TRESK Background K$^+$ Channel Is Inhibited by Phosphorylation via Two Distinct Pathways*[s]

Gábor Czirják[1] **and Péter Enyedi**[2]

From the Department of Physiology, Semmelweis University, H-1444 Budapest, Hungary

The two-pore domain K$^+$ channel, TRESK (TWIK-related spinal cord K$^+$ channel, KCNK18) is directly regulated by the calcium/calmodulin-dependent phosphatase calcineurin and 14-3-3 adaptor proteins. The calcium signal robustly activates the channel via calcineurin, whereas the anchoring of 14-3-3 interferes with the return of the current to the resting state after the activation in *Xenopus* oocytes. In the present study, we report that the phosphorylation of TRESK at two distinct regulatory regions, the 14-3-3 binding site (Ser-264) and the cluster of three adjacent serine residues (Ser-274, Ser-276, and Ser-279), are responsible for channel inhibition. The phosphorylation of Ser-264 by protein kinase A accelerated the return of the current of S276E mutant TRESK to the resting state after the calcineurin-dependent activation. In the presence of 14-3-3, the basal current of the S276E mutant was reduced, and its calcineurin-dependent activation was augmented, suggesting that the direct binding of the adaptor protein to TRESK contributed to the basal inhibition of the channel under resting conditions. Unexpectedly, we found that 14-3-3 impeded the recovery of the current of S264E mutant TRESK to the resting state after the calcineurin-dependent activation, despite of the mutated 14-3-3 binding site. This suggests that 14-3-3 inhibited the kinase phosphorylating the regulatory cluster of Ser-274, Ser-276, and Ser-279, independently of the direct interaction between TRESK and 14-3-3. In conclusion, two distinct inhibitory kinase pathways converge on TRESK, and their effect on the calcineurin-dependent regulation is differentially modulated by the functional availability of 14-3-3.

The two-pore domain K$^+$ channel, TRESK[3] (TWIK-related spinal cord K$^+$ channel, KCNK18) is a major background K$^+$ channel of pseudounipolar sensory neurons (1–3). Abundant expression of TRESK mRNA and protein has been detected in dorsal root ganglia (DRG) (1, 2). TRESK cDNA was originally cloned from human spinal cord (4), and subsequently from mouse cerebellum (5) and testis (6). The distribution of the immunolocalization of TRESK in different central nervous system regions has also been recently reported (7). In DRG neurons, TRESK current was reliably identified at the single channel level (1). It was found to be the most prominent determinant of the resting K$^+$ conductance of the plasma membrane together with another two-pore domain K$^+$ (K2P) channel, TREK-2 (1, 2). TRESK has recently attracted particular attention, because of its efficient inhibition by the paresthesia-inducing pungent agents of the medicinal herb Szechuan pepper (8) and because of its probable role in nociception (9–11).

Although TRESK is abundant in DRG neurons, its coexpression with other two-pore domain K$^+$ (K2P) channels (against which no selective inhibitors exist at present) impedes its examination as a whole cell current. Therefore we embarked on the investigation of this important K$^+$ channel in a heterologous system, in *Xenopus laevis* oocytes. We demonstrated that, unlike the other K2P channels (12–17), TRESK is activated by the calcium signal (5). Calcineurin, a calcium/calmodulin-dependent protein phosphatase, was responsible for the activation (5). We have shown that calcineurin directly interacts with a Nuclear Factor of Activated T cells (NFAT)-like docking motif of TRESK (18). This interaction with calcineurin via the NFAT-like motif is unique for TRESK among the ion channels. In addition to the enzymatic activation of the phosphatase, the calcium/calmodulin complex also stimulated the docking of calcineurin to the NFAT-like motif of TRESK (18). A surface of the phosphatase distinct from the active site of the enzyme is involved in the interaction (19), and the affinity of TRESK for calcineurin is the highest among the known protein ligands containing an NFAT-like motif (20).

TRESK activation in response to the stimulation of G$_q$ protein-coupled receptors has also been reported in DRG neurons (3). Cell-attached patches containing TRESK channels were selected, and the application of acetylcholine, glutamate, or histamine to the bath increased the single channel average current by about 30–100% (3). Although the mechanism of activation has not been examined in this painstaking experimental setup, the efficiency of the agonist applied outside the examined membrane patch also suggested the involvement of a diffusible cytoplasmic messenger in DRG neurons, in accordance with the possible contribution of calcium and calcineurin (3).

We have recently identified another interacting partner of TRESK, 14-3-3 adaptor protein (21). 14-3-3 binds to the RSN**S**CPE motif (a typical mode 1 site) in the intracellular loop of the channel, if the second (*underlined*) serine (Ser-264 in mouse TRESK) is phosphorylated (21). The interaction between TRESK and 14-3-3 has a functional consequence dif-

ferent from that of the previously described association of TASK-1 or TASK-3 K2P channels with the adaptor protein. TASK channels (containing an atypical C-terminal mode 3 binding site) are redirected to the cell surface as the binding of 14-3-3 overrides endoplasmic reticulum retention signal(s), *i.e.* the expression of the channels is enhanced (22–25). According to a recent study, *N*-glycosylation modulates the surface expression of TRESK (26), and we have shown that 14-3-3 regulates TRESK channel activity (21). After the calcineurin-dependent activation, return of TRESK current to the resting state is impeded by 14-3-3 (21). Originally, we proposed that the binding of 14-3-3 to the phosphorylated Ser-264 obstructed the rephosphorylation of Ser-276 after its calcineurin-mediated dephosphorylation, and thus 14-3-3 interfered with the recovery (21). However, analysis of the effect of 14-3-3 on TRESK channels mutated at distinct regulatory serine residues indicated a more complex mechanism of action of the adaptor protein. The picture, emerging from the detailed analysis of the mutants, unraveled two distinct types of inhibitory phosphorylation with different relations to 14-3-3.

EXPERIMENTAL PROCEDURES

Plasmids, Reagents, and in Vitro Site-directed Mutagenesis—The cloning of human and mouse TRESK cDNAs and their subcloning to the plasmid suitable for *in vitro* cRNA synthesis and expression in *Xenopus* oocytes was previously described (5). Different mutant versions of these plasmids were produced with QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). The primer sequences for the S264A, S264E, S274E, S276E, and S279A mutations of mouse TRESK were previously reported (5).

