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The Two-pore Domain K^+ Channel, TRESK, Is Activated by the Cytoplasmic Calcium Signal through Calcineurin*

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Gábor Czirják‡, Zsuzsanna E. Tóth§, and Péter Enyedi‡¶

From the ‡Department of Physiology and §Laboratory of Neuromorphology, Semmelweis University, H-1444 Budapest, Hungary

Agonist-induced cytoplasmic calcium signals often have profound effects on the membrane potential during cellular activation. In the present study, we report that cytoplasmic calcium elevation can regulate the membrane potential by a novel mechanism. TRESK, a recently described member of the two-pore domain potassium ($2PK^+$) channel family, was activated 5–15-fold after stimulation of various Ca^{2+}-mobilizing receptors in *Xenopus* oocytes. Extracellular application of ionomycin, as well as the microinjection of inositol 1,4,5-trisphosphate or calcium, also evoked TRESK activation, whereas microinjection of EGTA or pretreatment of the oocytes with thapsigargin prevented the receptor-mediated effect. These data indicate that TRESK is activated by increased cytoplasmic calcium concentration. However, application of Ca^{2+} to inside-out membrane patches failed to influence TRESK single channel activity, suggesting that cytoplasmic factors are also required for the regulation. Cyclosporin A and FK506, specific inhibitors of the calcium/calmodulin-dependent protein phosphatase (calcineurin), completely eliminated TRESK activation. Coexpression of a constitutively active form of calcineurin with TRESK increased the basal background K^+ current and attenuated the response of the channel to the calcium signal, indicating that TRESK was activated by the permanent calcineurin activity. Serine 276 was identified as the major functional target of calcineurin in TRESK by alanine-scanning mutagenesis. This is the first example of calcineurin being involved in the regulation of a two-pore domain K^+ channel, and thus, TRESK channels may regulate the excitability of neurons and other cell types in response to Ca^{2+}-mobilizing hormones and neurotransmitters in a manner that is sensitive to immunosuppressive drugs.

Background (leak) K^+ channels drive the membrane potential incessantly toward the K^+ equilibrium potential, stabilize the negative resting membrane potential of nonexcitable cells, and counterbalance the excitability and adjust the firing rate of neurons. Background K^+ conductance has been assigned to cloned two-pore domain potassium ($2PK^+$)[1] channels (for reviews, see Refs. 1 and 2). Two-pore domain K^+ channels are classified as a family based on their similar molecular architecture in addition to amino acid sequence similarity. Each subunit contains four transmembrane segments and two pore-forming domains, one located between the first and second and another between the third and fourth transmembrane segments. (This topology assumes that both the N and C termini of the subunits are intracellular.)

The activity of $2PK^+$ channels is not influenced by membrane potential changes in general; however, distinct physicochemical parameters (intra- and extracellular pH (3–5), temperature (6), membrane tension (7, 8)), hypoxia (9–12), and signaling pathways (protein kinase A and C (13, 14), arachidonic acid (15), and phospholipase C induced reduction of membrane phosphatidylinositol 4,5-bisphosphate (16)) were reported to regulate different $2PK^+$ channels. Considering the robust expression of $2PK^+$ channels in many cell types, the above regulatory mechanisms have major impact on the control of membrane potential and cellular function.

Recently, cloning of a novel member of the $2PK^+$ channel family, human TRESK (TWIK-related spinal cord K^+ channel) was reported (17). TRESK shows limited (<34%) amino acid sequence similarity to other $2PK^+$ channels; accordingly, it belongs to a novel $2PK^+$ channel subfamily. Expression of TRESK in L929 cells resulted in a slightly outwardly rectifying background potassium current, which was inhibited by unsaturated free fatty acids (arachidonic, docosahexaenoic, and linoleic acid), some nonspecific K^+ channel blockers (quinine, quinidine, triethanolamine, and glyburide), and extreme acidification (40% inhibition by pH 5.6) (17).

