Targeting of Calcineurin to an NFAT-like Docking Site Is Required for the Calcium-dependent Activation of the Background $K^+$ Channel, TRESK

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The two-pore domain $K^+$ channel, TRESK (TWIK-related spinal cord $K^+$ channel) is activated in response to the calcium signal by the calcium/calmodulin-dependent protein phosphatase, calcineurin. In the present study we report that calcineurin also interacts with TRESK via an NFAT-like docking site, in addition to its enzymatic action. In its intracellular loop, mouse TRESK possesses the amino acid sequence, PQIVID, which is similar to the calcineurin binding consensus motif, PXIXIT (where X denotes any amino acid), necessary for NFAT (nuclear factor of activated T cells) activation and nuclear translocation. Mutations of the PQIVID sequence of TRESK to PQIVIA, PQIVAD, or PQAVAD increasingly deteriorated the calcium-dependent activation in the listed order and correspondingly reduced the benzocaine sensitivity. Increasingly deteriorated the calcium-dependent activation in the PQIVID sequence of TRESK to PQIVIA, PQIVAD, or PQAVAD is necessary for NFAT (nuclear factor of activated T cells) activation and nuclear translocation. The intracellular loop of TRESK, expressed as a GST fusion protein, binds constitutively active calcineurin in vitro. PQAVAD mutation as well as addition of VIVIT peptide to the reaction abrogated this calcineurin binding. Wild type calcineurin was recruited to GST-TRESK-loop in the presence of calcium and calmodulin. These results indicate that the PQIVID sequence is a docking site for calcineurin, and its occupancy is required for the calcium-dependent regulation of TRESK. Immunosuppressive compounds, developed to target the NFAT binding site of calcineurin, are also expected to interfere with TRESK regulation, in addition to their desired effect on NFAT.

Two-pore domain potassium (2PK)$^3$ channels give rise to background (leak) potassium conductance and their diverse regulatory mechanisms control cellular function by adjusting both the resting membrane potential and excitability (for review, see Refs. 1 and 2). TRESK (TWIK-related spinal cord $K^+$ channel) is uniquely regulated by the calcium signal among the 2PK$^+$ channels. We have recently reported that TRESK, expressed heterologously in Xenopus oocytes, is activated about 10-fold by the calcium/calmodulin-dependent protein phosphatase, calcineurin (3). TRESK was cloned from human spinal cord (4) and mouse cerebellum (3), and its expression was also demonstrated by reverse transcription-PCR in cerebrum, brainstem, testis, pancreas, and placenta (5–7). A massive signal was detected by Northern blot in rat thymus and spleen (7). Single channel activity of TRESK has recently been demonstrated in dorsal root ganglion neurons (8). In the absence of specific antibodies and inhibitors, TRESK has neither been identified at the protein level nor has it been detected as an endogenous whole cell current. However, the robust activation of TRESK by the calcium signal implicates that it can influence substantially the function of the native cells expressing the channel.

The calcineurin-mediated regulation suggests that TRESK activation is the target of the widely used immunosuppressive drugs, cyclosporine A and FK506, as it was, in fact, demonstrated in Xenopus oocytes (3). These drugs inhibit calcineurin (and consequently NFAT activation and interleukin-2 production of T lymphocytes) by forming inhibitory complexes with the ubiquitous immunophilin proteins (9, 10). However, general inhibition of calcineurin also causes several undesired effects. Therefore, a novel direction of drug development focuses on the NFAT-docking site of calcineurin, which is considered to be a more specific target than the phosphatase activity (11, 12). The binding of calcineurin to the PXIXIT consensus motif (where X denotes any amino acid) is required for NFAT activation. Apart from NFAT, the only known mammalian proteins possessing similar calcineurin binding sites are calcineurin inhibitors/modulators or anchoring proteins (13, 14). In the present study we report for the first time that an ion channel, TRESK, has an NFAT-like calcineurin binding consensus sequence and the binding of the phosphatase to this docking site is indispensable for the regulation of the channel. Therefore, the inhibitors, designed to block the calcineurin-NFAT interaction specifically, may also interfere with TRESK activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—VIVIT peptide (NFAT inhibitor) and calcineurin autoinhibitory peptide (ITSFEEAKGLDRINERMPPPRDAMP) were purchased from Calbiochem, bovine calcineurin and calmodulin from Sigma. Enzymes and kits of molecular biology applications and all other chemicals of analytical grade were obtained from the companies listed elsewhere (3).

