# TREK and TRESK channels are the major background potassium channels of the primary somatosensory neurons

Doctoral theses

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## Introduction

The high resting potassium permeability of the plasma membrane was first demonstrated by Hodgkin and Huxley in the 1950's. Two pore domain potassium channels ($K_{2P}$), the molecular correlates responsible for this conductance were the last group of potassium channels to be identified. $K_{2P}$ channels contain 2 pore domains per subunit, so in contrast with other $K^+$ channels, they dimerize to form functional channels. They are open at all membrane potential values and are the major determinants of the resting membrane potential. Their activity is regulated by a variety of intra- and extracellular stimuli. Increased $K_{2P}$ channel activity leads to hyperpolarization and decreased cellular excitability.

The primary somatosensory neurons of the dorsal root- and trigeminal ganglia play a crucial role in the detection and processing of stimuli such as mechanical force, changes in temperature and a wide variety of endo- and exogenous chemical mediators. A subset of these sensory neurons called nociceptors are responsible for the detection of stimuli capable of causing tissue damage.

Activation of these neurons leads to the sensation of pain. The $K_{2P}$ channels TREK-1, TREK-2 (TREK: TWIK-Related $K^+$ channel) and TRESK (TWIK-Related Spinal cord $K^+$ channel) are abundantly expressed in these sensory neurons. Accordingly, they have been implicated in the pathophysiology of migraine and other disorders of nociception.

Heteromeric assembly is a widespread phenomenon in the other families of $K^+$ channels. Heteromeric channels have unique properties compared to the parent homomers, which increases the diversity of $K^+$ currents. To date, only a few examples of heteromerization have been described in the $K_{2P}$ family. The first $K_{2P}$ heterodimer (TASK-1/TASK-3) was identified by our laboratory. TREK-1 and TREK-2 are closely related, however, their biophysical and pharmacological properties are different. Coexpression of TREK-1 and TREK-2 has been described in multiple organs. One of the goals of my research was to examine the heterodimerization of TREK-1 and TREK-2, characterize the properties of the heterodimer and identify the heterodimeric channel in native tissue.

Examination of the physiological role of $K_{2P}$ channels is impeded by a lack of specific modulators. A high-throughput study has identified cloxyquin (an antiamoebic drug) as a TRESK activator. However, the selectivity and mechanism of action was not examined in detail. The other major goal of my PhD studies was to determine the selectivity of cloxyquin and elucidate its mechanism of action. If cloxyquin is adequately selective for TRESK, it would be valuable pharmacological tool to identify TRESK current in native cells in patch clamp experiments and to activate TRESK in animal models of migraine and nociceptive disorders. We speculated that we could obtain further TRESK modulators by chemically modifying cloxquin. These modulators can be of interest as research tools to examine the role of TRESK in migraine and nociception. A sufficiently potent TRESK activator can also be of therapeutic interest.

## Aims

The goals of my doctoral work were the following:

**1.** Examine the heterodimerization of TREK-1 and TREK-2 subunits, characterize the TREK-1/TREK-2 heterodimer in heterologous expression systems and identify the heterodimer in native cells.

**2.** Determine the selectivity of cloxyquin and elucidate the mechanism of the action of the drug.

**3.** If cloxyquin is selective for TRESK, it can serve as a parent compound for novel TRESK modulators. These new compounds can be screened on channels expressed in *Xenopus* oocytes.

**4.** Determine the effect of cloxyquin and any promising TRESK modulators on the electrophysiological properties of isolated dorsal root ganglion (DRG) neurons.

## Methods

### Preparation and injection of *Xenopus* oocytes

Oocytes were isolated from African clawed frogs (*Xenopus laevis*) and injected with channel cRNA. Experiments were performed 2-3 days after injection.

### Isolation of adult DRG neurons

Adult FVB/Ant mice (wild type or TRESK KO) were killed humanely by $CO_2$ exposure. The isolated ganglia were digested using collagenase at 37 °C and mechanically dissociated. After centrifugation, the cell suspension was plated on cell culture dishes treated with poly-L-lysine. Cells were cultured at 37 °C, in an incubator containing 5% $CO_2$. Cells were used within 48 hours after isolation.

### Culturing and transient transfection of HEK293T cells

HEK293T cells were cultured in DMEM medium containing 10% FBS and antibiotics. Cells were transfected with channel DNA using Lipofectamine 2000 and used for patch clamp experiments 24-48 hours after transfection.