Alanine-scanning mutagenesis of all the 13 intracellular serines and 4 threonines of mouse TRESK was previously performed for the identification of putative phosphorylation sites participating in the calcineurin-dependent regulation (5, 18). Serine 264, 274, and 276 were identified as probable regulatory residues. The average of calcineurin-dependent activation of the S279A mutant was also smaller by about 30% than that of the wild type, although this difference was not statistically significant in that study (5). To verify the importance of the residues identified by alanine-scanning mutagenesis and examine the ensemble contribution of other serines/threonines of TRESK to the calcineurin-dependent regulation, we combined mutations of Ser-264, Ser-274, Ser-276, and Ser-279. Phosphoserine, in the respect of its negative charge, is more similar to glutamate than to alanine, and we previously found that substitution of Ser-276 with alanine induced a more drastic effect than the substitution with glutamate. (Calcium activated S276A or S276E mutant TRESK by about 10% or 2-fold, respectively (5)). Therefore, glutamate substitutions were used in the experiments of combined mutations (with the exception of S279A, which altered the regulation moderately by itself (5)).

The sequence of the sense primer for the S252A mutation of human TRESK was 5′-GGAGAGGAGTAACGCATGC-CCCGAACTGGTGTTGG-3′, also including a silent mutation coding for a novel SphI restriction enzyme site in addition to the desired mutation. The construction of the TRESKloop-H$_8$ protein from the intracellular loop of mouse TRESK, the pro-

duction of the substrate protein containing only three serines (274, 276, and 279) by repeated site-directed mutagenesis, and its expression in *Escherichia coli* was previously described (21). The cloning of mouse 14-3-3γ, and its conversion to the R57,61A mutant, dominant negative form was also reported in our recent study (21).

Ionomycin (calcium salt, Sigma) was dissolved in DMSO as a 5 mM stock solution and diluted to 0.5 μM before measurement in all experiments. Benzocaine and forskolin were dissolved in ethanol, IBMX in DMSO. The pS-Raf259 phosphopeptide, LSQRQRSTpSTPNVHA, was purchased from Sigma as a custom-synthesized peptide (99% purity). Chemicals of analytical grade were purchased from Sigma, Fluka, or Merck. Enzymes and kits of molecular biology applications were purchased from Ambion (Austin, TX), Fermentas (Vilnius, Lithuania), New England Biolabs (Beverly, MA), and Stratagene (La Jolla, CA).

Animals, Tissue Preparation, Xenopus Oocyte Microinjection, and Two-electrode Voltage Clamp Measurements—Mouse brain for cytosol preparation derived from the NMRI mouse strain (Toxicop, Hungary). The oocytes were prepared, the cRNA was synthesized and microinjected, and two-electrode voltage clamp measurements were performed as previously described (5, 27). Oocytes were injected 1 day after defolliculation. Fifty nanoliters of the appropriate RNA solution were delivered with the Nanoliter Injector (World Precision Instruments, Saratosa, FL). Electrophysiological experiments were performed three or 4 days after the injection. Low [K$^+$] solution contained (in mM): NaCl 95.4, KCl 2, CaCl$_2$ 1.8, HEPES 5 (pH 7.5 adjusted with NaOH). High [K$^+$] solution contained 80 mM K$^+$ (78 mM Na$^+$ of the low [K$^+$] solution was replaced with K$^+$). TRESK background K$^+$ current was measured at the ends of 250- or 300-ms voltage steps to -100 mV applied every 3 or 4 s in all experiments. All treatments of animals were conducted in accordance with state laws and institutional regulations. The experiments were approved by the Animal Care and Ethics Committee of Semmelweis University.

Preparation of Mouse Brain Cytosol and in Vitro Radioactive Phosphorylation—For the preparation of mouse brain cytosol, one brain of a female mouse was homogenized in 1.2 ml of a solution containing (in mM): phenylmethylsulfonyl fluoride 2, benzamidine 0.5, β-mercaptoethanol 9, Tris-Cl 40, pH 7.6. The lysate was centrifuged at 35,000 $\times$ g for 20 min, supplemented with NaCl (to a final concentration of 100 mM) and centrifuged again at 35,000 $\times$ g for 10 min. The supernatant was loaded onto a Sephadex G-25 column (1.5-cm diameter, 23-cm height) equilibrated with solution A containing (in mM): phenylmethylsulfonyl fluoride 0.5, benzamidine 0.5, β-mercaptoethanol 2, Tris-Cl 40, pH 7.6. The fractions of high optical density at 280 nm and low conductance (containing the ATP-, and salt-free proteins of the cytosol) were pooled. The gel-filtrated cytosol was diluted 8-fold with solution A, and 10 μl was added to the mutant TRESKloop-H$_8$ protein (containing only serines 274, 276, and 279) immobilized on 10 μl of Ni-nitrilotriacetic acid resin and resuspended in 40 μl of solution containing (in mM): KCl 50, MgCl$_2$ 10, imidazole 75, β-glycerolphosphate 20, β-mercaptoethanol 4, sodium orthovanadate 0.2, HEPES 60 (pH adjusted to 7.4 with NaOH), supplemented with 1% Triton X-100, 20 μM Na$_2$ATP, and 50 kBq of [γ-^{32}P]ATP. The immo-
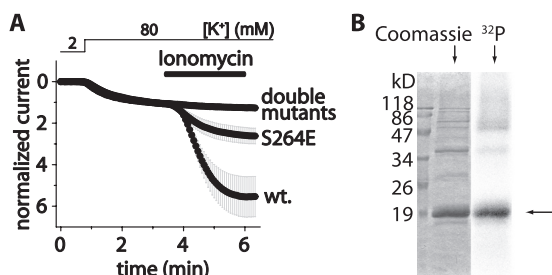
FIGURE 1. **The S264E mutation reduces, whereas the combined mutations, S264E/S274E, S264E/S276E and S264E/S279A, abolish the calcineurin-dependent activation of TRESK.** The cluster of serines 274, 276, and 279 is phosphorylated by mouse brain cytosol *in vitro*. *A*, currents of *Xenopus* oocytes expressing the wild type ($n = 15$), S264E ($n = 10$), S264E/S274E ($n = 9$), S264E/S276E ($n = 7$), or S264E/S279A ($n = 6$) mutant (mouse) TRESK channels were measured at −100 mV. Extracellular [K^+] was increased from 2 to 80 mM, and the cells were subsequently challenged with ionomycin (*Iono.*, 0.5 μM) as indicated by the *horizontal black bar*. Normalized currents were plotted (the current measured in 2 mM EC [K^+] was normalized to 0 and the current measured in 80 mM EC [K^+], before the stimulation with ionomycin, to 1). Note that the curves of the S264E/S274E, S264E/S276E, and S264E/S279A mutants (*double mutants*) run on each other. (The *gray* error bars represent S.E. The regulation of the S264E and double mutant channels was promoted by the coexpression of dominant negative 14-3-3γ.) *B*, mutant TRESKloop-H$_8$ protein (corresponding to amino acids 185–292 of mouse TRESK extended with an octahistidine tag) was phosphorylated with mouse brain cytosol in the presence of [γ-^{32}P]ATP. The mutant protein contained only serines 274, 276, and 279, whereas all the other serines (and threonine) were mutated to alanine. The *left side* of the panel shows the SDS-PAGE gel stained with Coomassie Blue, whereas the autoradiogram of the same gel is on the *right side* of the figure. Note that the mutant TRESKloop-H$_8$ protein (indicated with the *horizontal arrow*) was phosphorylated by the cytosol.

bilized protein was phosphorylated at 37 °C for 1 h. The protein was run on a 15% SDS-PAGE gel; the gel was stained with Coomassie Brilliant Blue, and its radioactivity was detected with a phosphorimager (GS-525, Bio-Rad).

