Investigation of the two pore domain potassium channels TREK-2 and TRESK expressed in the dorsal root ganglion neurons

Ph.D. thesis

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INTRODUCTION

Pseudounipolar neurons of the dorsal root and trigeminal ganglia (DRG, TRG) play a central role in the afferentation of potentially painful physicochemical and thermal stimuli. Ion channels of these neurons have been extensively investigated for a long time, since they are potential targets of analgetics.

The hyperpolarizing effect of the currents of potassium channels reduces excitability. Therefore, activation of potassium currents results in the attenuation of pathological hyperexcitability and hyperactivity of neurons.

With respect to the two-pore (2P) domain potassium channels (K_{2P}), the interest of our research group was mainly focused on TRESK and TREK-2, which are abundantly expressed in DRG neurons. It has been suggested that TRESK plays a role in neuropathic pain.

The name of the family "two-pore domain" potassium (K_{2P}) channels stems from the distinguishing topology of the K^+ channel containing two K^+ channel pore-forming (P) domains per subunits. Accordingly, K_{2P} channel subunits function as dimers, in contrast to the other K^+ channel families characterized as tetramers. Being open and active at the negative voltage values, K_{2P} channels establish the resting membrane potential and regulate excitability, thus influence many important functions in both excitable and non-excitable cell types.

The localization of TRESK compared to the other K_{2P} channels is more restricted: its mRNA is expressed mainly in the primary sensory neurons of the dorsal and trigeminal ganglia, and to a lesser extent in the neurons of vegetative ganglia. Although the exact physiological role of TRESK is unknown, it is supposed to relieve chronic pain, and to be responsible for other (patho)physiological processes, since it has been reported recently, that dominant-negative mutation of TRESK is linked to familial migraine with aura in a large pedigree.

Our research group reported the several-fold calcium-dependent activation of TRESK current by calcineurin in the *Xenopus* oocyte heterologous expression system. Calcineurin dephosphorylates specific serine residues, in mouse TRESK Ser 264 and the adjacent serines 274, 276 and 279 (so-called Ser^{276} cluster) of the intracellular loop of TRESK, the latter three being more important in the activation of TRESK current. Efficient stimulation of TRESK current has also been reported in mammalian cells, however, the extent of these activations are limited compared to those detected in the *Xenopus* expression system, and the role of calcineurin has yet to be verified.

After the substantial (5- to 10-fold) activation in the *Xenopus* oocyte, the current of TRESK slowly decreases and returns to the resting state, thus the dephosphorylated serines of TRESK-loop are (re)phosphorylated by intracellular kinase(s) resulting in the cessation of the activated state of TRESK and the inhibition of its current.

Our workgroup has also demonstrated that the phosphorylation of the Ser^{276} cluster (the most important serines in the activation of TRESK current) is inhibited by the 14-3-3 adaptor protein irrespectively of the direct binding of 14-3-3 to the channel. The less relevant regulatory serine is phosphorylated by PKA.

Excitable cells possessing background K^+ currents, for instance neurons of the DRG or the cerebellar granule cells, express many different K_{2P} channels. However, in order to unravel the clear function and role of the distinct K_{2P} channels expressed in the same cell using whole-cell patch-clamp method, adequate differentiation of their current is essential. So far hardly any compounds are available which can be used as a specific activator or inhibitor among K_{2P} channels.

Based on the previous result of our research group, the polycationic dye ruthenium red (RR) is widely used in native cells in order to differentiate between the currents of the closely related TASK-1 and TASK-3 K_{2P} channels.

In spite of RR not being a specific inhibitor of TASK-3, since it acts on several other channels, RR has been used successfully to separate the current of the two similar TASK channels in motoneurons, cerebellar granule cells, the glomus cells of the carotid body and several other cell types.

Hence, the question was raised whether RR is able to influence the potassium currents of other K_{2P} channels.

OBJECTIVES

1. In HEK293 mammalian cell line, we planned to investigate the degree and mechanism of calcineurin-dependent current activation of TRESK K_{2P} channel responsible for one of the most significant background potassium currents in DRG and TRG neurons.