**Two-electrode voltage clamp**

Current flowing across the membrane of *Xenopus* oocytes was measured with the two-electrode voltage clamp technique. The extracellular (EC) solution contained 2 or 80 mM [$K^+$] (for the high [$K^+$] solution, 78 mM NaCl was substituted with KCl). Resistance of the recording electrodes was 0.3-1 MΩ when filled with 3 M KCl. Data was recorded and analysed using pClamp 10.3 software. Background $K^+$ currents were measured as an inward current at the end of 300 ms voltage step to -100 mV in the high [$K^+$] solution. The nonspecific leak current measured in the low [$K^+$] solution was subtracted from the value measured in high [$K^+$] solution. Experiments were performed at room temperature (21 $^oC$).

**Whole cell patch clamp**

Whole cell patch clamp experiments were performed on HEK293T cells and DRG neurons The EC soluton contained 3.6 or 30 mM [$K^+$] (for the high [$K^+$] solution, 26.4 mM NaCl was substituted with KCl). Data were recorded and analysed using pClamp software.

Background potassium current was determined as described in the case of the two-electrode voltage clamp experiments.

**Excised patch patch clamp**

Excised patch patch clamp experiments were performed using the same patch clamp setup used for the whole cell experiments. Data were acquired at 20 kHz and filtered at 2 kHz. Inside-out recordings were performed on patches excised from *Xenopus* oocytes at a membrane potential of +60 mV. Recordings in the outside-out configuration were performed on patches excised from *Xenopus* oocytes and DRG neurons at a membrane potential of -60 mV. Analysis was performed using pClamp software. The single channel conductance, open propability ($P_o$) and channel activity ($NP_o$) was determined from the recordings.

**Plasmids and cRNA synthesis**

For expression in *Xenopus* oocytes, channel cDNAs were cloned into the pXEN *Xenopus* expression vector.

Plasmids were linearized and used as template for *in vitro* cRNA synthesis reaction performed with the mMESSAGE mMACHINE T7 Transcription kit. RNA integrity was checked using denaturing agarose gels and quantified using a spectrophotometer. Point mutations were generated using the QuikChange Site-Directed Mutagenesis Kit. For expression in HEK cells, channels were subcloned into an appropriate vector (pcDNA3.1 or pIRES-CD8). Fidelity of the constructs was confirmed using automated sequencing.

**Isolation of membrane fraction, Western blot**

Membrane fractions were isolated from *Xenopus* oocytes two days after the injection with channel cRNA. The samples were solubilized in a solution containing 1% Triton and mixed overnight with beads conjugated with anti-V5 antibodies. Proteins bound to the beads were eluted with a sample buffer containing SDS. Proteins were seperated using SDS-PAGE and visualized with Western blot using a monoclonal anti-FLAG antibody and an anti-mouse IgG antibody conjugated with horseradish peroxidase.

**Statistical analysis**

Statistical analysis was performed with either Origin8 or Statistica software. In most cases, results were compared with Student's t test or one way ANOVA and Tukey's post hoc test. If data couldn't be compared with parametric statistical tests, the appropriate non-parametric test was used. P values less than 0.05 were considered to be significant.

**Results**

The expression of TREK-1 and TREK-2 subunits overlaps in several organs, so it is possible that they can form functional heterodimers. To examine the properties of the TREK-1/TREK-2 heterodimer, I generated a construct in which TREK-1 and TREK-2 are covalently linked to each other to enforce heterodimerization. This artifical heterodimer can be distinguished from the TREK-1 and TREK-2 homodimers based on it's EC pH- and ruthenium red (RR) sensitivity. When TREK-1 and TREK-2 subunits were coexpressed in *Xenopus* oocytes or HEK293T cells, the EC pH- and RR-sensitivity of the whole cell current suggested the formation of TREK-1/TREK-2 heterodimers. Formation of the heterodimer was also demonstrated in pull-down experiments.

Multiple single channel conductance levels were described for both TREK-1 and TREK-2. The different conductance levels are the consequence of isoforms with differing N-terminal length, caused by the phenomenon of alternative translation initiation.

In order to obtain uniform conductance levels for TREK-1 and TREK-2, respectively, I generated mutant TREK-1 and TREK-2 channels, possessing only one translation initiation site. This lead to the loss of multiple conductance levels. I also created a tandem channel from these mutants. The mutant channels were expressed in *Xenopus* oocytes and their single channel conductances were determined. The homodimers and the tandem channel could be identified based on their distinct single channel conductance values. When the mutant TREK-1 and TREK-2 subunits were coexpressed, channels with single channel conductances corresponding to the tandem channel were recorded. Therefore, the heterodimerization of the two subunits was confirmed on the single channel level.

