Investigation of TRESK background K^+ channel regulation by protein kinase C

Ph.D. thesis

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INTRODUCTION

TRESK (TWIK-Related Spinal Cord K^+ Channel; $K_{2P}18.1$; KCNK18 gene) belongs to the two-pore domain K^+ channel family, and it is the only member of the TRESK subfamily. In contrast to the widely distributed TREK and TASK channels, TRESK is specifically expressed only in few tissue types. The most important locations of the channel are the dorsal root and trigeminal ganglia, where the primary sensory neurons express TRESK. In the trigeminal ganglion (TRG), the expression of TRESK mRNA is about three-fold higher than that in the dorsal root ganglion (DRG). In addition, TRESK can also be found in certain sensory and effector neurons of the autonomic nervous system, e.g. in the nodose ganglion of the vagus nerve, or in the superior cervical ganglion of the sympathetic system. Human TRESK was shown to be the most selectively expressed K^+ channel in primary sensory neurons among all K^+ channel types by using RNA sequencing methods, when the expression levels were compared to the brain and several peripheral tissues. (Among all the ion channels, TRESK proved to be the second most selectively expressed channel type in both (DRG and TRG) tissues, after $Na_v1.8$ voltage-gated Na^+ channel.)

TRESK channel induces a typical macroscopic background K^+ current, characterized by slight outward rectification of the voltage-current relationship. The current is only weakly sensitive to the changes of the extra-, or intracellular pH, or temperature, it is not much affected by mechanical forces, and it is not activated by the application of polyunsaturated free fatty acids, i.e. TRESK is not influenced by the factors, which generally modulate TREK and TASK channel activity.

The stimulation of G_q protein-coupled receptors also regulates TRESK differently from the previously examined members of the TREK and TASK subfamilies. Whereas the other K_{2P} channels are inhibited, or not affected by the stimulation of G_q protein-coupled receptors, TRESK current is increased several-fold. This increase of TRESK current is evoked by the stimulation of coexpressed M_1 muscarinic receptor or angiotenzin receptor, and it is also induced if the endogenous lysophosphatidic acid (LPA) receptors of the *Xenopus* oocyte are stimulated. These LPA receptors are also coupled to G_q protein, and they can also increase TRESK current 5 to 10-fold, although they are not overexpressed but present at their normal endogenous level. TRESK channel is also activated by the stimulation of the endogenous LPA receptors in DRG neurons, and this effect is missing in the control cells isolated from TRESK-deficient animals.

The substantial activation of TRESK is evoked by the increase of the cytoplasmic calcium concentration in response to the stimulation of G_q protein-coupled receptors. The microinjection of the calcium chelator EGTA into the cells expressing TRESK completely prevented the receptor-mediated activation of the channel. The calcium-dependent TRESK activation is induced by the calcium/calmodulin-dependent protein phosphatase calcineurin. Calcineurin binds directly to the PQIIIS and LQLP sequence motifs of TRESK, and dephosphorylates the serine residues in the positions 262, 264 and 267 of the intracellular loop region of the channel.

In the TRESK knock-out (KO) mouse model, the sensitivity to wide range of painful stimuli was substantially increased on the head/face region. The KO animals were more sensitive to painful mechanical, heat,

and chemical stimuli than their wild type (WT) littermates. The excitability was increased in the TRG neurons from the KO mice, compared to the cells from the WT animals. These experimental results are in good accordance with observation that a certain frameshift mutation of human TRESK (F139WfsX24) is associated with a rare form of inherited migraine headache. Interestingly, the response to painful stimuli was not (or was only slightly) increased on the body of the animal, in the regions innervated by the DRG neurons. Accordingly, the excitability of DRG neurons isolated from the KO animals was not much different from the WT, although the TRESK current was undoubtedly eliminated. Most probably, the compensatory overexpression of other K^+ channel types were more pronounced in the DRG neurons of the KO mice than in the TRG neurons, and this could have been responsible for the distinct changes of sensitivity to painful stimuli in the different body regions.