Statistics and Calculations—Data are expressed as means ± S.E. Statistical significance was estimated by Student's *t* test for independent samples, with the exception of a one-way analysis of variance (ANOVA) and *Scheffé* posthoc test for multiple comparisons (as indicated under "Results"). The Statistica 8.0 program package (StatSoft, Tulsa, OK) was used for the analysis. The difference was considered to be significant at $p < 0.05$.

RESULTS

Serine 264 and the Cluster of Serines 274, 276, and 279 Are the Major Regulatory Sites of Calcineurin-dependent Activation—Combined mutations, S264E/S274E, S264E/S276E, and S264E/S279A completely eliminated the calcineurin-dependent activation of TRESK (Fig. 1*A*). The response of all three double mutants was statistically different from the activation of S264E mutant channel ($p < 0.01$, ANOVA, *Scheffé* posthoc test). This indicates that the cluster of adjacent serines 274, 276, and 279 (hereafter called the S276 cluster) constitutes an important regulatory focal point in addition to Ser-264. The mutation of any single serine in the S276 cluster was sufficient to completely abrogate the regulatory function of the entire locus, probably because of the close proximity of the serines in the cluster. Because the combined mutation of the two identified regulatory regions (S264 and the S276 cluster) abolished the calcineurin-dependent activation, the phosphorylation of other serines/threonines of TRESK was not likely involved in the regulation.

In vitro phosphorylation of Ser-264 by protein kinase A was previously demonstrated (21). However, under identical conditions, the substrate protein (corresponding to a part of the intracellular loop region, amino acids 185–292 of mouse TRESK, extended with a His tag for purification from *E. coli*), containing serines 274, 276, and 279, and alanines instead of all the other serines, has not been phosphorylated (21). Therefore we tested the phosphorylation of this substrate protein with mouse brain cytosol. The cytosol was gel-filtrated on a Sephadex G-25 column to remove ATP, and incubated with the substrate protein immobilized on Ni-nitrilotriacetic acid agarose in the presence of [γ-^{32}P]ATP. The cytosol phosphorylated the substrate protein (Fig. 1*B*), indicating that the S276 cluster was indeed the target of a kinase activity different from protein kinase A.
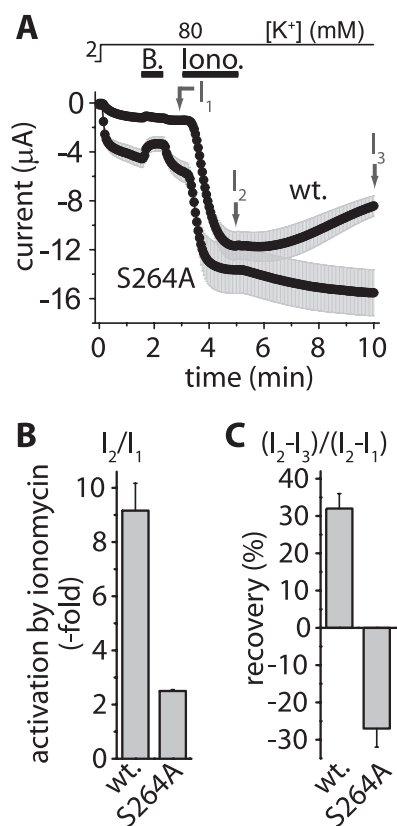
Recovery of S264A Mutant to the Resting State after the Calcineurin-dependent Activation Is Slow—The return of the current of wild-type TRESK to the resting state after the calcineurin-dependent activation (hereafter called recovery) is inhibited by 14-3-3 (21). If 14-3-3 inhibits recovery via its direct interaction with the channel then the loss-of-function mutation (S264A) of the interaction site should accelerate recovery. (We previously demonstrated that S264A mutation eliminated the binding of 14-3-3 (21), as it was expected for a mode 1 motif.) Therefore we examined the kinetics of calcium-dependent regulation of S264A mutant TRESK (Fig. 2). In accordance with the permanently missing phosphorylation at one of the major regulatory sites, the S264A mutant was partially activated under resting conditions. Benzocaine sensitivity is a property discriminating activated wild-type TRESK channels from those showing basal activity in *Xenopus* oocytes (18). The benzocaine sensitivity of the basal current of S264A mutant was higher than that of the wild-type channel ($22 \pm 1\%$ ($n = 8$) *versus* $10 \pm 1\%$ ($n = 14$) inhibition, respectively, by 1 mM at 105 s, $p < 10^{-5}$, Fig. 2*A*). The partial activation under resting conditions also resulted in an apparently reduced calcineurin-dependent stimulation (2.5 ± 0.05-fold, in contrast to the 9.2 ± 1.0-fold activation in the wild-type group, $p < 10^{-4}$, Fig. 2*B*).

Interestingly, however, the recovery of S264A mutant TRESK was not accelerated at all. The current even increased during the washout period ($-27 \pm 5\%$ recovery, Fig. 2*C*), in contrast to the control cells expressing the wild-type channel ($32 \pm 4\%$ recovery, $p < 10^{-8}$). The recovery of the S264E mutant was also slower than that of the wild-type TRESK (data not shown; see the *blue curve* in Fig. 3*A*). These results indicated that the direct interaction between TRESK and 14-3-3 did not exclusively determine the kinetics of recovery. The slow recovery of Ser-264 mutants urged us to examine how the functional availability of 14-3-3 influenced the calcium-dependent regulation of these mutants unable to directly interact with the adaptor protein.