The sensitivity of single TREK channels to different inhibitors (EC pH, RR, spadin) was determined in the outside-out configuration. The TREK-1/TREK-2 tandem was sensitive to spadin (a selective inhibitor of TREK-1) and RR. Therefore, the heterodimer can be differentiated from the TREK-1 and TREK-2 homodimers based on this unique inhibtiory profile.

In membrane patches excised from DRG neurons, we found $K^+$ channels sensitive to both RR and spadin, thus demonstrating TREK-1/TREK-2 heterodimer formation in native cells.

Cloxyquin was identified as a TRESK activator during a high-throughput screen, however the selectivity and mechanism of action wasn't determined. During the course of my PhD studies, I addressed these questions using our collection of mouse $K_{2P}$ channels and TRESK mutants. Cloxyquin had no effect on other $K_{2P}$ channels expressed in *Xenopus* oocytes and activated mouse TRESK current by 4.5-fold. The $EC_{50}$ value for mouse TRESK was 26.4 μM. The effect of cloxyquin was dependent on the activation state of channel: cloxyquin had no effect on TRESK being activated via the cytoplasmic calcium signal or on a channel converted to be constitutively active by point mutations. These results raised the possibility that cloxyquin might activate TRESK by stimulating the calcium-calcineurin pathway.

However, chelation of the cytoplasmic calcium signal by EGTA buffer injection or pharmacological inhibition of calcineurin by FK506 did not prevent activation of TRESK by cloxyquin. Furthermore, cloxyquin activated a TRESK mutant unable to bind calcineurin. Based on these results, cloxyquin is a direct activator of TRESK. In isolated DRG neurons, application of cloxyquin increased the background potassium current, therefore cloxyquin can be used to identify (and activate) TRESK in native cells.

To activate TRESK *in vivo* (in animal experiments, or as a potential therapeutic drug), more potent activators than cloxyquin would be required. We started a collaboration with the Department of Organic Chemistry (Prof. Péter Mátyus and colleagues), to produce chemically modified cloxyquin analogues (or compounds with a similar structure to cloxyquin). These novel compounds were screened on mouse $K_{2P}$ channels expressed in *Xenopus* oocytes in a way similar to cloxyquin. We were unable to obtain an activator with better properties than cloxyquin.

However, multiple analogues turned out to be efficient inhibitors of TRESK. Two compounds (A2764, A2793) were chosen for more detailed analysis. Both compounds showed state dependence, the degree of inhibition was higher when TRESK current had been activated before the application of the inhibitor. This state-dependence was more pronounced in the case of A2793. However, A2793 also inhibited TASK-1 with an efficiency similar to TRESK. Among all $K_{2P}$ channels, A2764 inhibited TRESK most efficiently, therefore we used this compound for our patch clamp studies on DRG neurons.

In DRG neurons isolated from wild type animals, a subpopulation of neurons had background potassium currents which was inhibited by A2764. This subpopulation sensitive to A2764 was missing from DRG neurons isolated from TRESK KO animals. Accordingly, the A2764-sensitive current corresponds to the TRESK current. In current clamp experiments, A2764 depolarized the membrane potential and decreased the rheboase of wild type DRG neurons.

These parameters were not modified by A2764 in DRG neurons isolated from TRESK KO animals, which

implies that the changes in excitability observed in wild type animals were a consequence of TRESK inhibition.

## Conclusions

**-TREK-1 and TREK-2 form functional heterodimers in heterologous expression systems. The TREK-1/TREK-2 heterodimer can be identified on the basis if its pharmacological profile.**

**-TREK-1 and TREK-2 form functional heterodimers in DRG neurons.**

**-Cloxyquin is a selective activator of TRESK. Cloxyquin directly activates the channel, the calcium-calcineurin pathway does not play a role in the effect of the drug. The effect of cloxyquin is state-dependent, preactivated channels cannot be further stimulated by cloxyquin.**

**-Cloxyquin can activate the background $K^+$ current in isolated DRG neurons.**

**-The cloxyquin derivative A2764 is a selective inhibitor of TRESK.**

**-The inhibition is state-dependent, the degree of inhibition is larger when the channel is in the active state.**

**-A2764 inhibits the background $K^+$ current of isolated DRG neurons. Inhibition of the background $K^+$ current leads to depolarization of the cells and a decrease in the rheobase. A2764 had no effect on DRG neurons isolated from TRESK KO mice. These results indicate that the effects of A2764 are mediated by inhibition of TRESK.**

**List of publications**
**Publications related directly to the theses**

**Lengyel M**, Czirják G, Enyedi P
Formation of Functional Heterodimers by TREK-1 and TREK-2 Two-pore Domain Potassium Channel Subunits.
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