In the present study, we report that TRESK is a target of receptor-mediated regulation. We have cloned the TRESK channel from another species and tissue (mouse cerebellum) and demonstrate its mRNA expression also in the testis. Therefore, the expression pattern of TRESK seems to be more extensive than originally suggested (also by the denomination of the channel). Here, we show that the stimulation of various Ca^{2+}-mobilizing receptors substantially activates the K^+ current when mouse (or human) TRESK is expressed in *Xenopus* oocytes. We also demonstrate that this receptor-mediated activation is dependent on the cytoplasmic Ca^{2+} signal, and the rise of $[Ca^{2+}]$ is sufficient for TRESK activation regardless of the source of the ion. Thus, TRESK is the first $2PK^+$ channel reported to be regulated by cytoplasmic Ca^{2+}. Unlike the classical Ca^{2+}-activated K^+ channels (having six transmembrane segments), TRESK activation by Ca^{2+} does not involve the binding of the ion directly to the channel protein, but it is

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY325301.

¶ To whom correspondence should be addressed: Dept. of Physiology, Semmelweis University, P. O. Box 259, H-1444 Budapest, Hungary. Tel.: 36-1-266-2755; Fax: 36-1-266-6504; E-mail: enyedi@ puskin.sote.hu.

[1] The abbreviations used are: $2PK^+$, two-pore domain potassium; LPA, lysophosphatidic acid; $InsP_3$, inositol 1,4,5-trisphosphate; CsA, cyclosporin A; RT, reverse transcriptase.

prevented by the inhibition of the calcium/calmodulin-dependent protein phosphatase, calcineurin. Consequently, TRESK may hyperpolarize the membrane potential in response to different stimuli increasing the cytoplasmic calcium concentration, depending on the signal transduction system of the given cell in the spinal cord and also in other tissues. Also, this regulatory mechanism may be considered as a means by which neuronal excitability is affected by immunosuppressive drugs of the calcineurin inhibitor class.

EXPERIMENTAL PROCEDURES

Materials—Enzymes and kits of molecular biology applications were purchased from Ambion (Austin, TX), Amersham Biosciences, Fermentas (Vilnius, Lithuania), New England Biolabs (Beverly, MA), Promega (Madison, WI), and Stratagene (La Jolla, CA). All other chemicals of analytical grade were obtained from Calbiochem, Fluka (Buchs, Switzerland), Promega, and Sigma. Lysophosphatidic acid and cyclosporin A were dissolved in ethanol (0.5 mg/ml and 0.5 mM, respectively), and FK506 was dissolved in Me_2SO (2 mM).

Cloning of Human and Mouse TRESK—A novel K^+ channel pore domain was found in the human genome data base with a computer program developed in our laboratory (source code available upon request). The conceptual coding region of the new channel was calculated from the neighboring genomic sequences by the FGENESH program at the Softberry site on the World Wide Web. Since no expressed sequence tag information was available, the three human exons were PCR-amplified one by one from genomic DNA and assembled (primer sequences and PCR conditions not shown). This resulted in a functional $2PK^+$ channel having essentially the same properties as its mouse counterpart described extensively in this paper (data not shown). The sequence of our exon-concatenated human $2PK^+$ channel is identical to the recently reported sequence of human TRESK (17).

Since the exon boundaries of our artificially constructed human $2PK^+$ channel were only probable at the time of its cloning, we cloned TRESK also from a natural source, mouse cerebellar RNA, by RT-PCR. Total RNA was extracted from different mouse tissues as previously described (18). The complete coding region of mouse TRESK was PCR-amplified with −8s (5′-ATCgaattcCAAGAGGATGGAGGCTGAGG-3′) and 1185a (5′-GCGctcgagTTACCAAGGTAGCGAAACTTCCCTTTG-3′) primers (designed on the basis of mouse genomic sequences) from cerebellar RNA after reverse transcription with Moloney murine leukemia virus reverse transcriptase enzyme (Promega, Madison, WI). Taking advantage of the EcoRI and XhoI sites (marked with lowercase letters) of the −8s and 1185a primers, respectively, the TRESK coding region was ligated into pEXO vector. The sequence of TRESK was determined by automatic sequencing. The PCR-derived original clone contained two mutations: a silent one, which was not corrected, and another, which was corrected with QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the final clone was resequenced. Since the correcting primers (not shown) were designed to contain two further silent mutations (thus creating an easily detectable Bsp119I site), there were three silent mutations (C42T, T720C, and G723A) in our final construct, not affecting the amino acid sequence of mouse TRESK.