Ann-Kathrin Rahm et al. reported that human TRESK current is increased about 3-fold by phorbol 12-myristate 13-acetate (PMA, 100 nM) in about 30 minutes, in the *Xenopus* oocyte heterologous expression system. This effect was reduced by different protein kinase C (PKC) inhibitors, but was not affected by the inhibition of calcineurin. The mutations of the PKC phosphorylation consensus sequences of TRESK also failed to influence the degree of activation by PMA. These data suggested that the TRESK activation by PKC represented a new calcineurin-independent mechanism of regulation, thus we embarked on the investigation of the interaction between TRESK and PKC by pharmacological and molecular biological methods.

OBJECTIVES

1. The investigation of the relationship between the mechanisms of TRESK activation induced by phorbol 12-myristate 13-acetate (PMA) and calcineurin, by using mutant TRESK channel constructs, or by the successive stimulation of the two regulatory pathways.

2. The identification of PKC isoforms, which reproduce the effects of PMA and activate TRESK, when they are coexpressed with the channel.

3. The examination of the effect of PMA or the coexpression of PKC with TRESK under conditions, when the channel is phosphorylated by the appropriate microtubule-affinity regulating kinase (MARK) construct.

4. The adaptation of a biochemical method for the estimation of the phosphorylation state of TRESK channel. The direct detection of TRESK dephosphorylation by calcineurin and the investigation of the effect of PMA on the phosphorylation of TRESK.

METHODS

Plasmid constructs and cRNA synthesis

The *Xenopus* oocyte expression plasmid containing human TRESK channel cDNA was available in our laboratory. The novel-type PKC isoenzymes (η és ε) were amplified by polymerase chain reaction using Pfu polymerase, following reverse transcription (RT-PCR). The total RNA for this reaction was extracted from mouse brain with TRIzol reagent. PKCη cDNA was cloned in the pXEN *Xenopus* oocyte expression vector (GenBank EU267939.1) with EcoRI and XhoI restriction enzymes, while PKCε with Eco81I and XhoI. The cloning of MARK2 isoform 2 was previously reported by our workgroup, the appropriate kinase cDNA was available at the beginning of my work. MARK2Δ construct was produced by truncating MARK2 kinase at residue 361, using a PCR method.

The different mutant versions of TRESK channel and PKC enzymes were produced by QuikChange *in vitro* site-directed mutagenesis kit (Stratagene), according to the protocol of the manufacturer. The sequences of the primer pairs for the mutations were designed with the SeqHandler software developed by my supervisor. In addition to the desired mutation, the primers sequences also contained silent mutations, which did not change the encoded amino acid sequence, but introduced a new restriction enzyme site in the DNA for the identification of the mutant clones.

For the efficient detection of TRESK protein with the Western blot method, a double influenza hemagglutinin (HA) epitope tag was inserted in the N-terminus of the channel. The MEVSGHP N-terminal sequence of wild type TRESK was modified to MEVSG-(YPYDVPDYA)-

GG-(YPYDVPDYA)-GHP, where the two epitope tags are separated by two glycines and their sequences are indicated in brackets. In the HA_2-N70Q-hTRESK construct, the N70Q mutation was also introduced in order to prevent the N-glycosylation of the channel.

The cRNAs were synthesized *in vitro* by the mMESSAGE mMACHINE T7 Transcription kit, according to the manual of the manufacturer (Ambion). The integrity of the cRNA was verified by denaturing formaldehyde agarose gel electrophoresis and ethidium-bromide staining.

Preparation and microinjection of *Xenopus* oocytes

The oocytes were prepared from *Xenopus laevis* frogs, the follicular layer was removed mechanically from the surface of the cells by fine tweezers, after the digestion with collagenase. The cRNA was microinjected one day after the isolation of the cells. The electrophysiology measurements and biochemical experiments were performed three days after the injection of the cRNA.

Two-electrode voltage clamp

The TRESK current expressed in *Xenopus* oocytes was measured by the two-electrode voltage clamp method, using OC-725C amplifier (Warner Instrument Corp.). The data of the electrophysiology measurements were digitized by a Digidata Interface (Axon Instruments, Foster City, CA), and recorded by the components of the pClamp software package (Molecular Devices). The low $[K^+]$ extracellular solution contained in mM: NaCl 95.4, KCl 2, CaCl2 1.8, HEPES 5 (pH 7.5, NaOH). In the high (80 mM) $[K^+]$ solution, $[Na^+]$ was appropriately reduced, in

order to maintain the sum of [K^+] and [Na^+] as a constant value. The recording chamber was continuously perfused with the extracellular solutions by using a gravity-driven perfusion system.

