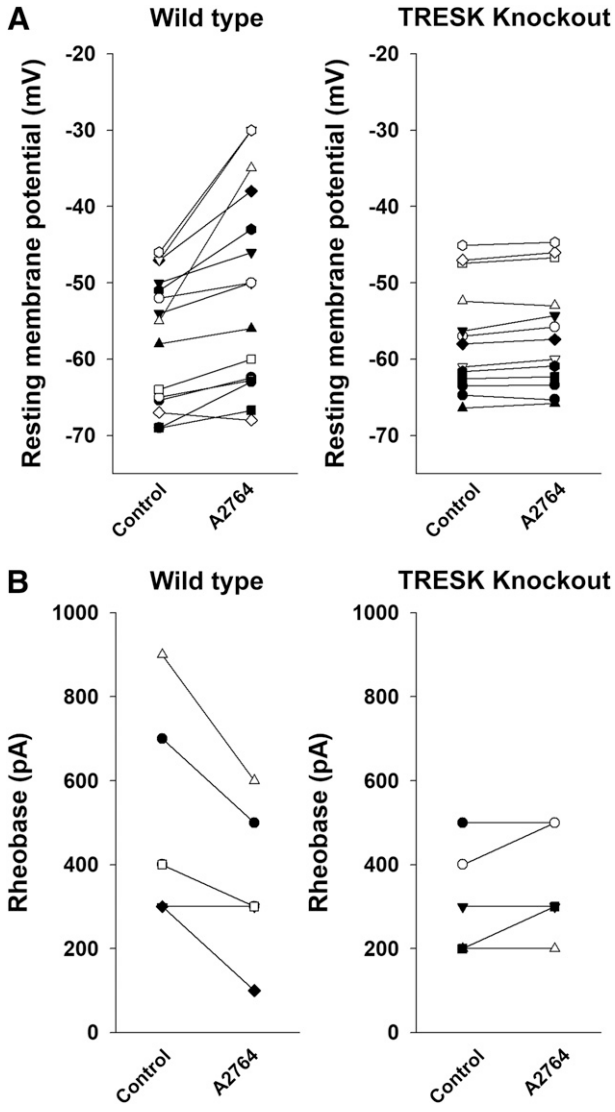

Fig. 7. Summary of the effect of A2764 on wild-type and TRESK KO DRG neurons. (A) The resting membrane potential of 15 wild-type (left panel) and 13 TRESK KO (right panel) DRG neurons was measured before (Control) and after (100 μM) A2764 application. Data points corresponding to one neuron are connected with a straight line. (B) The rheobase of six wild-type (left panel) and five TRESK KO (right panel) DRG neurons was measured before (Control) and after (100 μM) A2764 application. Data points corresponding to one neuron are connected with a straight line.

These data consistently indicate that the enhanced excitability induced by A2764 relied on the inhibition of the TRESK channel, which is a major determinant of action potential generation in DRG neurons.

Discussion

K_{2P} channels are expressed in a wide variety of cell types, where they play a major role in the determination of the resting membrane potential and in the regulation of cellular excitability (for review, see Enyedi and Czirják, 2010; Feliciangeli et al., 2015). In comparison with the other K_{2P} channel subunits, the expression pattern of TRESK is more restricted. The channel is abundantly and specifically expressed in the primary sensory neurons of the DRG and trigeminal ganglion. In accordance with this localization, TRESK has been implicated in the modulation of both physiological and pathophysiological nociception (for recent reviews, see Enyedi and Czirják, 2015; Mathie and Veale, 2015). Important functional data highlighting TRESK as a regulator of cellular excitability in primary sensory neurons were obtained by eliminating TRESK (by transient knockdown or by using KO animal strains), transiently overexpressing the channel, or by using pharmacological modulators of channel activity (Dobler et al., 2007; Tulleuda et al., 2011; Guo and Cao, 2014). An accumulating body of evidence also indicates that TRESK current alterations may contribute to the pathologic mechanism of neuropathic, migraine, and cancer pain (Lafrenière et al., 2010; Tulleuda et al., 2011; Yang et al., 2018).