2. Identification and examination of the kinase responsible for the inhibitory phosphorylation of the Ser^{276} cluster of TRESK *in vitro* and in the *Xenopus* expression system.

3. Comprehensive analysis of the interaction between the polycationic ruthenium red (RR) and different K_{2P} channels expressed in *Xenopus* oocytes in order to uncover whether the dye differentiates among distinct K_{2P} currents.

4. Qualitative and quantitative examination of the effect of RR on the identified RR-sensitive K_{2P} channels expressed in *Xenopus* oocytes. Investigation of the RR-sensitive current component in native cells expressing endogenous K_{2P} currents.

METHODS

Isolation and injection of *Xenopus* oocytes

Oocytes were prepared from the frogs *Xenopus laevis*, and one day after defolliculation cRNAs were microinjected. Experiments were performed 2−4 days after the microinjection.

Isolation of dorsal root ganglion (DRG) neurons

DRGs were dissected from 40–70-day-old NMRI mice (euthanized with CO_2) and ganglia were incubated with gentle shaking at 37°C in PBS containing collagenase. Then gentle trituration was performed in DMEM-F-12 containing 10% FBS and antibiotics resulting in suspension of neurons and glias. For purification cells were centrifuged and washed with culture medium, and plated on plastic culture dishes treated with poly-L-lysine. They were incubated in 95% air–5% CO_2 mixture at 37°C, and measured within the following 3 days after adhesion of neurons to the coated plate.

HEK293 cell culture, and transient transfection

Human embryonic kidney (HEK293) cell line was maintained in culture medium DMEM containing 10% FBS and antibiotics. Mouse TRESK was subcloned to pIRES-CD8 vector for transient transfection (Lipofectamin 2000) of HEK293 cells, experiments were performed within 36-72 hours after tranfection.

Two-electrode voltage clamp recording

The currents flowing across the membrane of Xenopus oocytes were measured by two-electrode voltage clamp technique. A gravity-driven perfusion system was applied for the continuous exchange of extracellular (EC) solutions containing K^+ at low or high concentrations. The sum of $[K^+]$ and $[Na^+]$ was constant. Intracellular borosilicate glass microelectrodes (0,3-1 MΩ) were filled with 3 M KCl. Data were transferred through an analog-to-digital converter to PC, then pClamp 10.1 program was used for the registration and evaluation of the data.

Background K^+ currents were measured in the high $[K^+]$ solution at the end of a 300 ms long voltage step to -100 mV applied every 4 sec at room temperature (21 °C). The amplitude of background K^+ current was corrected by the non-specific leak current measured in the low $[K^+]$ solution under the same conditions.

Patch clamp recording

For current measurements on HEK293 cells and DRG neurons the whole-cell patch clamp technique was applied. The borosilicate glass microelectrode (3-9 MΩ) was connected to the headstage of a patchclamp amplifier (Axopatch-1D). Data acquisition and analysis was performed similarly to that of two-electrode voltage clamp method. Likewise extracellular (EC) solutions contained K^+ at low or high concentrations, and the sum of $[K^+]$ and $[Na^+]$ was constant. TRESK current was measured on attached, separated HEK293 cells at 21 °C. Before the stimulation of

current by ionomycin calcium free EC solution was applied, but during the administration of ionomycin Ca^{2+} (2 mM) was supplemented. Patch clamp measurements on DRG neurons were performed at 37 °C and in EC solutions containing Ca^{2+} (2 mM). Other parameters of the measurement were the same as the parameters of the HEK293 cell protocol.

Plasmids and cRNAs

The coding region of the appropriate proteins were cloned to pXEN *Xenopus* oocyte expression vector. *In vitro* synthesis of cRNAs were carried out by the mMESSAGE mMACHINE T7 Transcription kit. Products were verified on a denaturing agarose gel. Complementary DNAs of the AMPK-related kinases (e.g. MARK2) and tau were amplified by RT-PCR from the total RNAs of mouse tissues. All kinase cDNAs were cloned to pXEN vector, and verified by automatic sequencing. Different mutant versions of the kinases were produced with QuikChange site directed mutagenesis kit (Stratagene).