Coexpression of Dominant Negative 14-3-3 Augments the Calcineurin-dependent Activation of S264E Mutant TRESK, but Wild-type 14-3-3 Augments That of the S276E Mutant—In the following experiments, the current kinetics of mutant channels was analyzed by estimating the basal current measured under resting conditions, the normalized activation evoked by the elevation of cytoplasmic calcium concentration and the

FIGURE 2. **The recovery of S264A mutant TRESK to the resting state after the calcineurin-dependent activation is not accelerated, compared with the wild-type channel.** *A*, average currents of *Xenopus* oocytes expressing wild-type (mouse channel, $n = 14$) or S264A mutant ($n = 8$) TRESK were plotted. Extracellular [K$^+$] was increased from 2 to 80 mM, and the oocytes were challenged with benzocaine (*B.*, 1 mM) and ionomycin (*Iono.*, 0.5 μM) as indicated by the *horizontal black bars*. (The partially activated state of the basal current was examined by the application of benzocaine, which inhibits the resting and calcineurin-activated wild-type channel by 10–15% and 50–60%, respectively, Ref. 18.) *B*, normalized activation of the current of each cell, in response to ionomycin, was calculated as I_2/I_1 (see the *gray arrows* in *panel A*; I_1 and I_2 were corrected for the small nonspecific current measured in 2 mM [K$^+$]). *C*, recovery was calculated for each cell as $(I_2 - I_3)/(I_2 - I_1)$, corresponding to the fraction of activated current returning to the resting state during the washout period (see the *gray arrows* in *panel A*). The negative recovery value of the S264A mutant indicated that the current did not recover toward the resting state but it even increased during the washout of ionomycin.

recovery to the resting state during the washout period. A brief introduction to the basic theory of background K$^+$ current regulation by single phosphorylation and the integrated evaluation of these three parameters can be found in supplemental materials S1.

Because the phosphorylation of Ser-264 and also the binding of 14-3-3 to the channel were abrogated in S264E mutant TRESK, the regulation via the S276 cluster was clearly manifested in these experiments. To manipulate functional 14-3-3 levels, wild-type or dominant negative (R57,61A mutant) mouse 14-3-3γ was coexpressed with S264E mutant TRESK. (As a control group, cells expressing only the mutant channel were also measured. The results of these control cells represented the effect of endogenous 14-3-3 and have not been included in the statistical evaluation.) Three important differences were observed between the groups with elevated or diminished functional 14-3-3 levels. First, the resting K$^+$ cur-

: only channel   :+ 14-3-3γ :+ dom. neg. 14-3-3γ
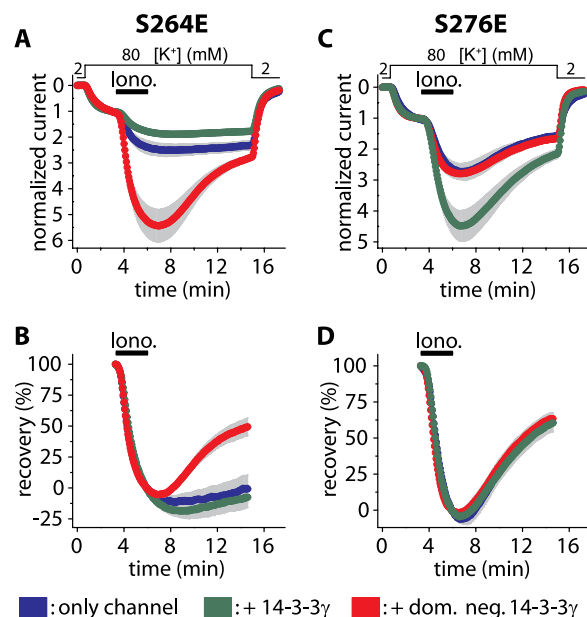
FIGURE 3. **The coexpression of wild-type or dominant-negative 14-3-3 evokes substantially different effects on the S264E and S276E mutant versions of mouse TRESK.** *A*, normalized currents of three groups of oocytes expressing only S264E mutant TRESK (*blue*), or coexpressing the S264E mutant channel with wild-type (*green*) or dominant negative (*red*) 14-3-3γ. The cells were stimulated with ionomycin (*Iono.*, 0.5 μM, as indicated by the *horizontal black bar*) in 80 mM extracellular [K$^+$]. Note the high activation in the cells coexpressing dominant-negative 14-3-3 with the S264E mutant channel (*red*). *B*, recovery was calculated from the same recordings as presented in *panel A*. Note the accelerated recovery in the cells coexpressing dominant-negative 14-3-3 with the S264E mutant channel (*red*). *C*, same coexpression experiment as in *panel A* was performed with the S276E mutant version of TRESK. Note the high activation in the cells coexpressing wild-type 14-3-3 with the S276E mutant channel (*green*). *D*, recovery was calculated from the same recordings as shown in *panel C*. Note that the recovery was identical in the presence of wild-type and dominant negative 14-3-3.

rent of S264E mutant TRESK was 1.1 ± 0.2 μA ($n = 12$) when 14-3-3 was suppressed with the coexpression of dominant negative 14-3-3γ (dn14-3-3). This value was significantly smaller than the basal current of the cells coexpressing wild-type 14-3-3 (wt14-3-3) with the S264E mutant TRESK (2.0 ± 0.2 μA ($n = 10$), $p < 0.01$). Second, the calcineurin-dependent activation was augmented in the dn14-3-3 group (5.2 ± 0.6-fold at 356 s, *red curve* in Fig. 3*A*), compared with the wt14-3-3 group (1.8 ± 0.1-fold, *green curve* in Fig. 3*A*, $p < 10^{-4}$). Third, the recovery was accelerated in the dn14-3-3 group ($49 \pm 7\%$ at 876 s, *red curve* in Fig. 3*B*), but it was slow in the wt14-3-3 group ($-8 \pm 8\%$, *green curve* in Fig. 3*B*, $p < 10^{-4}$).

The reduced basal current and the augmented activation together with a modest acceleration of recovery indicated that the kinase phosphorylating the S264E mutant TRESK was activated in the cells coexpressing dn14-3-3 with the channel. Because Ser-264 was not present (and thus evidently could not be phosphorylated), 14-3-3 inhibited the kinase phosphorylating the functional S276 cluster of the S264E mutant. As the 14-3-3 binding site of TRESK was disintegrated, the adaptor protein evoked this effect independently of its direct interaction with the channel. Dominant negative 14-3-3 accelerated the recovery by alleviating the inhibitory effect of 14-3-3 on the kinase phosphorylating the S276 cluster.