The borosilicate glass microelectrodes (micropipettes) were filled with 3 M KCl, thus their resistance was in the range of 0.3-1 MΩ, and this salt solution was connected via Ag/AgCl electrodes to the recording apparatus. TRESK current was measured in the high [K^+] solution, at the end of 300 ms long voltage steps to -100 mV, repeated in each 4 s, at room temperature (21 °C). From this current, the current amplitude measured in the low [K^+] solution was subtracted. The endogenous K^+ current of the *Xenopus* oocytes injected with distilled water was at least an order of magnitude smaller than the current expressed in response to the injection of TRESK cRNA.

Investigation of the phosphorylation state of TRESK channel by using the Phos-tag™ SDS-PAGE method followed by anti-HA immunoblot

The plasma membrane fraction was isolated from different groups of *Xenopus* oocytes (containing equal numbers of cells), expressing the HA_2-N70Q-hTRESK construct, by applying the appropriate centrifugation steps after the homogenization of the cells in a glass potter homogenizer in lysis solution. The plasma membrane fraction was dissolved in SDS loading buffer, stored at -80 °C, and used in further experiments.

The different groups of oocytes were treated in different ways before the preparation of the plasma membrane fraction. The effect of PMA on TRESK phosphorylation was examined after the treatment of the cells with PMA (100 nM) for 45 min, in the presence of cyclosporin A (CsA, 1 μM) and FK506 (tacrolimus, 1 μM) calcineurin inhibitors. The

negative control cells, in which the phosphorylation of TRESK was maintained, were treated similarly in the presence of calcineurin inhibitors, but without PMA. In addition, two positive control groups were examined. In the first one, the plasma membrane preparation was *in vitro* dephosphorylated with λ phosphatase. In the second positive control group, the oocytes were incubated with ionomycin (1 μM) for 3 minutes, in the absence of calcineurin inhibitors.

The TRESK proteins, deriving from the oocyte groups after the different pretreatments, were separated by the Phos-tag™ SDS-PAGE method (Wako Pure Chemical Industries, Osaka, Japan). The Zn^{2+}-Phos-tag method was used in 8 % polyacrilamide gels with the neutral (Tris-MOPS) buffer system. The concentration of Phos-tag reagent was 15 μM. Before loading the samples, they were supplemented with $ZnCl_2$ and NaOH, in order to saturate free EDTA and to restore alkaline pH. In the case of the Phos-tag SDS-PAGE, the voltage for electrophoresis was 50 V.

After the separation on the Phos-tag gel, the proteins were blotted on nitrocellulose membrane by the wet/tank blotting method in Towbin buffer (25 mM Tris, 192 mM glicin, pH 8.6 ± 0.2) containing 20 % methanol and 0.05 % SDS. The nonspecific binding sites of the membrane were blocked with the mixture of 0.2 g/10 ml bovine serum albumin, 0.5 g/10 ml non-fat dry milk and 0.2 % Tween-20 detergent in phosphate-buffered saline (PBS). The primary antibody was monoclonal anti-HA IgG1 (26183, Clone 2-2.2.14, Thermo Scientific), which was diluted 10000-fold in PBS containing 10 % blocking buffer. The secondary antibody was goat anti-mouse IgG (R-05071-500; Advansta) conjugated to horseradish peroxidase (HRP). The membrane was washed once after blocking, and 4-6 times for 5-10 min after the antibodies with 20-30 ml

PBS containing 0.1% Tween-20. TRESK protein was detected with the enhanced chemoluminescence method, according to the instructions of the manufacturer (WesternBright ECL HRP, Advansta).

Evaluation of the data and statistical analysis

Data are expressed as mean ± S.E. The statistical difference was considered significant at $p<0.05$. The type of the statistical test used for the analysis was decided on the basis of the experimental model by also considering the properties of the obtained data with a predetermined algorithm. Normality of data distribution was estimated by the Shapiro-Wilk test and the homogeneity of variance by the Levene test. The data were most often anlysed by using the homo- or heteroscedastic Student's t test, one-way (Fisher) ANOVA followed by Tukey HSD post hoc test, or Welch analysis of variance followed by Games-Howell post hoc test. Mann-Whitney U test was used as a nonparametric test. Statistical calculations were performed with Statistica 13.4 (TIBCO Software, Tulsa, OK) or SPSS Statistics 25.0 (IBM Corporation). Analysis of densitometry data was performed with Wayne Rasband's ImageJ 1.47v software.