**GST-TRESK Loop Fusion Proteins**—To produce GST-TRESK loop fusion protein, the cDNA encoding the intracellular loop of mouse TRESK (amino acids 164–292) was PCR-amplified from our pEXO-TRESK plasmid (3), applying consecutively Loop-1/Loop-a1 and Loop-s2/Loop-a1 primer combinations (see the sequences and cloning sites in Table 1). The PCR product was ligated into the XhoI site of pGEX-4T-1 (Amersham Biosciences, Little Chalfont, UK). To obtain the longer GST-TRESKLoop-TAPtag protein, TRESK loop was amplified from our pEXO-TRESK plasmid (3), applying consecutively Loop-s1 and Loop-a2 oligonucleotides. The PCR product was cloned...
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TABLE 1
Oligonucleotide sequences for PCR and in vitro site-directed mutagenesis

| Primer              | Sequence (5' → 3')          | Restriction enzyme |
|---------------------|-----------------------------|--------------------|
| Loop-s1             | CCA GCC ATC CCC ACC TGA TAT ATT CTC GCC ACC ATC | KpnI               |
| Loop-s2             | CCA GCC GCC ACC TGA TAT ATT GCC TCA ACC TGT | XhoI               |
| Loop-a1             | GGG GCC ATG TCA GCC CTC TCC ACC TGT | XhoI               |
| Loop-a2             | GGG GCC ATG TCA GCC CTC TCC ACC TGT | EcoRI               |
| TAP-a               | CAC CAA TAA CTA ATT GGG GCC GAC ACC | XhoI               |
| mCnA-s              | CC GCT GGC GAG CAT ACT AT A T CG T CG T GAC | NdeI               |
| mCnA-d              | GGC CTA TAT GCT GAT GAC TGG TCT TTC CGG G | XhoI               |
| hCnB-s              | GGC CTA GAG GAG CAT ACT AT A T CG T GC T GAC | XhoI               |
| hCnB-a              | GGC CTA GAG GAG CAT ACT AT A T CG T GC T GAC | BamHI               |
| PQIVIA-s            | CAG AGT CCT GCT GGT GAG AAC CAC CTC | PvuII               |
| PQIVAD-s            | CCA CCT CTC GAT CTA GCT GGT GAG AAC CAC CTC | Cfr10I             |
| PQAVAD-s            | GAA GCT CTC CCT GGT GTC CTC GAG TGC GAT GTA AAC | Cfr10I             |

into the pcDNA3-TAPtag plasmid (SérAPHIN Laboratory, EMBL, Heidelberg, Germany). The fused TRESKloop-TAPtag coding region was amplified with the Loop-s2/TAP-a primer combination and ligated into the Sall site of pGEX-6P-3 (Amersham Biosciences). QuikChange™ in vitro site-directed mutagenesis was performed as described previously (3).

The GST-TRESKloop fusion constructs were expressed in BL21 strain of Escherichia coli. Bacteria were sonicated in buffer A, containing in mM: 50 NaCl, 50 KCl, 1 EDTA, 0.2% Triton X-100, 1% Tween 20, 50 Tris-HCl (pH 7.6). The lysate was centrifuged and the GST fusion protein was affinity-purified from the supernatant by glutathione-agarose (Sigma).

Recombinant, Constitutively Active Calcineurin—The hexahistidine-tagged constitutively active calcineurin was produced by a modification of the methods reported previously (15). Coding regions of the truncated mouse calcineurin A (α-isoform, amino acid residues 1–398) and human calcineurin B subunits were amplified from pBluescript-KS-FL and pBluescript-KS-B plasmids (16), applying the mCnA-s/mCnA-del and hCnB-s/hCnB-del primer combinations, respectively. The calcineurin A and B products were ligated into the same pET15b vector (Novagen), between the Ndel and BamHI sites. BL21 strain of E. coli was cotransformed with pBluescript-KS-FL containing myristoyl-CoA:protein N-myristoyltransferase (17, 18) to modify calcineurin B post-translationally and the bicistronic plasmid encoding calcineurin A and B. Bacteria were grown in LB containing 50 μg/ml kanamycin and 130 μg/ml ampicillin and induced with isopropyl β-D-thiogalactopyranoside (0.5 mM) in the presence of myristic acid (0.24 mM) for 3 h. Bacteria were sonicated in buffer B, containing: 200 NaCl, 2 MgCl₂, 1 EGTA, 5.5 β-mercaptoethanol, 30 phosphate (pH 7.8 with NaOH), supplemented with 1 PMSF, 0.2 benzamidine, 5 imidazole (in mM) and 30 soybean trypsin inhibitor (type II, Sigma), 16 leupeptin, 1 aprotinin (in μg/ml). The lysate was centrifuged, and the protein was purified from the supernatant by nickel-nitrilotriacetic acid-agarose (Qiagen); after binding for 1 h, washing with buffer containing 0, 20, and 50 mM imidazole, in the first, second, and third pair of washing steps, respectively. Calcineurin was eluted by 5 × 0.5 ml of 250 mM imidazole in buffer B and dialyzed against 140 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 0.1 mM diithiothreitol, 20 mM Tris-HCl (pH 7.3) and stored at −70°C.