Localizing TRESK with RT-PCR—Tissue localization of TRESK was determined by RT-PCR from different mouse RNAs, with −172s (5′-AGCCAAACATGATGGGTTCC-3′, annealing to the 5′-untranslated region of TRESK) and 453a (5′-TCCAAAGAGCGCATACAGC-3′) primers. (The PCR protocol was as follows: 15-min initial denaturation and Hot Star *Taq* (New England Biolabs, Beverly, MA) activation at 94 °C; 40 cycles of 60-s denaturation at 94 °C, 60-s annealing, 90-s extension at 72 °C); and a final 6-min extension at 72 °C. The annealing temperature was 64, 62, and 60 °C in the first five, second five, and final 30 cycles, respectively.) The control 532-bp glyceraldehyde-3-phosphate dehydrogenase PCR product was amplified for 30 cycles with sense (5′-TGGGTGGAGCCAAACGGGTC-3′) and antisense (5′-GGAGTTGCTGTTGAAGTCGCA-3′) primers.

Subcloning of the Calcineurin Subunits—The wild type and constitutively active calcineurin A coding regions were amplified by *Pfu* polymerase from pBJ5-CnA-FL, applying the sense primer (5′-CAGgaattCTGGAGATGTCCGAGCCCAAGGCGATTG-3′), and antisense primers (5′-GCGctcgagCAATCCCATCATGCCCTGCAGCTCAA-3′) and (5′-GCGctcgagCTATTTCCCGCCAGAGAGCAC-3′), respectively. The two PCR products were ligated between the EcoRI and XhoI sites of pEXO, taking advantage of the restriction enzyme sites incorporated into the primers (indicated by lowercase letters). The calcineurin B coding region was amplified from pBJ5-CnB with the primers (5′-CAGGAATTCGAGCAAAATGGGAAATGAGGCAAG-3′) and (5′-GCGctcgagTCACACATCTACCACCATC-3′). The product was digested with XhoI and cloned into pEXO digested with EcoRI, treated with Klenow polymerase, and digested with XhoI.

In Vitro Site-directed Mutagenesis—*In vitro* site-directed mutagenesis was performed according to the manufacturer's instructions using the QuikChange™ site-directed mutagenesis kit (Stratagene). Complementary primer pairs were designed, coding for the desired mutation together with discriminating silent mutations (introducing or eliminating restriction enzyme sites). The primer sequences of the sense oligonucleotides in the 5′ to 3′ direction were the following: S192A, CTTCAAATGGCGAGCTCTCCCGCTCTGC; S264A, GAGAGGAGCAACGCGTGTCCCGAGCTG; S175A, AGGGCTTACGCTCGATTCCAGGCTCTCCTTTG; S227A/S232A/S234A, AACCCGCAGCCGGCCAAGGACCCCCCCGCTCCGGCATGCAATGTGGAGCTG; T256A/S262A, CTACAACCACCCGCGCGTCCCGTGGAGAGGGCCAACTCCTGTCCCGAG; S274A/S276A/S279A, CTGGGGCGACTGGCATGCGCTATTCTCGCGAATCTGGATGAAGTGG; S274A, GCTGGGGCGACTGGCATGCTCTATTCTCAGCAATC; S276A, GGCGACTGTCCTGTGCGATTCTCAGCAATCTGG; S279A, CCTGTTCTATTCTCGCGAATCTGGATGAAGTGG; S264E, GAGAGGAGCAACGAATGCCCCGAGCTGGTGCTGG; S274E, GTGCTGGGGCGACTCGAGTGTTCTATTCTCAGCAATC; S276E, GGCGACTGTCCTGTGAGATTCTCAGCAATCTGG. The S391A mutant of TRESK was amplified by PCR with the −8s (see above) sense and (5′-GAGCTCGAGTTACCATGGTAGTGCAACTTCCCTTTG-3′) antisense primers. The mutant clones were identified by restriction enzyme mapping and automatic sequencing.