Because the above mechanism may mask it, the functional significance of the direct interaction between TRESK and

14-3-3 cannot be simply deduced from the measurement of the effect of 14-3-3 on wild-type TRESK. Therefore, the question was raised whether the direct interaction between TRESK and 14-3-3 modified channel activity and/or the calcineurin-dependent regulation. To address this question, we examined the effect of 14-3-3 on the S276E mutant, in which the S276 cluster (and its 14-3-3-dependent regulatory phosphorylation) was disabled. In this case, the basal K^+ current was smaller in the cells coexpressing wt14-3-3 (1.3 ± 0.1 μA, $n = 12$) than in the cells coexpressing dn14-3-3 (2.9 ± 0.5 μA, $n = 12$, $p < 0.01$). The calcineurin-dependent activation was augmented in the wt14-3-3 group (4.3 ± 0.4-fold at 356 s, *green curve* in Fig. 3*C*), compared with the dn14-3-3 group (2.7 ± 0.2-fold, *red curve* in Fig. 3*C*, $p < 0.01$). Most importantly, however, the recovery was not changed by the different 14-3-3 levels (the *green*, *blue*, and *red* curves practically overlap in Fig. 3*D*).

The unaltered recovery indicated that 14-3-3 did not influence the kinase phosphorylating Ser-264. With respect to the direct interaction between the channel and 14-3-3, this was anticipated. The activated fraction of S276E mutant channels are dephosphorylated at Ser-264. These dephosphorylated channels are the substrate of the kinase and determine the kinetics of recovery; however, they cannot bind 14-3-3, because the binding of 14-3-3 to TRESK depends on the phosphorylation of Ser-264. Nevertheless, the unaltered recovery indicated that an effect of 14-3-3 on the kinase, independent of the direct interaction of TRESK and 14-3-3, was also highly unlikely. The reduced basal current and augmented calcineurin-dependent activation with unaltered recovery in the presence of 14-3-3 suggest that the direct binding of the adaptor protein to the channel contributes to the basal inhibition of K^+ current under resting conditions.

Microinjection of pS-Raf259 Phosphopeptide Accelerates the Recovery of S264E Mutant but Does Not Influence That of the S276E Mutant—The phosphopeptide, pS-Raf259 occludes the peptide-binding groove of 14-3-3, and inhibits the binding of the adaptor protein to its natural partners (28, 29). For example, pS-Raf259 competes with TRESK for binding to 14-3-3 both *in vitro* and *in vivo* (21). As an alternative approach to reduce the functional 14-3-3 levels at a short timescale, pS-Raf259 was microinjected into the cells coexpressing S264E mutant TRESK channels with 14-3-3γ. The basal K^+ current was inhibited by the microinjection of the phosphopeptide (1.1 ± 0.3 μA, $n = 5$), compared with the control cells microinjected with water (2.7 ± 0.5 μA, $n = 6$, $p < 0.05$). The calcineurin-dependent activation was small in the pS-Raf-injected group (1.6 ± 0.2-fold at the end of the stimulation with ionomycin, not significantly different from that of the control cells, Fig. 4*A*). However, the recovery was drastically accelerated and was complete at the end of the measurement ($164 \pm 58\%$ at 876 s in the pS-Raf group, while $-50 \pm 14\%$ in the water-injected cells, $p < 0.01$, Fig. 4*B*). The reduced basal current and the small normalized activation together with the highly accelerated recovery indicated that the kinase phosphorylating the S276 cluster was robustly up-regulated in response to the neutralization of 14-3-3 by the phosphopeptide. (We obtained identical results when the same pS-Raf microinjection was performed with cells coexpressing S264A mutant TRESK and 14-3-3γ; see
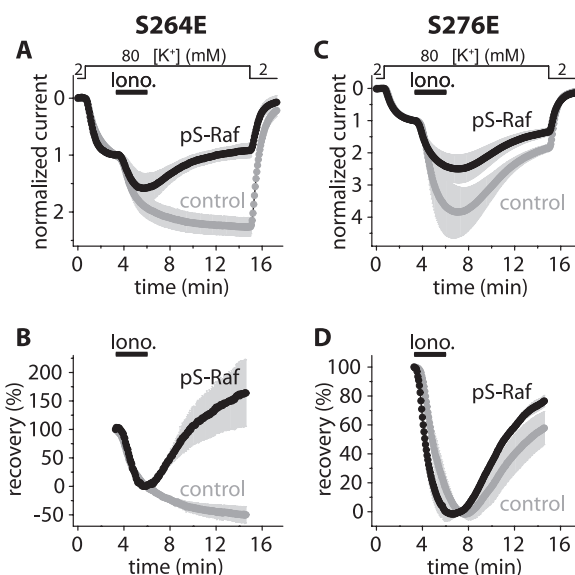
FIGURE 4. **The microinjection of the 14-3-3 inhibitor pS-Raf259 accelerates the recovery of S264E mutant TRESK but does not change that of the S276E mutant.** *A*, normalized currents of oocytes microinjected with the phosphopeptide (*pS-Raf, black*, 50 nl, 10 mм, 70–150 min before the application of ionomycin) or with water (*control, gray*). The cells were coexpressing S264E mutant (mouse) TRESK with 14-3-3γ. (Experimental protocol and current normalization were the same as in Fig. 3*A*.) *B*, recovery was calculated from the same recordings as presented in *panel A*. Note that the recovery was complete in about 5 min after the withdrawal of ionomycin in the cells microinjected with pS-Raf259 phosphopeptide (*black*). *C*, same microinjection experiment as in *panel A* was performed with S276E mutant TRESK. The current kinetics were highly similar to those obtained in the coexpression experiment (compare the curves to Fig. 3*C*). *D*, recovery was calculated from the same recordings as presented in *panel C*. The recovery was not significantly changed by the microinjection of the phosphopeptide.

supplemental materials S2 for the comparison of average S264A and S264E currents. For further comments on the data about the regulation of S264E mutant TRESK, see supplemental materials S3). These microinjection experiments confirmed the results obtained with the coexpression of dn14-3-3; *i.e.* the adaptor protein inhibited the kinase phosphorylating the S276 cluster, independently of its direct interaction with TRESK.

To selectively examine the effect of pS-Raf259 on the regulatory mechanism acting via Ser-264, we performed the microinjection experiment with cells coexpressing S276E mutant TRESK with 14-3-3γ. Elimination of functional 14-3-3 with pS-Raf increased the basal K^+ current to 4.3 ± 0.9 μA ($n = 5$), compared with the water-injected control cells (1.4 ± 0.1 μA, $n = 5$, $p < 0.02$). The calcineurin-dependent activation was 2.4 ± 0.4-fold in the pS-Raf-injected and 3.6 ± 0.9-fold in the control group at the end of the stimulation with ionomycin. (These averages (Fig. 4*C*) were similar to those obtained in the coexpression experiments (see the *green* and *red* curves in Fig. 3*C*), although the difference was not statistically significant due to the higher variation of data in this experiment.) The recoveries of the control and pS-Raf-injected cells were not different (Fig. 4*D*). The elevated basal current and the unaltered recovery of S276E mutant TRESK in the cells injected with pS-Raf259 were compatible with the results obtained with the coexpression of dn14-3-3. These data also support the conclusion that 14-3-3 does not influence the kinase phosphorylating Ser-264,

but the binding of the adaptor protein to TRESK contributes to the inhibition of the resting K^+ current.