GST Pulldown Assay—Ten μl of the 50% resin suspension of the appropriate GST fusion protein was incubated for 1 h with or without full-length bovine calcineurin (1.5 μg), recombinant constitutively active calcineurin (5–10 μg), calmodulin (2.5 μg), VIVIT peptide (200 μM), calcineurin autoinhibitory peptide (200 μM), CaCl₂ (0.25 mM), in the presence of 1% Triton X-100. The final volume was adjusted with a buffer containing (in mM): 50 NaCl, 50 KCl and 50 Tris-HCl (pH 7.6) (plus 1 mM EDTA in the reactions containing recombinant calcineurin). The resin was washed twice with 150 μl of assay buffer, also containing 1% Triton X-100 in the first wash, and 0.3 mM CaCl₂ if full-length calcineurin was used. The bound proteins were analyzed on 12% polyacrylamide gels stained with Coomassie Brilliant Blue or silver, according to Heukeshoven and Dernick (19).

Handling of Xenopus laevis Oocytes and Two-electrode Voltage Clamp Measurements—The oocytes were prepared, the CRNA was synthesized, and microinjected and two-electrode voltage clamp measurements were performed as described previously (3). The low [K⁺] solution was replaced with K⁺ (in mM): 50 NaCl, 2 KCl, 1.8 CaCl₂, 5 HEPES (pH 7.5 with NaOH). The high [K⁺] solution contained 80 mM K⁺ (78 mM Na⁺) of the low [K⁺] solution was replaced with K⁺. The voltage protocol for measuring TRESK current and the calcium-activated chloride current simultaneously as increasing: −100 mV for 300 ms, 0 mV for 250 ms, and +20 mV for 300 ms, applied every 1 s from a holding potential of 0 mV. TRESK current and the calcium-activated chloride current (IₗCaCl) were measured at the end of the voltage steps to −100 mV and +20 mV, respectively. As we reported previously (20, 21), IₗCaCl at −100 mV in 80 mM [K⁺] was negligible compared with the robust K⁺ current expressed 2P(K) channels. Thus IₗCaCl was estimated by applying voltage steps to +20 mV, which is close to the K⁺ equilibrium potential. The initial IₗCaCl could be measured independently from the K⁺ current, since TRESK activation was delayed compared with the onset of IₗCaCl.

All treatments of the animals were conducted in accordance with state laws and institutional regulations. The experiments were approved by the Animal Care and Ethics Committee of the Semmelweis University.

Statistics and Calculations—Data are expressed as means ± S.E. Statistical significance was estimated by t test for dependent or independent samples, one-way ANOVA or two-way repeated measures ANOVA and Scheffe’s test for pairwise post hoc comparisons using the Statistica 6.0 program package (StatSoft, Tulsa, OK). The difference was considered to be significant at p < 0.05. Free [Ca²⁺] was calculated with Checlator program (written by Theo J. M. Schoenmakers, University of Nijmegen, Nijmegen, The Netherlands). Some sequence operations (e.g. automatic design of silent mutations to QuikChange primers for selecting the mutant clones) were performed with the sequence-handling program “SeqHandler” developed in our laboratory.