Synthesis of Ion Channel, Receptor, and Calcineurin cRNA—The cRNAs coding for TRESK, the different receptors and calcineurin subunits were synthesized using the Ambion mMESSAGE mMACHINE™ T7 *in vitro* transcription kit (Ambion, Austin, TX). The template for the wild type or mutant TRESK ion channel cRNAs was the XbaI-linearized pEXO-TRESK or its mutant counterparts, respectively. AT_{1a} angiotensin (19), M_1 (20), and M_2 muscarinic (21) receptor cRNAs were synthesized as previously described (16).

Animals, Tissue Preparation, and Xenopus laevis Oocyte Injection—Oocytes were prepared as previously described (22). Oocytes were injected with 50 nl of the appropriate cRNA solution 1 day after defollicluation. Electrophysiological experiments were performed 3 or 4 days after the injection. The tissues for RNA preparation derived from NMRI strain of *Mus musculus* (Toxicop). 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 Semmelweis University.

Two-electrode Voltage Clamp Measurements—Whole oocyte currents were recorded by two-electrode voltage clamp (OC-725-C; Warner Instrument Corp., Hamden, CT) using microelectrodes made of borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK) with a resistance of 0.3–1 megaohms when filled with 3 M KCl. Currents were filtered at 1 kHz and digitally sampled at 1–2.5 kHz with a Digidata Interface (Axon Instruments, Foster City, CA). Recording and data analysis were performed using pCLAMP software version 6.0.4 (Axon Instruments). Experiments were carried out at room temperature. Different solutions were applied by a gravity-driven perfusion system. Low $[K^+]$ solution contained 95.4 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 5 mM HEPES (pH 7.5 with NaOH). High $[K^+]$ solution contained 80 mM K^+ (78 mM Na^+ of the low $[K^+]$ solution was replaced with K^+).

The volume of microinjections was 50 nl, and the pH of Ca^{2+} + EGTA + HEPES, EGTA + HEPES, and HEPES solutions was adjusted to 7.3 with KOH. The injected 50-nl volume was diluted to ~10-fold in the cytoplasm of the oocyte. The steady state free $[Ca^{2+}]$ was calculated to fall from the original 30 μM (in 5 mM total $[Ca^{2+}]$ plus 5 mM [EGTA]) to about 100 nM during the reduction of the total $[Ca^{2+}]$ from 5 to 2 mM. (At 4.9 and 4.1 mM total $[Ca^{2+}]$, the free $[Ca^{2+}]$ was <10 μM and <1 μM in this calculation, respectively.) Background K^+ currents were measured in high EC $[K^+]$ at the end of 300-ms-long voltage steps to −100 mV applied every 3 s. The holding potential was 0 mV. For estimating the amplitude of the background K^+ current, the inward current in high $[K^+]$ was corrected for the small nonspecific leak measured in 2 mM EC $[K^+]$ at −100 mV.

Single Channel Recordings—Single channel recordings were performed with an Axopatch 1D amplifier (Axon Instruments) using microelectrodes made of borosilicate glass (Clark Electromedical Instruments) with resistance of 30–80 megaohms when coated with R-6101 elastomer (Dow Corning, Midland, MI), fire-polished and filled with pipette solution, containing 140 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 10 mM HEPES (pH 7.4 with NaOH). Bath solutions contained 2 mM $MgCl_2$,

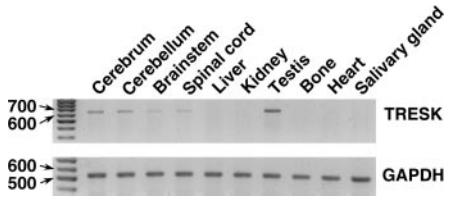

FIG. 1. **Localization of TRESK mRNA in different mouse tissues by RT-PCR.** One microgram of total RNA was reverse transcribed, and TRESK PCR product was amplified with −172s and 453a primers, as detailed under "Experimental Procedures." Marker bands on the *left* represent DNA fragments from 300 to 900 bp in 100-bp increments. The *lower panel* shows the control, 532-bp glyceraldehyde-3-phosphate dehydrogenase PCR products, amplified from the same RNAs as the TRESK fragment. Marker bands on the *left* represent DNA fragments from 400 to 700 bp in 100-bp increments.