Production and purification of recombinant fusion proteins

The coding region of the different TRESK-loop, MARK2 and tau fusion protein constructs tagged with Glutathione S-transferase (GST) or thioredoxin-hexahistidine (Trx-His6) were subcloned into distinct pGEX and pET plasmids and expressed in BL21 strain of *E. coli*. The proteins were affinity-purified with Ni-NTA or glutathione-agarose, then the bound Trx-His6-tagged and

GST fusion proteins were eluted by solutions supplemented with 300 mM imidazole or 20 mM reduced glutathione, respectively.

In vitro radioactive phosphorylation

The different TRESK-loop fusion constructs immobilized on glutathione or Ni-NTA resin were phosphorylated at 30 °C for 1 hour with continuous shaking with the appropriate constitutively active MARK2-T208E supplemented with 20 μM ATP és 50-100 kBq ^{32}P-γ-ATP. The reaction was quantitatively evaluated according to the appearance of the radioactive ^{32}P in the substrate protein of the kinase by the Phosphorimager (BioRad GS-525).

Isolation and purification of rutheniumviolet (RV)

In order to purify RV we applied cation exchange chromatography using carboxymethyl cellulose resin. Crude old RR preparation (produced in 1996) was dissolved in ammonium acetate (AA; 10 mM). First bulk RR was eluted followed by washing steps. Subsequently, RV was eluted with a linear gradient of AA. Purity and RV content of these fractions was established according to their absorbance spectra. Fractions containing RV were lyophilized, immediately dissolved in 10 mM AA, and stored at -20 °C.

Statistical analysisis

Data are expressed as means±SEM. Data were subjected to Student's t-test for independent samples, and one-way ANOVA followed by Tukey HSD or Scheffe's post hoc tests for multiple comparisons, and Pearson's product–moment correlation analysis. Normalized dose–response curves were fitted by a modified Hill equation. The differences were evaluated by the software STATISTICA, except for the curve fitting and correlation analysis for which ORIGIN was used. The difference was considered to be significant at $p<0.05$. Sample size of each group in all experiments was at least 5.

RESULTS

TRESK, one of the most important background potassium channels of DRG neurons, could be activated by the cytoplasmic Ca^{2+} signal only moderately (by 20-80%), when expressed in mammalian cell lines. During my PhD work I have determined the conditions of whole-cell patch clamp measurement under which the activation of TRESK current is remarkable similarly to that in the *Xenopus* expression system.

Both the extracellular administration of ionomycin and the stimulation of G_q protein-coupled receptors were suitable for the several-fold (3- to 6-fold) activation of TRESK current. We also verified the role of endogenous calcineurin as the direct activator of TRESK current in mammalian cells (like in *Xenopus* oocytes). Specific calcineurin inhibitors FK506 and cyclosporin A prevented the activation of the current, even at low concentrations. Therefore, the mechanism of activation of TRESK current expressed in *Xenopus* oocytes or in mammalian cells is the same.

Our research group's previous result, namely that the adaptor protein 14-3-3 inhibits the effect of the unknown regulatory kinase on TRESK, led us to the identification of the AMPK related MARK kinases as TRESK regulators. Since MARK kinases ubiquitously controll cell polarity, it is posible that they are also present in the cells expressing endogenous TRESK current. Among the four MARK kinases MARK1, MARK2 and MARK3 accelerated

the return of the activated TRESK current to the resting state in Xenopus oocytes, while MARK4 does not influence TRESK current at all. Other examined AMPK related kinases did not affect TRESK current recovery.

Hererafter we investigated only MARK2 isoform 2, briefly MARK2. We demonstrated that MARK2 not only accelerated the return of activated human and mouse TRESK currents, but also inhibited the resting currents. The effect on the recovery could also be detected if TRESK current was activated by receptor stimulation instead of ionomycin.