RESULTS

The Local Anesthetic Benzocaine Distinguishes Activated TRESK from the Resting Channels—Reduced stimulation of a mutant TRESK channel by the calcium signal may reflect the impaired mechanism of calcineurin-dependent activation. However, a constitutively active mutant channel, which is nearly maximally active even under basal conditions, is also expected to be unresponsive to the calcium signal. To
Sincethemutationofaprolineresidueoftenresultsinmajorstructural
changes and apart from the proline only the two isoleucines match
directly to the PQXIT consensus motif, we mutated one or both of these
isoleucines (PQIVAD and PQAVAD mutants). In addition, we also
examined the PQIVIA mutant to elucidate whether the aspartate
residue, which does not correspond to the NFAT consensus motif,
can substitute for the threonine and strengthen the binding to calcineurin.
Expression of all the mutants yielded functional channels in Xenopus
oocytes; however, their responses to the calcium signal, evoked by the
calcium ionophore ionomycin, were affected (Fig. 2A). The response of
the PQIVIA mutant was attenuated (4.1 ± 0.3-fold activation (n = 5)
in contrast to the 7.5 ± 0.6-fold (n = 6) increase of the wild type), suggest-
ing that the aspartate residue promoted the binding of calcineurin, and
its mutation to alanine decreased the affinity to the phosphatase. Mut-
tion of the second isoleucine of the motif (PQIVAD mutation) strongly
reduced the ionomycin-evoked activation (only 1.6 ± 0.1-fold increase,
n = 5). The mutation of both critical isoleucines (PQAVAD) had an
even stronger effect; the calcium-dependent regulation was completely
eliminated (1.1 ± 0.1-fold current after the stimulation, n = 5, Fig. 2A).
The reduced response of the mutant channels to the calcium signal
suggests that the PQIVAD sequence of TRESK, similarly to the PQXIT
motifs of NFAT proteins, is a functionally important calcineurin dock-
ing site.

MutationsofthePQIVADSequenceofTRESKPreventtheCalcium-
dependentActivation—Initsintracellularloop,mouseTRESKpos-
poses the PQIVAD sequence (amino acids 210–215), which is highly
similar to the calcineurin binding consensus motif (PQXIT) of NFAT
proteins. To examine the role of this motif in the calcium-dependent
regulation, different mutants of TRESK channel were created by replac-
ing distinct amino acid residues of the PQIVAD sequence with alanines.
Since the mutation of a proline residue often results in major structural

![Figure 1](image1.png)

**FIGURE 1.** Differential inhibition of resting and activated TRESK channels by benzocaine. A, currents of an oocyte expressing mouse TRESK were measured at the end of 300-ms voltage steps to −100 mV applied every 3 s from 0-mV holding potential. (The extracellular [K⁺] was changed from 2 to 80 mM and back, as indicated.) TRESK current was stimulated with ionomycin (0.5 μM, gray bar). Sensitivity of TRESK to benzocaine (1 μM) was tested by brief applications of the anesthetic, as indicated by black bars. B, statistical representation of the inhibition of wild type TRESK evoked by benzocaine before and after ionomycin stimulation. The inhibition of the current of S276A constitutively active mutant of TRESK by benzocaine is also shown. (The current of the S276A mutant has not been stimulated with ionomycin.) The numbers in the bars indicate the number of measured oocytes.

![Figure 2](image2.png)

**FIGURE 2.** Mutations of the PQIVAD motif reduce the activation induced by ionomycin, stimulation of M₁ muscarinic acetylcholine receptor, or microinjection of a saturated calcium buffer. A, TRESK currents of the oocytes expressing the wild type (wt, PQIVAD), PQIVIA, PQIVAD, or PQAVAD mutant channels (as indicated on the right side) were stimulated with ionomycin (Iono., 0.5 μM, gray bar). After ionomycin application, the currents were inhibited with benzocaine (Benzo., 1 μM, black bar). TRESK currents were normalized to the resting value measured at 0 min. All the mutant channels were statistically different from the wild type in the respect of their ionomycin activation and benzocaine sensitivity (n = 5–6, p < 0.001). B, wt type or the same mutant TRESK channels as in A were coexpressed with M₁ muscarinic receptor. The oocytes were stimulated with carbachol (1 μM) as indicated by the gray bar. The activation of the PQIVAD and PQAVAD mutants was significantly smaller than that of the wild type (n = 5 each, p < 0.01). C, the oocytes expressing the wt type or the mutant channels were microinjected (as indicated by the vertical arrow) with 50 nl of a saturated calcium buffer (50 mM EGTA, 50 mM CaCl₂, 50 mM HEPES (pH 7.3 with KOH); calculated free [Ca²⁺] = 30 μM after an estimated 10-fold dilution in the cytoplasm). The activation of the PQAVAD mutant was reduced significantly, compared with the wild type (n = 5 each, p < 0.05). (Statistical difference of the activation of the distinct channels was determined with ANOVA followed by pairwise comparisons with Scheffe’s test.)
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In the same experiment, by applying our newly discovered pharmacological tool, benzocaine, we also demonstrated that the mutations of the PQIVID sequence really interfered with the process of activation and did not render TRESK constitutively active. The benzocaine sensitivity, measured after the ionomycin stimulation, was reduced in line with the smaller responsiveness to the calcium signal. Whereas the wild type TRESK current was inhibited by 57 ± 2% (n = 5), the sensitivity of the PQIVIA, PQIVAD, and PQAVAD mutants was attenuated (44 ± 1% inhibition, n = 5), strongly reduced (23 ± 1%, n = 5), and decreased nearly to the level characteristic for the resting channels (18 ± 1%, n = 5), respectively (Fig. 2A). These pharmacological data confirm the conclusion that the more severe is the perturbation of the binding motif, the more impaired is the interaction with calcineurin, and consequently, the higher fraction of the mutant channels remains in the resting state after the calcium signal.