The Inhibitory Phosphorylation of the S276 Cluster of Mouse TRESK Is Conserved in the Human Channel—The ERSNSCPELVL sequence in the neighborhood of Ser-264 (*underlined*; Ser-252 in human TRESK), is absolutely conserved in the human, mouse, and rat orthologs. However, the region of the S276 cluster of mice, RLSCSILSNL differs from the RLSYSI-ISNL sequence in the human channel. The discrepancy of the amino acids located between the serines of the cluster, especially the cysteine to tyrosine substitution, raises the question whether the inhibitory phosphorylation of the cluster is operational in human TRESK. Therefore we have examined the calcineurin- and 14-3-3-dependent regulation of the S252A mutant version of the human channel.

All three major characteristics of the regulation were reproduced with the human ortholog (data not shown). The basal current was inhibited by the coexpression of dn14-3-3 with the channel (Fig. 5*A*). The calcineurin-dependent activation was augmented by dn14-3-3 (Fig. 5*B*). The recovery after the cessation of the stimulation was accelerated by dn14-3-3 (Fig. 5*C*). These results indicate that the inhibitory mechanism, characteristic for the S276 cluster of the mouse channel, also operates in human TRESK. The inhibition of recovery by 14-3-3 makes feasible that the same kinase phosphorylates the human and mouse orthologs, despite the discrepancy of the amino acid sequences.

The Activation of Protein Kinase A Accelerates the Recovery of S276E Mutant TRESK but Does Not Affect That of the S264E Mutant—Because protein kinase A phosphorylated Ser-264 but not the S276 cluster *in vitro*, this kinase was also expected to distinguish between the two pathways of inhibitory phosphorylation in addition to 14-3-3. Therefore, we examined the regulatory kinetics of the S264E and S276E mutants during the stimulation of protein kinase A in the cell. Forskolin (50 μM) plus 3-isobutyl-1-methylxanthine (IBMX, 1 mM) (an activator of adenylate cyclase plus an inhibitor of phosphodiesterase, respectively) was used to elevate [cAMP] (Fig. 6). Because this pharmacological treatment inhibited TRESK nonspecifically (presumably via a direct action on the channel, see [supplemental materials S4](#)), the real effect of phosphorylation could only be evaluated after the washout of these compounds, at the end of the measurement. The current of the S264E mutant was not different at 876 s in the treated and control groups (Fig. 6*A*).

In turn, the S276E mutant rapidly recovered in response to forskolin plus IBMX. Accordingly, its current was smaller at the end of the measurement than that of the control untreated S276E mutant channels (1.1 ± 0.1 μA ($n = 11$) *versus* 2.0 ± 0.3 μA ($n = 7$), $p < 0.01$, Fig. 6*B*). The acceleration of the recovery was also detectable, when lower concentrations of forskolin (5 μM) plus IBMX (0.2 mM) were applied (see [supplemental materials S5](#)). As the recovery of the S276E mutant (containing functional Ser-264) was accelerated, but that of the S264E mutant (retaining only the S276 cluster as an intact regulatory region) was not altered, the functional data corresponded well to the *in vitro* specificity of protein kinase A.
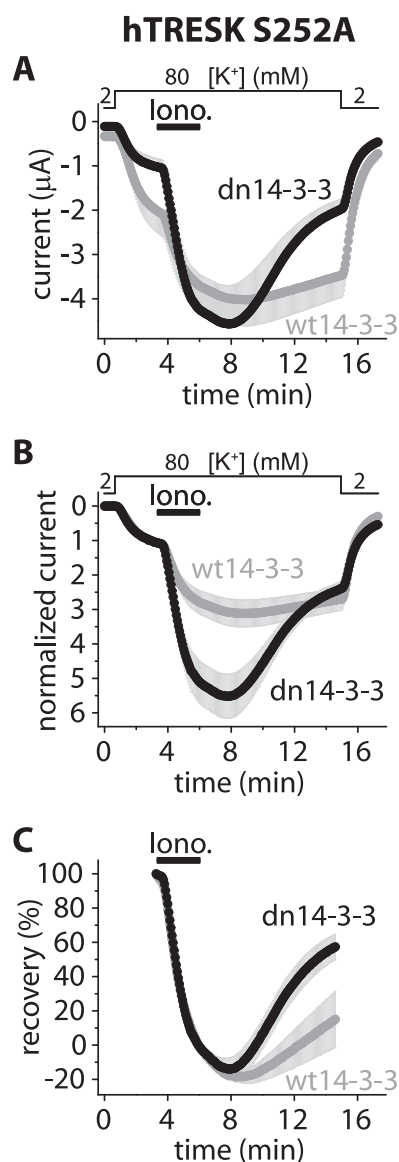
FIGURE 5. **The regulation of the S252A mutant human TRESK is similarly modulated by 14-3-3 as that of the S264E (or S264A) mutant mouse channel.** *A*, average currents of two groups of oocytes coexpressing wild-type (*gray, wt14-3-3, n = 12*) or dominant-negative (*black, dn14-3-3, n = 11*) 14-3-3γ with S252A mutant human TRESK. The basal current (measured in 80 mM [K^+], before the stimulation with ionomycin) was significantly smaller in the dn14-3-3 than in the wt14-3-3 group. *B*, normalized currents were calculated from the same recordings as presented in *panel A*. Note that the normalized activation of the S252A mutant was significantly higher in the dn14-3-3 group than in the wt14-3-3 group at the end of the stimulation with ionomycin. *C*, recovery was calculated from the same recordings as presented in *panel A*. The recovery was accelerated by dn14-3-3, compared with the cells coexpressing wt14-3-3 with the channel.