5 mM EGTA, 10 mM HEPES (pH 7.3 with NaOH) plus 140 mM KCl in high or 4 mM KCl, 136 mM NaCl in low $[K^+]$ solution. Experiments were performed at room temperature after oocytes were devitellinized manually in a hyperosmotic solution, containing 200 mM DL-aspartic acid, 20 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES (pH 7.4 with KOH). For low noise recordings, seal resistance was above 40 gigaohms. The cut-off frequency of the eight-pole Bessel filter was adjusted to 2 kHz, and data were acquired at 10 kHz with Digidata 1200. Recordings shown in Fig. 5 were not filtered further.

Statistics and Calculations—Data are expressed as means ± S.E. Statistical significance was estimated by t test for independent samples, and the difference was considered to be significant at $p < 0.05$. Multiple comparisons (in Figs. 7 and 8) were performed by the nonparametric Kruskal-Wallis analysis of variance. The subsequent planned pairwise comparisons of the groups were performed with the nonparametric Mann-Whitney U test, and the difference was considered to be significant at $p < 0.05/k$ (according to the Bonferroni adjustment), where k was the number of the pairwise comparisons. The Statistica 6.0 program package (StatSoft, Tulsa, OK) was used for the analysis. Single channel current histograms were fitted to the sum of $n + 1$ normal (Gauss) distributions, $y = \Sigma(a_i/(w_i \times \sqrt{\pi/2}) \times \exp(-2 \times ((x - q_i)/w_i)^2))$, where i runs from 1 to $n + 1$ (n is the maximum number of simultaneously open channels), a_i is the amplitude, w_i is width, and q_i is the position of the peak along the abscissa.

RESULTS

TRESK Is Expressed in the Central Nervous System and Also at the Periphery—The coding regions of mouse and human TRESK were cloned from human genomic DNA and mouse cerebellar mRNA, respectively (see "Experimental Procedures"). Mouse TRESK is composed of 394 amino acid residues (10 residues longer than the human channel), and its amino acid sequence is 67% identical to its human counterpart. The mouse TRESK gene is located on chromosome 19 in the 19D3 region. The BAC clone, RP-292H20 (AC139040), includes the whole coding sequence between 168,324 and 152,449 bp in the negative strand (three exons of 256, 129, and 800 bp interspersed by two introns of 5421 and 9270 bp, respectively). The sequence of mouse TRESK cDNA was deposited to GenBank™ (accession number AY325301). The expression of TRESK mRNA was examined in different mouse tissues by RT-PCR. The specificity of the PCR product was verified by restriction enzyme mapping (not shown). The specific 626-bp product was amplified from cerebrum, cerebellum, brainstem, spinal cord, and testis (Fig. 1).

TRESK Is Activated by the Stimulation of Calcium-mobilizing Receptors—TRESK current was measured in 80 mM extracellular K^+ concentration at −100 mV with a two-electrode voltage clamp. (The $[K^+]$ was occasionally reduced to 2 mM to verify that the charge carrier was K^+.) Microinjection of the *in vitro* synthesized mouse TRESK cRNA induced the expression of a noninactivating, background K^+ current in *X. laevis* oocytes, not present in noninjected or water-injected cells. In oocytes coexpressing M_1 muscarinic receptor with TRESK, stimulation with carbachol (1 μM) evoked substantial K^+ current activation (10.4 ± 1.4-fold, $n = 5$). The effect was slowly reversible (>20 min) after the withdrawal of the stimulus and was reproducible (see Fig. 2 for a representative curve).