RESULTS

PMA significantly increased TRESK K^+ current in all examined *Xenopus* oocyte preparation, the activation was typically from 2 to more than 6-fold on average. In the first series of experiments we were curious about the relationship between the PMA-induced and calcineurin-dependent TRESK activation mechanisms. Therefore, we examined the effect of PMA on different mutant TRESK channels with defective calcineurin-dependent regulation.

We tested the effect of PMA on the TRESK-PQAAAS-AQAP construct, in which the PQIIIS and LQLP calcineurin-binding sequences were nonfunctional because of the alanine substitutions. In the oocytes expressing TRESK-PQAAAS-AQAP channel, after the application of PMA, the increase of the K^+ current was similar to that of the cells expressing the wild type channel. This indicates that elimination of the calcineurin-dependent activation mechanism does not prevent the effect of PMA on the channel, thus calcineurin does not mediate the PKC-dependent TRESK activation.

The same experiment was also performed by using a TRESK mutant, in which the serine 264 amino acid was replaced with alanine. This channel is constitutively active, since it mimics the dephosphorylated state at one of the most important regulatory sites of the channel. The K^+ current of the S264A mutant was not increased at all by PMA. If the phosphorylated state of the channels was imitated by glutamate substitution, then this S264E mutant TRESK channel was neither activated by PMA at all. This confirmed the result obtained with the S264A mutant;

if the phosphorylation-dependent regulation at the position S264 is compromised then this prevents the activation of TRESK by PMA.

We also examined whether the successive activation of protein kinase C and calcineurin (with PMA (100 nM) and ionomycin (0.5 μM), respectively) causes greater increase of the K^+ current than the activation of calcineurin alone, or these treatments result in the same degree of channel activation. If the effect of PMA relies on the phosphorylation state of S264, then the increase of TRESK current in response to the PMA+ionomycin treatment should not exceed that evoked by only ionomycin. Ionomycin applied after PMA did not result in larger final activation than the administration of ionomycin alone. This result is compatible with the hypothesis that the activation mechanisms induced by PMA and calcineurin converge on a common point, on the dephosphorylation of S264. This raised the question how the PMA treatment results in the dephosphorylation of serine 264 residue.

The return of TRESK current to the resting (inhibited) state after the calcineurin-dependent activation is caused by the function of the kinase(s) phosphorylating S264. The effect of PMA on this kinase was examined by measuring the recovery rate of the K^+ current after the activation of the channel with ionomycin. The oocytes expressing human wild type TRESK channel were stimulated with ionomycin (0.5 μM), or with the combination of ionomycin and PMA (100 nM), and the recovery rates of the K^+ current to the resting state were compared. If PMA was also applied in addition to ionomycin, then the recovery of the K^+ current to the resting state was much slower than that without PMA. This suggested that PMA induced the inhibition of the kinase being responsible for the phosphorylation of the regulatory residue S264 in the channel.

If the activation of PKC indirectly results in the dephosphorylation of S264, then this effect could be counterbalanced by the coexpression of another kinase, which directly phosphorylates the S264 residue. Our workgroup previously demonsrated that the region of S264 in TRESK channel is phosphorylated by three members of the microtubule-affinity regulation kinase family (MARK1-3). Thus we attempted to prevent the effect of PMA by the coexpression of MARK2 kinase with TRESK. We applied the MARK2Δ construct truncated at its regulatory region, since the activity of this construct was not affected by the PMA treatment. When the MARK2Δ construct was coexpressed with TRESK, the channel was not activated by PMA at all. However, the coexpression of MARK2Δ did not prevent channel activation in general. Ionomycin, applied at the end of the experiment, activated TRESK similarly in the presence of MARK2Δ as in the control group expressing only the channel. The coexpression of MARK2Δ with TRESK maintained the phosphorylation of S264 against the slow dephosphorylating effect induced by PMA; however, it can not counteract the overwhelming phosphatase activity of calcineurin binding directly to the channel.