To verify that the different levels of activation of TRESK mutants were not due to the difference in the calcium signal, we measured the endogenous calcium-activated chloride current (IClCa), reflecting the cytoplasmic [Ca2+] of the oocytes. The amplitude of IClCa, measured at +20 mV in 80 mM extracellular [K+], 9–15 s after the administration of ionomycin, was not significantly different in the oocytes expressing the different TRESK mutants (data not shown). This indicates that the reduced activation of TRESK mutants was not the consequence of the altered Ca2+ homeostasis of the cell, but it was the inherent property of the mutant channels.

The Activation of the PQIVID Mutants Is Reduced Irrespective of the Mechanism of the Calcium Signal—The levels of activation of the wild type and mutant TRESK channels were also compared, when the calcium signal was induced in a more physiological manner, by stimulation of M1 muscarinic ACh receptors, coexpressed with the channel. Whereas carbachol (1 μM) activated wild type TRESK efficiently, the response of the PQIVIA, PQIVAD, and PQAVAD mutants were attenuated, strongly reduced and eliminated, respectively (Fig. 2B), as in the case of stimulation with ionomycin. These results suggest that the receptor-mediated activation of TRESK also depends on the binding of calcineurin to the PQIVID motif.

In Xenopus oocytes, ionomycin increases the cytoplasmic [Ca2+] mainly by releasing the ion from intracellular stores (22), and the stimulation of M1 receptor also results in calcium release. To activate TRESK independently from the calcium stores, the cytoplasmic [Ca2+] was controlled directly by microinjecting a saturated calcium buffer of high (50 mM Ca2+ plus 50 mM EGTA) concentration. The order of responsiveness of wild type TRESK and the PQIVID mutants to the microinjection was the same as those obtained with ionomycin and carbachol (Fig. 2C). This indicates that the activation of TRESK may generally rely on the intact PQIVID motif in the case of calcium signals of different source and kinetics.

Microinjection of VIVIT Peptide Eliminates the Calcineurin-dependent Activation of TRESK—The sequence of VIVIT peptide (MAGPHPVIVITGPHEE) was optimized for the highest affinity toward the NFAT binding site of calcineurin (23). To provide a further in vivo evidence for TRESK-calcineurin interaction in addition to TRESK mutants, we also attempted to block the interaction by saturating the NFAT binding site of calcineurin with VIVIT peptide. Xenopus oocytes expressing wild type TRESK were microinjected with the peptide and subsequently challenged with ionomycin. These oocytes failed to respond with TRESK activation to the stimulation (Fig. 3A). The effective inhibition indicated that VIVIT peptide occupied the NFAT-docking site of calcineurin and thus prevented the binding of the intracellular loop of TRESK, which would have normally interacted with the same region of the phosphatase.

The dependence of TRESK regulation on calcineurin raised the question whether the basal K+ current was also the result of the calcineurin activity in the resting cell. To suppress the resting calcineurin activity by reducing the cytoplasmic calcium concentration to subphysiological levels, EGTA was microinjected to the oocytes expressing TRESK, and the background K+ current was measured before and after the microinjection. It was reported previously (3) that the 50-nl injection of a high (50 mM) concentration of EGTA was sufficient (even after the estimated 10-fold dilution of the chelator in the cytoplasm) to eliminate the calcium-dependent TRESK activation. However, an identical EGTA injection failed to influence the basal TRESK current (Fig. 3B), indicating that a calcium-dependent calcineurin activity did not contribute to the maintenance of the basal TRESK current. As an alternative approach to extinguish the possible basal calcineurin action on TRESK independently from calcium, the TRESK-interacting surface of calcineurin was blocked with VIVIT peptide. The microinjection of VIVIT peptide did not reduce the basal TRESK current (Fig. 3B), also confirming that calcineurin was not required for the resting TRESK activity.