By coexpressing constitutively active and kinase inactive ('kinase dead') mutants of MARK2 along with TRESK, we verified that the enzymatic activity of the kinase was indispensable for its effect on the channel. In *Xenopus* oocytes MARK2 also accelerated the return of current after the calcium-dependent activation if Ser 264 – a less significant determinant of regulation and the binding site of 14-3-3 adaptor protein – is mutated. This indicates that the effect of MARK2 on TRESK is independent from the phosphorylation of Ser 264, and the subsequent binding of 14-3-3 to the channel. It also suggests that the Ser^{276} cluster of TRESK could be the target of the kinase, and the inhibitory effect of MARK2 on TRESK might be mediated by the phosphorylation of the Ser^{276} cluster.

We analyzed the effect of constitutively active recombinant MARK2 protein produced in *E. coli* and microinjected into the oocytes a few hours before the measurement of TRESK current. Under these conditions MARK2 also accelerated the return of the

activated TRESK current to the resting state. So MARK2 kinase is 'acutely' effective after its diffusion from the site of injection in the cytoplasm of the oocyte, i.e. it does not have to be present long before the experiment for the inhibitory effect on TRESK.

In vitro radioactive phosphorylation revealed that MARK2 directly and efficiently phosphorylates the Ser^{276} cluster in the intracellular loop of TRESK but does not affect Ser 264.

The effect of the polycationic ruthenium red (RR) was analyzed on distinct K_{2P} channels. RR inhibited the current of TREK-2 potently (IC_{50}=0,23 μM) and rapidly, whereas the closely related TREK-1 proved to be insensitive to the compound.
Dose-response relationship of TREK-2 current and RR was characterized by a Hill coefficient of 1.2, implying that a single RR molecule binds simultaneously to both subunits in the functional TREK-2 dimer.

Since RR contains many positive charges, the RR interaction site in TREK-2 is expected to consist of extracellular, negatively charged or (deprotonated) histidine residues. In order to explore the target of the dye we mutated the extracellular negatively charged or histidine residues of TREK-2 which are non-conserved in TREK-1, and replaced them with the corresponding, homologous residues of TREK-1. All these residues are in the first extracellular loop, or the so-called *cap* domain. The RR sensitivity of D133A-D135I mutant TREK2 was abolished by the mutations. Therefore, we created the appropriate point mutant channels and it was shown that the mutation of the aspartate (D) 135 alone was enough to

eliminate the effect of RR on TREK-2. This conserved D135 of TREK-2 is homologous to Glu 70, the major determinant of RR sensitivity in TASK-3. The mutation of the corresponding residue of the RR-resistant TREK-1 to negatively charged aspartate (I110D) was sufficient to render the channel highly RR-sensitive, thus illustrating the importance of this specific residue in RR-sensitivity. We designed tandem (concatenated) constructs expressing the two TREK-2 subunits as a continuous polypeptide. Tandem TREK-2 was similarly sensitive to RR as the wild-type channel, but the absence of D135 only in one subunit mitigated the effect of RR. Thus both Asp contribute to the full effect of TREK-2 by RR.

Based on recently revealed crystal structures of the human TREK-2 channel, these conserved D135 residues line the wall of the extracellular ion pathway (EIP), a tunnel structure through the extracellular cap domain characterising K_{2P} channels. This position is also at the entrance of the channel pore, thus the electrostatic and/or steric interaction of RR molecule with this site could limit the movement of the K^+ cations, i.e. the K^+ current.

Intriguingly TRAAK current appeared to be less inhibited by RR in this study than we previously reported using an older and less pure preparation of the dye. We provided evidence, that ruthenium violet (RV), a contaminant in the old commercial RR product was partly responsible for the difference, since RV inhibited TRAAK current more potently (IC_{50}=0,11 μM), than RR (IC_{50}=1,7 μM). Hill coefficients of TRAAK for RR and RV (1.1, 2.0 respectively) suggest a different mechanism of action of the two

ruthenium compounds. The inhibitory profile of RV on the other K_{2P} currents turned out to be consistent with that of RR.