The novel-type protein kinase C isoforms PKCδ, PKCθ, PKCη and PKCε were cloned from mouse brain RNA by polymerase chain reaction following reverse transcription (RT-PCR), and the effect of their coexpression with TRESK was investigated. PKCθ did not influence the properties of TRESK current, while PKCδ caused relatively small effect; therefore these isoforms have not been furher examined. PKCη and PKCε clearly influenced TRESK current and its regulation.

The coexpression of PKCη increased the TRESK current about two-fold on average, but the difference was not statistically significant owing to the large variation of channel expression in this experiment. Nevertheless, TRESK was activated more strongly by ionomycin in the control cells expressing only the channel (7.3 ± 0.3-fold, n=7) than in the cells coexpressing PKCη (4.2 ± 0.6-fold, n=5, $p<0.001$, Student's t test). The reduced apparent activation in response to ionomycin in the cells coexpressing PKCη with TRESK channel, and the approximately equal maximum current values in the two groups, suggest that TRESK channel was already activated by the coexpression of PKCη under basal conditions, before the application of ionomycin.

The coexpression of PKCη with TRESK also significantly influenced the return of the K^+ current to the resting state from the calcineurin-dependent activation. In the control cells, expressing only TRESK, the activation of the current recovered to 30 ± 5 % of the maximum value at the end of the measurement. In contrast, in the oocytes coexpressing PKCη with TRESK, 73 ± 8 % of the current remained active at the end of the measurement ($p<0.001$, Student's t-test). This result suggests that PKCη substantially reduced the inhibitory (re)phosphorylation of TRESK channel. The enhanced basal TRESK activity and the reduced recovery rate after ionomycin in response to PKCη are in good accordance with the pharmacological results obtained in the case of the PMA treatment, and confirms that novel-type PKC contributes to the regulation of TRESK by the inhibition of the kinase phosphorylating the channel.

The above observations were independently reproduced in another experiment by the coexpression of the human TRESK-S252A mutant with wild type PKCε isoform. In this experiment, the coexpression of the kinase-dead K437R mutant PKCε with the channel did not affect the regulation of TRESK. Thus the function of the S252 regulatory site and the anchoring of the 14-3-3 adapter protein are not required for the PKC-dependent TRESK activation; however, the enzyme activity of PKCε kinase is indispensable for the effect.

We have also examined whether MARK2Δ prevents the effect of the coexpression of PKC on TRESK, similarly to the elimination of the PMA-evoked channel activation, as described above. In this experiment, the constitutively active A159E mutant version of PKCε was used, which has a higher kinase activity than that of the wild type enzyme under resting conditions. If this PKCε-CA construct of augmented activity was coexpressed with TRESK, then a high amplitude basal K^+ current was measured (6.5 ± 1.2 μA, n=10). The channel was activated to such an extent that ionomycin could increase the current further only to 1.5 ± 0.1-fold. Moreover, the K^+ current did not recover at all toward the resting state after the withdrawal of ionomycin; the activation reached 154 ± 25 % level at the end of the measurement.

However, if MARK2Δ kinase was also coexpressed with TRESK in addition to PKCε-CA (triple coexpression), then the basal K^+ current was reduced to 2.6 ± 0.6 μA (n=9, $p<0.01$, t-test). This smaller current was more strongly activated by ionomycin (3.2 ± 0.3-fold, $p<0.002$, t-test). Furthermore, the K^+ current completely recovered to the resting state in 5 minutes after the withdrawal of ionomycin, only 9 ± 5 % of the activation

remained at the end of the measurement ($p<0.0005$, Mann-Whitney U test). These results indicate that the constitutively active PKCε exceedingly increased TRESK basal activity. The majority of the channels were already in the dephosphorylated state, since the constitutively active PKCε inhibited the endogenous kinase phosphorylating S264. However, MARK2Δ, which was designed not to be regulated by PKCε, restored the basal phosphorylation of S264 and the resting inhibited state of TRESK, and also accelerated the rephosphorylation of the channel after the withdrawal of ionomycin.