The recovery of S276E mutant TRESK was also accelerated when cAMP (50 nl, 0.1 mM) was microinjected into the cell at the beginning of the recovery period. The normalized current in 80 mM EC [K^+] at the end of the measurement was 1.9 ± 0.2-fold ($n = 5$) in the cAMP-injected, whereas it was 2.5 ± 0.2-fold ($n = 6$, $p < 0.05$, Fig. 7*A*) in the water-injected cells. The recovery, calculated from the same data (Fig. 7*B*), was $75 \pm 1\%$ in the cAMP-injected cells and $57 \pm 4\%$ in the control cells ($p < 0.005$). The microinjection of high concentration of cAMP evoked a robust acceleration of recovery compared with the
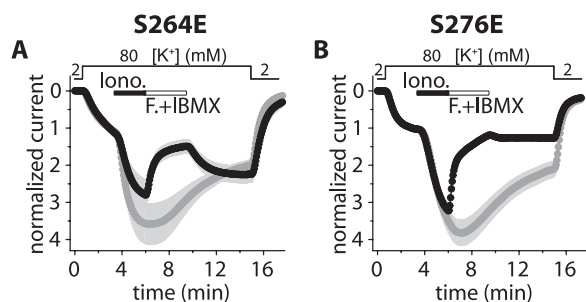
FIGURE 6. **The application of forskolin plus IBMX accelerates the recovery of the current of S276E mutant TRESK but does not alter that of the S264E mutant channel.** *A*, normalized currents of oocytes coexpressing S264E mutant (mouse) TRESK with dominant-negative 14-3-3γ. The cells were stimulated with ionomycin (*Iono.*, 0.5 μM, as indicated by the *horizontal black bar*), and subsequently were (*black curve*) or were not (*gray curve*) treated with forskolin (50 μM) plus IBMX (1 mM, *F.+IBMX*, as indicated by the *horizontal empty bar*) during the washout of ionomycin. Note that the recovery of the S264E mutant, measured in 80 mM [K$^+$] at the end of the washout period of ionomycin (after the cessation of the direct inhibition of the current by the drugs) was not altered by treatment with forskolin plus IBMX. *B*, same experiment as depicted in *panel A* was performed with cells coexpressing S276E mutant TRESK with wild-type 14-3-3γ. The recovery of the current rapidly became complete in response to the forskolin plus IBMX treatment, as the *black curve* was horizontal at the end of the measurement in 80 mM [K$^+$]. Accordingly, the currents measured before switching back to 2 mM [K$^+$] were significantly different between the treated (*black*) and untreated (*gray*) groups.

water-injected cells (see supplemental materials S6). These results verified that the elevation of [cAMP] accelerated the phosphorylation of Ser-264 irrespectively of the nonspecific effects of forskolin and IBMX. If protein kinase A is stimulated then TRESK is inhibited by the phosphorylation of Ser-264, whereas the other inhibitory pathway (phosphorylating the S276 cluster) is not affected under these conditions.

Ionomycin had no effect on the background K$^+$ current in DRG-neuron-derived F11 cells under whole-cell patch clamp conditions, in experiments where the current of TRESK was not separated from that of the other endogenous K$^+$ channels. In F11 cells transfected with TRESK, the K$^+$ current of high amplitude was also unresponsive to ionomycin under whole-cell patch clamp conditions (data not shown). The regulatory mechanism may be absent in the plasma membrane of cell bodies, may be prevented during the whole-cell patch clamp configuration, or further, presently unidentified, factors may be required for its operation in this system.

DISCUSSION

Physiological significance of TRESK has recently been proposed in two major areas. Abundant expression of TRESK in the sensory neurons of dorsal root and trigeminal ganglia (1, 2, 7), and the high sensitivity to anesthetics (30–35) and the paresthesia-inducing hydroxy-α-sanshool (8) led to the hypothesis that the channel is important in the somatosensory function with special respect to pain sensation (9–11). On the other hand, TRESK expression was reported in rat thymus and spleen (6), and recently in Jurkat human leukemic T lymphocytes (36, 37). This localization and the unique regulation of the channel by calcineurin (a major target of extensively used immunosuppressant drugs) led to the suggestion that TRESK could be involved in T-cell-mediated immune functions (38). Whereas TRESK current is undoubtedly important in the sensory neu-
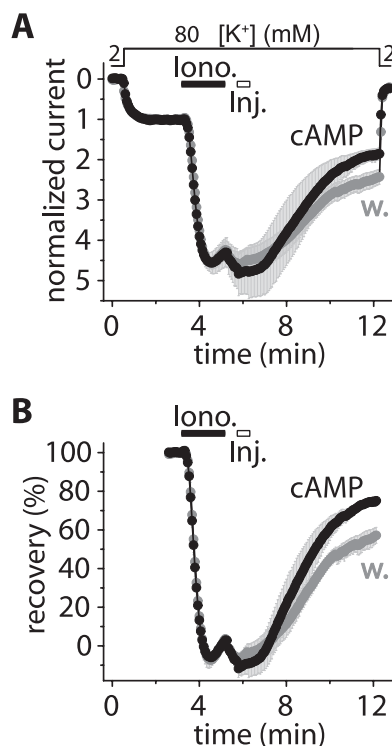

FIGURE 7. **The microinjection of cAMP accelerates the recovery of the current of S276E mutant TRESK.** *A*, normalized currents of oocytes coexpressing S276E mutant TRESK with 14-3-3γ were plotted. TRESK current was activated with ionomycin (*Iono.*, 0.5 μM, as indicated by the *horizontal black bar*), and after the beginning of the washout of ionomycin, the cells were microinjected with cAMP (50 nl, 0.1 mM, *black curve*) or water (*w.*, *gray curve*). A third (injection) microcapillary was inserted into the oocyte during the two-electrode voltage clamp measurement, and the injection was performed in the period indicated by the *horizontal empty bar* (*Inj.*). The insertion of the capillary and/or the injection caused a small increase of the nonspecific leak current. Note that the current in 80 mM EC [K$^+$] at the end of the measurement was significantly reduced in the cAMP-injected cells compared with the water-injected control group. *B*, recovery was calculated from the same recordings as presented in *panel A*. In accordance with the identical activation in the two groups, the kinetics of the recovery curves was highly similar to those of the normalized currents in *panel A*, and also showed that the microinjection of cAMP accelerated the recovery of the K$^+$ current.

rons of dorsal root ganglia, further studies are required to confirm its functional significance at other locations.

To elucidate the general aspects of TRESK regulation, we have searched for mechanisms directly affecting the channel protein. In two previous studies we reported two proteins directly interacting with well-defined binding motifs of TRESK. Calcineurin bound to the NFAT-like PQIVID motif (18), while 14-3-3 to the mode 1 RSN<u>S</u>CP sequence (21). Because the docking of these proteins is objectively determined at the amino acid sequence level and require a relatively low number of auxiliary conditions (*e.g.* calmodulin and calcium signal in the case of calcineurin; or the phosphorylation of the underlined regulatory serine in the 14-3-3-binding site), these interactions likely develop in different cell types.