The examination of the role of TRESK in primary sensory neurons is impeded by the lack of specific modulators. Several pharmacological agents, including inorganic modulators as zinc or mercuric ion (Czirják and Enyedi, 2006); the natural compounds hydroxy-α-sanshool from Szechuan peppers (Bautista et al., 2008); and aristolochic acid from *Aristolochiaceae* plants (Veale and Mathie, 2016), approved medicines such as lamotrigine or verapamil (Kang et al., 2008; Park et al., 2018); and also the insecticide pyrethroids (Castellanos et al., 2018) were demonstrated to inhibit the TRESK current.

Although these agents are known to have other pharmacological targets in addition to TRESK, and in most cases they were also shown to influence the activity of other K_{2P} channels, they provided the first options to experimentally manipulate the TRESK current of sensory neurons. As another tool to achieve this goal, cloxyquin, an antiamoebic drug, was identified as a TRESK activator in a high-throughput screening (Wright et al., 2013). We have recently shown that cloxyquin is selective for TRESK in the K_{2P} family and can activate the background K^+ current in mouse DRG neurons (Lengyel et al., 2017). Nevertheless, a high concentration of cloxyquin (in the 50–100 μM range) was required for TRESK activation. Therefore, we have synthesized 28 chemically modified analogs of cloxyquin to see whether we can find more potent activators of TRESK. After a pilot screening on an mTRESK channel expressed in *Xenopus* oocytes, it became apparent that the potency of these derivatives for TRESK activation was not increased by the chemical modification; however, their pharmacological profile has been changed substantially. Some of the analogs turned out to be inhibitors of TRESK, in sharp contrast to the activator parent compound cloxyquin. We have chosen the most potent analogs (one activator and two inhibitors) for further analysis.

The A2797 compound activated mTRESK current about 4-fold at a 100 μM concentration. This degree of activation was similar to the previously reported effect of cloxyquin (Lengyel et al., 2017). However, as opposed to the relative selectivity of cloxyquin for TRESK in the K_{2P} family, A2797 also activated TREK-2, TASK-2, and TRAAK channels. This indicates that the modification of the aromatic ring of cloxyquin may substantially influence the effect of the compound on various members of the background potassium channel family.

Modification of the hydroxyl sidechain in the chlorinated hydroxyquinoline parent molecule resulted in inhibitory derivatives. We have recently reported that cloxyquin showed state dependence in its effect; it was only able to activate TRESK in the resting state, but not when the channel was activated by the Ca^{2+}/calcineurin pathway (Lengyel et al., 2017).

Intrigued by the possibility that our inhibitors also show state-dependent effects, we determined the degree of inhibition by A2793 for both the resting mTRESK and the channels preactivated by application of the calcium ionophore ionomycin. Interestingly, the state dependence of the effect of A2793 proved to be the mirror image of that of cloxyquin. The inhibition by the compound was definitely more pronounced when TRESK current was stimulated with ionomycin before the application of A2793 than under the resting conditions of the channel.

The effect of A2793 was also tested on the other K_{2P} channels in addition to TRESK. It proved to be an efficient inhibitor of TASK-1, which was also reported to be expressed in some primary sensory neurons (Cooper et al., 2004). The effect on TASK-1 may limit the potential usage of A2793 to selectively inhibit TRESK in the native cells coexpressing these two specific K_{2P} channels. However, A2793 may be considered as a tool to discriminate between the resting and activated channels in heterologous expression systems, and to block TRESK activated by calcineurin in the native cells, which do not express TASK-1.

The other cloxyquin derivative A2764 was also found to exert a state-dependent effect. Similar to A2793, inhibition by this compound was larger in the case of the activated TRESK than at the resting channel. The concentration-response relationship between A2764 and the mTRESK current was also shifted to the left by the calcineurin-dependent dephosphorylation of the channel induced by the application of the Ca^{2+} ionophore ionomycin.