DRG neurons have been reported to express predominantly TREK-2, TRESK, TREK-1 and TRAAK background K^+ channels in rodents based on mRNA content and electrophysiological data. By patch clamp technique it was previously demonstrated that the dominant background K^+ current of rat DRG neurons was provided by TREK-2 at 37°C, and to a lesser extent by TRESK, and the TREK-1 and TRAAK currents were negligible, while at room temperature TRESK current was prevailing, whereas the TREK-2, TREK-1 and TRAAK currents were scarcely detectable.

We performed whole-cell patch clamp recordings on mouse DRG neurons at 37°C to investigate the RR-sensitive TREK-2 current. We were the first to detect a RR-sensitive leak K^+ current component in DRG neurons.

The neurons with small potassium current amplitude (<0,5 nA, in 30 mM [K^+] EC at -100 mV) were characterized by relatively high RR sensitivity (60-80%), whereas neurons with large background K^+ current (>0,5 nA) were usually weakly sensitive to RR. Significant negative correlation has been established between the K^+ current amplitude and the RR sensitivity in 20 DRG neurons, suggesting that neurons with small K^+ current at -100 mV expressed relatively more RR-sensitive component. Presumably, the RR-sensitive component corresponds to TREK-2, although TRAAK and perhaps TASK-3 may contribute as well.

CONCLUSIONS

1. The several-fold activation of TRESK by the calcium/calmodulin-dependent protein phosphatase calcineurin has also been demonstrated in a mammalian cell line (HEK293) by applying the whole-cell patch clamp method. The role of endogenous calcineurin was also verified, suggesting that the regulatory mechanisms might be similar in native, TRESK-expressing cells.

2. We have shown that MARK2 kinase accelerates the recovery from the calcium-dependent activation, and also inhibits the basal TRESK current. MARK2 phosphorylates the regulatory Ser^{276} cluster of the channel *in vitro*. It remains to be established whether MARK2 directly exerts its effect on TRESK *in vivo*, and how the microtubule dynamics and cell polarity, which are controlled by MARK kinases are related to the regulation of TRESK current.

3. TREK-2 is robustly and rapidly inhibited by the extracellular application of ruthenium red (RR), whereas TREK-1 is not affected.

4. Ruthenium red interacts with aspartate 135 located in the *cap* (first EC-loop) domains of TREK-2 subunits. Both of these aspartates are indispensable for the efficient inhibition of TREK-2 (dimer) by RR.

5. A ruthenium red sensitive background K^+ current component was detected in mouse DRG neurons at 37 °C, indicating that RR can also be applied for the characterization of K_{2P} currents in native cells. The negative correlation between K^+ current amplitudes and RR sensitivity is in good accordance with a recent publication reporting that principally small DRG neurons show abundant TREK-2 immunoreactivity.

PUBLICATIONS

Publications relevant to the dissertation:

Braun G, Nemcsics B, Enyedi P, Czirják G. (2011) TRESK background K^+ channel is inhibited by PAR-1/MARK microtubule affinity-regulating kinases in Xenopus oocytes. *PLoS One,* 6: e28119.
IF: 4,092

Braun G, Lengyel M, Enyedi P, Czirják G. (2015) Differential sensitivity of TREK-1, TREK-2 and TRAAK background potassium channels to the polycationic dye ruthenium red. *Br J Pharmacol,* 172: 1728-1738.
IF: ~4,842 (2014)

Other publications:

Enyedi P, **Braun G**, Czirják G. (2012) TRESK: The lone ranger of two-pore domain potassium channels. *Mol Cell Endocrinol,* 353: 75-81. Review.
IF: 4,039

Enyedi P, Veres I, **Braun G**, Czirják G. (2014) Tubulin binds to the cytoplasmic loop of TRESK background K^+ channel in vitro. *PLos One,* 9: e97854.
IF: 3,234