The PQIVID Motif of TRESK Binds Constitutively Active Calcineurin Permanently and Wild Type Calcineurin Calcium/Calmodulin-dependently—To demonstrate the direct binding of the PQIVID motif of TRESK to calcineurin, GST pulldown experiments were performed with a GST-TRESKloop-TPepTag fusion protein (see “Experimental Procedures”), immobilized on glutathione-agarose beads. The constitutively active form of calcineurin (A subunit lacking the autoinhibitory domain and the calmodulin binding site but complexed with B subunit) was incubated with this resin. The characteristic band of the truncated calcineurin A could be easily detected with Coomassie Blue staining (compare lanes 1 and 2 in Fig. 4A) if all proteins bound to the resin were
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The Calcineurin Autoinhibitory Peptide Diminishes TRESK Activation but Does Not Interfere with the Binding of Calcineurin to the PQIVID Motif—As the binding of calcineurin to the PQIVID motif was indispensable for TRESK activation, the question emerged whether this binding was sufficient on its own or whether the phosphatase activity of calcineurin was also required for the regulation. To suppress the phosphatase activity without occluding the protein–protein interaction, a peptide corresponding to the autoinhibitory domain (467–491 amino acids) of calcineurin was microinjected. This microinjection inhibited the ionomycin-induced activation of wild type TRESK (Fig. 5A), suggesting that the phosphatase activity of endogenous calcineurin was required for TRESK activation.

To demonstrate that the autoinhibitory peptide did not influence the binding of calcineurin to the PQIVID motif, recombinant constitutively active calcineurin was used. We reported previously that the coexpression of the truncated calcineurin A subunit (1–398 amino acids, devoid of the autoinhibitory domain) and full-length calcineurin B with TRESK resulted in permanent activation of the channel (3). Inhibition of the phosphatase activity by the reintroduction of the autoinhibitory domain by peptide microinjection substantially reduced the otherwise permanently stimulated TRESK current (Fig. 5B). On the other hand, the autoinhibitory peptide, blocking only the active site of the phosphatase, did not influence the binding of the constitutively active calcineurin to the PQIVID motif in our GST pulldown experiments (compare lanes 3 and 4 in Fig. 5C). The reduction of TRESK current by the selective inhibition of the phosphatase activity supports the conclusion that dephosphorylation is also required for TRESK regulation in addition to the binding of calcineurin to the PQIVID motif.
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DISCUSSION

Several proteins are substrates of calcineurin (24); however, only a small subset of them interacts directly with the calcium/calmodulin-dependent phosphatase through an NFAT-like docking site (13, 14, 25–27). Previously, we demonstrated unequivocally that the calcium-dependent regulation of the two-pore domain K+ channel, TRESK, is mediated by calcineurin (3). Alanine scanning mutagenesis of all intracellular serine and threonine residues of TRESK indicated that Ser276, located in the intracellular loop, is a likely candidate for dephosphorylation, since the S276A mutation, mimicking the dephosphorylated state, rendered the channel constitutively active (3).

In the present study we report another aspect of TRESK-calcineurin interaction. In addition to the enzyme-substrate relation, calcineurin also binds to the NFAT-like PQIVID motif of mouse TRESK. This binding motif does not resemble the sites dephosphorylated by calcineurin at all and it has been shown in the case of NFAT proteins that a surface of the phosphatase, other than the substrate binding region, connects to PXIXIT motifs (28). Interestingly, our GST pulldown experiments indicated that the regulation of calcineurin activity essentially influenced TRESK-calcineurin interaction. The constitutively active calcineurin devoid of the autoinhibitory domain bound to the PQIVID motif permanently. In contrast, the binding of the wild type enzyme required (or at least it was enhanced drastically by) the presence of calcium/calmodulin complex. This substantial change of affinity may result in the shuttling of calcineurin between the cytoplasm and the PQIVID site during the calcium signal.

Beyond the in vitro results, we provided compelling evidence that the association of the two proteins is indispensable for the regulation of TRESK in Xenopus oocytes. The channel was activated efficiently by the calcineurin A (pBJ5-CnA-FL) and calcineurin B (pBJ5-CnB), and Jill Manchester and Jeffrey Gordon for the N-myristoyltransferase (pBB131) plasmid constructs. We thank Irén Veres and Beáta Busi for the expert technical assistance.

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