We have also determined the effect of A2764 on the other K_{2P} channels expressed in *Xenopus* oocytes. The substantial inhibition of TASK-1 (by A2793) was missing from the profile of A2764; thus, A2764 showed much better overall selectivity for TRESK among the K_{2P} channels than the other inhibitor. The degree of inhibition of the most affected other K_{2P} channels (TREK-1 and TALK-1) was moderate (21.0% ± 9.7% and 22.4% ± 10.3%, respectively), compared with the robust inhibition of dephosphorylated TRESK (77.8% ± 3.5%), suggesting that A2764 could be used as a selective inhibitor of TRESK in isolated cells in vitro.

To test the hypothesis that A2764 can be applied to inhibit TRESK and thus determine the contribution of the channel to the ensemble background K^+ current in native cells, we have performed whole-cell patch-clamp experiments in DRG neurons from both wild-type and TRESK KO mice. Based on previous single channel and immunocytochemical results, in the DRG neurons primarily the expression of two major K_{2P} channels, TRESK and TREK-2 was expected (Acosta et al., 2014; Kang and Kim, 2006). To estimate the contribution of these channels to the background K^+ current by a further independent method, we determined the degree of inhibition by A2764 and RR. We have previously described that RR inhibits background K^+ currents in DRG neurons (Braun et al., 2015). The molecular correlates of the RR-sensitive K^+ current may be TREK-2, TASK-3, or TRAAK homodimers, or heterodimers of the TREK subfamily (Braun et al., 2015; Blin et al., 2016; Lengyel et al., 2016). We found that in neurons derived from wild-type animals, A2764 strongly inhibited the background K^+ current in a subset of DRG neurons. However, this subset of A2764-sensitive neurons was completely missing from the DRG neurons prepared from TRESK KO animals. Based on these results from the wild-type and TRESK KO DRG neurons, we propose that A2764 is a useful selective inhibitor of TRESK.

To determine the contribution of TRESK and the RR-sensitive channels to the background K^+ current, the sensitivity of the current to the two inhibitors was determined in the same DRG neurons. We found an inverse correlation between the amplitude of the A2764-sensitive and RR-sensitive currents, indicating that the cells where TRESK is the major determinant of the background K^+ current express relatively less RR-sensitive K_{2P} channels and vice versa. In the DRG neurons analyzed, around 80% of the background K^+ current was sensitive to the combination of A2764 and RR. We have thus shown for the first time using whole-cell patch clamp that TRESK and various RR-sensitive K_{2P} subunits are the major determinants of the background K^+ current of isolated mouse DRG neurons. The application of A2764 offers a viable alternative to the previous methods for the investigation of TRESK and its regulation in the different functionally important subpopulations of primary sensory neurons.

In good accordance with the efficient inhibition of TRESK current by A2764, the application of this inhibitor also depolarized DRG neurons and appreciably increased their excitability in vitro, as indicated by the decreased rheobase in current-clamp experiments. Since these effects and also the inhibition of the background K^+ current was completely absent in the neurons isolated from the KO animals, we concluded that the current component blocked by A2764 corresponds to TRESK. We propose that cloxyquin and A2764 can be used to modulate the activity of TRESK channels in native cells, and that the TRESK KO mouse line can serve as a reliable negative control in these experiments. We hope that with this combined genetic and pharmacological approach the functional relevance of the enigmatic TRESK channel can be further elucidated.

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Authorship Contributions

Participated in research design: Lengyel, Bálint-Polonka, Hegedűs, Czirják, Enyedi.

Conducted experiments: Lengyel, Erdélyi, Pergel, Bálint-Polonka, Dobolyi, Bozsaki, Király.

Contributed new reagents or analytic tools: Erdélyi, Mátyus.

Performed data analysis: Lengyel, Pergel, Bozsaki, Király, Hegedűs, Czirják, Mátyus.

Wrote or contributed to the writing of the manuscript: Lengyel, Erdélyi, Pergel, Dobolyi, Bozsaki, Dux, Király, Hegedűs, Czirják, Mátyus, Enyedi.

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