By applying the Phos-tag™ SDS-PAGE method, we have also investigated the phosphorylation status of HA_2-N70Q-hTRESK protein in plasma membrane fractions prepared from *Xenopus* oocytes. This method is based on the Phos-tag™ reagent covalently incorporated into the SDS-PAGE gel matrix. The Zn^{2+} complex of Phos-tag™ reversibly binds the phosphate group of phosphoproteins, and thus reduces their migration during the electrophoresis. This method, after the partial compensation for its several initially unexpected disadvantages, was found to be suitable for the investigation of TRESK phosphorylation. We have demonstrated for the first time that the TRESK protein located in the plasma membrane of the cell is indeed dephosphorylated in response to the activation of calcineurin. In addition, we verified that the treatment of the cells with PMA undoubtedly results in the dephosphorylation of TRESK protein.

CONCLUSIONS

1. TRESK channel activation by phorbol 12-myristate 13-acetate (PMA) is not mediated via the calcium/calmodulin-dependent protein phosphatase calcineurin. However, the effect of PMA relies on the dephosphorylation of serine 264, which is located in the intracellular loop region of TRESK, and it is a major regulator of channel activity and also the substrate of calcineurin.

2. Novel-type protein kináz C epsilon (ε) and eta (η) isofroms activate human TRESK channel in the *Xenopus laevis* heterologous expression system. The pharmacological activation of protein kinase C with PMA and the coexpression of PKCε (or PKCη) similarly influence TRESK activity and the calcium-dependent regulation of the channel.

3. Protein kinase C results in the dephosphorylation of TRESK by the inhibition of the endogenous kinase phosphorylating serine 264. In the absence of the kinase activity phosphorylating S264, the channel is slowly dephosphorylated (in 30-60 minutes), and the amplitude of TRESK K^+ current increases.

4. The microtubule-affinity regulating kinase 2 (MARK2) phosphorylates serine 264 residue in TRESK channel, and accordingly the coexpression of the truncated version of MARK2 prevents the effect of novel-type PKC on the channel.

PUBLICATIONS

Publications relevant to the dissertation:

1. **Pergel E.**, Lengyel M., Enyedi P., Czirják G. (2019) TRESK (K2P18.1) Background Potassium Channel Is Activated by Novel-Type Protein Kinase C via Dephosphorylation. *Mol Pharmacol.* 95:661-672. **(**IF: 3.853)

2. Lengyel M., Erdélyi F., **Pergel E.**, Bálint-Polonka Á., Dobolyi A., Bozsaki P., Dux M., Király K., Hegedűs T., Czirják G., Mátyus P., Enyedi P. (2019) Chemically Modified Derivatives of the Activator Compound Cloxyquin Exert Inhibitory Effect on TRESK (K2P18.1) Background Potassium Channel. *Mol Pharmacol.* 95:652-660. **(**IF: 3.853)

Other publications:

3. Kubinyi E., Bence M., Koller D., Wan M., **Pergel E.**, Rónai Z., Sasvári-Székely M., Miklósi Á. (2017) Oxytocin and Opioid Receptor Gene Polymorphisms Associated with Greeting Behavior in Dogs. *Front Psychol.* 8:1520. **(**IF: 2.089)

4. Kolacsek O., **Pergel E.**, Varga N., Apáti Á., Orbán T.I. (2017) Ct shift: A novel and accurate real-time PCR quantification model for direct comparison of different nucleic acid sequences and its application for transposon quantifications. *Gene.* 598:43-49. **(**IF: 2.498)

5. Szebényi K., Füredi A., Kolacsek O., **Pergel E.**, Bősze Z., Bender B., Vajdovich P., Tóvári J., Homolya L., Szakács G., Héja L., Enyedi Á., Sarkadi B., Apáti Á., Orbán T.I. (2015) Generation of a Homozygous Transgenic Rat Strain Stably Expressing a Calcium Sensor Protein for Direct Examination of Calcium Signaling. *Sci Rep.* 5:12645. **(**IF: 5.228)

6. Kis A., Bence M., Lakatos G., **Pergel E.**, Turcsán B., Pluijmakers J., Vas J., Elek Z., Brúder I., Földi L., Sasvári-Székely M., Miklósi A., Rónai Z., Kubinyi E. (2014) Oxytocin receptor gene polymorphisms are associated with human directed social behavior in dogs (Canis familiaris). *PLoS One.* 9:e83993. **(**IF: 3.234)