We have shown that the calcium/calmodulin-dependent interaction with calcineurin and the resulting dephosphorylation was responsible for a robust activation of TRESK K$^+$ current (5, 18). However, the mechanism opposing the action of calcineurin and restoring the inhibited (resting) state of the channel has not been examined so far. In the present study, we
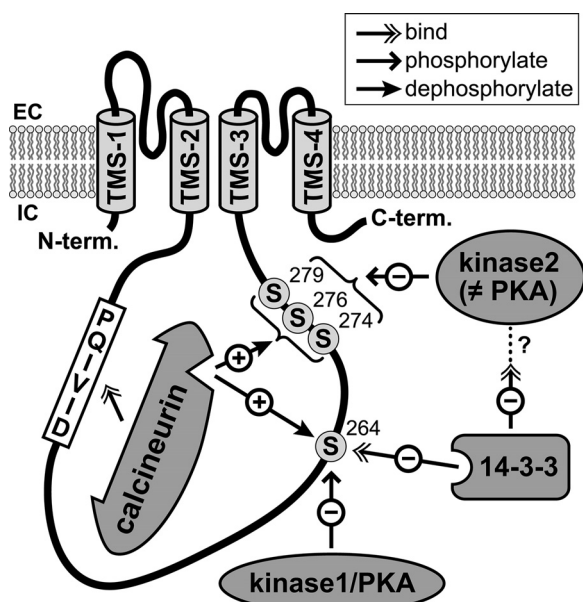
FIGURE 8. **Schematic representation of phosphorylation-dependent TRESK regulation.** Calcineurin (stimulated by the calcium/calmodulin complex, not shown) is recruited to the NFAT-like PQIVID motif in the intracellular loop of TRESK and activates the channel by the dephosphorylation of the two major regulatory regions, Ser-264 and the S276 cluster. After the decay of the calcium signal, two different kinase activities restore the phosphorylation at the two locations; *kinase1* acts at Ser-264 and *kinase2* at the S276 cluster. Protein kinase A (*PKA*) can play the role of kinase1 and accelerates the recovery of TRESK current to the resting (inhibited) state by the phosphorylation of Ser-264. Kinase1 is not affected by 14-3-3 adaptor protein; however, the docking of 14-3-3 to the phosphorylated Ser-264 in the channel loop contributes to the inhibition. Kinase2 is different from PKA, and is inhibited by 14-3-3, which effect may be direct or indirect (indicated by a *? mark*) and probably depends on the phosphorylation of kinase2 or its regulator, respectively.

have provided novel insight into this regulatory mechanism (in fact, we have identified two apparently independent inhibitory pathways) by delineating the sites of phosphorylation and clarifying the relation of these sites to the 14-3-3-dependent modulation of channel activity (Fig. 8). The identified regulatory mechanisms also rely directly on the channel protein, phosphorylation of Ser-264 and the S276 cluster is expected to inhibit TRESK irrespectively of the nature of the phosphorylating kinase activity. Futhermore, we have demonstrated that protein kinase A (PKA) phosphorylates Ser-264 not only *in vitro* but also as an endogenous enzyme of the living cell, and thus PKA can mediate one of the two inhibitory mechanisms.

In fact, it remains an open question whether PKA phosphorylates TRESK in native cells, and if so, in which cells and under what conditions. Answering these questions will most probably require the separation of TRESK whole cell current from the other currents (especially from the similar background K^+ currents of other K2P channels) with highly selective pharmacological agents. However, in the absence of these agents, an example of such a separation of TRESK current has not yet been reported.

In contrast to the relatively straightforward inhibition of TRESK by PKA-mediated phosphorylation and the subsequent binding of 14-3-3, the regulation of the S276 cluster was less resolved with *in vitro* site-directed mutagenesis. As the mutation of any of the serines diminished the function of the cluster, the exact location of phosphorylation(s) in the RL<u>S</u>C<u>S</u>IL<u>S</u>NL

motif could not be deduced. This interdependence of nearby residues in the respect of phosphorylation is plausible, considering that a typical kinase consensus sequence for substrate recognition includes the amino acids adjacent to the phosphorylated serine. The substitution of these nearby amino acids may substantially influence the affinity of the substrate for the kinase. If more than one serine is phosphorylated in the cluster then the apparent interdependence may even have interesting functional consequences. In this case, the kinase reaction phosphorylating a given residue likely depends on the phosphorylation state of the other serine(s). As the S276 cluster does not match the consensus sequence of known serine/threonine kinases (predicted by different programs), and because of the possible involvement of multiple kinase activities, the identification of the phosphorylation patterns of the cluster and the corresponding kinases are beyond the scope of the present study.

We previously reported the direct interaction between TRESK and 14-3-3 (21). Because the significance of the two parallel inhibitory pathways was not acknowledged at that time, the role of this direct interaction could not be completely elucidated by measuring the effect of the adaptor protein on wild-type TRESK. The most consistent effect of 14-3-3 on wild-type TRESK was the acceleration of the recovery of the K^+ current to the resting state after the calcineurin-dependent activation. (Retrospectively, 14-3-3 could modify the two simultaneously acting inhibitory mechanisms in the opposite direction with respect to the basal current and normalized activation parameters (see the *green* and *red* curves in Fig. 3, *A* and *C*). However, the adaptor protein did not influence the recovery via the pathway acting at Ser-264 (see Fig. 3*D*), and thus its effect on the mechanism targeting the S276 cluster (Fig. 3*B*) could become consistent.) The analysis of the Ser-264 and Ser-276 mutants indicated that the accelerated recovery was independent from the direct TRESK-14-3-3 interaction. Instead, direct or indirect inhibition of the kinase activity phosphorylating the S276 cluster was responsible for the accelerated recovery. The direct TRESK-14-3-3 interaction evoked another effect: the basal current of TRESK (phosphorylated at Ser-264) was inhibited by the binding of 14-3-3, although in the case of the wild-type channel this effect could be overridden by the inhibition of the kinase phosphorylating the S276 cluster. The combined (and thus obscured) action of 14-3-3 on the wild type channel mainly reflects that both inhibitory pathways were invariably active in *Xenopus* oocytes. Selective regulation of one or the other inhibitory pathway may manifest the effect of 14-3-3 specific for that pathway also in the case of wild-type TRESK.

In summary, two inhibitory kinase pathways converge on TRESK. One of them results in the phosphorylation of serine 264 (may be mediated by protein kinase A), and the subsequent binding of 14-3-3 to this residue contributes to channel inhibition. The other pathway regulates TRESK via the phosphorylation of the S276 cluster, and this mechanism is inhibited by 14-3-3. The effect of the two pathways is integrated by the channel, and the reduced background K^+ conductance may increase the excitability of the cell. It remains to be established what kind of biologically relevant information is transmitted to

TRESK via the two inhibitory pathways of phosphorylation in native cells.

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