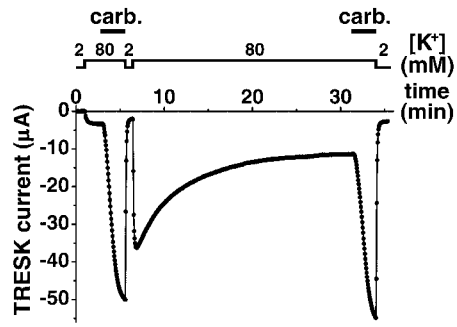

FIG. 2. **Stimulation of M_1 muscarinic receptor activates the two-pore domain K^+ channel TRESK expressed in *Xenopus* oocytes.** Currents of an oocyte coexpressing TRESK and M_1 muscarinic receptor were measured at the end of 300-ms voltage steps to −100 mV applied in every 3 s from a 0-mV holding potential. The oocyte was stimulated twice with carbachol (1 μM; *horizontal black bars*), and the extracellular $[K^+]$ was occasionally reduced from 80 to 2 mM, as indicated *above* the *graph*. Note the long time scale of the recording.

Stimulation with angiotensin II (10 nM) also activated TRESK in oocytes coexpressing another Ca^{2+}-mobilizing receptor, AT_{1a} angiotensin receptor, with the channel (13.3 ± 1.9-fold activation, $n = 5$). Since the stimulation of overexpressed receptors may generate nonphysiologically robust signals, we also examined an endogenous Ca^{2+}-mobilizing receptor of the oocyte. Stimulation of the lysophosphatidic acid (LPA) receptor with 0.5 μM LPA in oocytes expressing only TRESK also activated the K^+ current strongly (7.6 ± 2.5-fold, $n = 6$). In contrast to these three different Ca^{2+}-mobilizing receptor types, stimulation of the heterologously expressed M_2 muscarinic receptor (activating G_i protein instead of G_q) failed to activate TRESK (data not shown). Since the stimulation of the different receptors evoked the same effects on human TRESK (not shown), activation of TRESK turned out to be specific for Ca^{2+}-mobilizing receptors.

The Cytoplasmic Ca^{2+} Signal Is Necessary and Sufficient for TRESK Activation—Stimulation of Ca^{2+}-mobilizing receptors releases Ca^{2+} from intracellular stores through the formation of inositol 1,4,5-trisphosphate ($InsP_3$). Therefore, we have examined the effect of $InsP_3$ microinjection (10 ng) on TRESK current. $InsP_3$ (but not water) microinjection activated TRESK 12.2 ± 1.3-fold ($n = 5$; Fig. 3*A*), suggesting that $InsP_3$ was involved in the receptor-mediated TRESK activation. The calcium ionophore, ionomycin, evokes a receptor-independent increase of the cytoplasmic $[Ca^{2+}]$. Ionomycin (0.5 μM) activated TRESK current 6.2 ± 0.4-fold ($n = 57$; Fig. 3*B*).

Inhibition of the endoplasmic reticulum Ca^{2+}-ATPase by Tg pretreatment (1 μM, 5–6 h) and the following depletion of the intracellular Ca^{2+} store prevented the LPA-mediated TRESK activation. (In the Tg- and Me_2SO-treated (control) groups, TRESK was activated 1.2 ± 0.1- and 2.8 ± 0.4-fold by 0.5 μM LPA in 75 s, respectively ($n = 5$, $p < 0.003$). The currents after the Tg and Me_2SO pretreatment (before the stimulation with LPA) were not significantly different (11.5 ± 5.8 and 15.5 ± 4.4 μA, respectively). The efficiency of thapsigargin and ionomycin suggested that the elevation of cytoplasmic $[Ca^{2+}]$ is required and sufficient for the regulation of TRESK irrespective of the other branches of the divergent signaling pathways activated by Ca^{2+}-mobilizing receptors.

Next we designed an experiment to verify that the exogenously added Ca^{2+} can also activate TRESK. Since TRESK activation could not be reliably evoked by the microinjection of solutions containing millimolar free $[Ca^{2+}]$ (probably as a consequence of

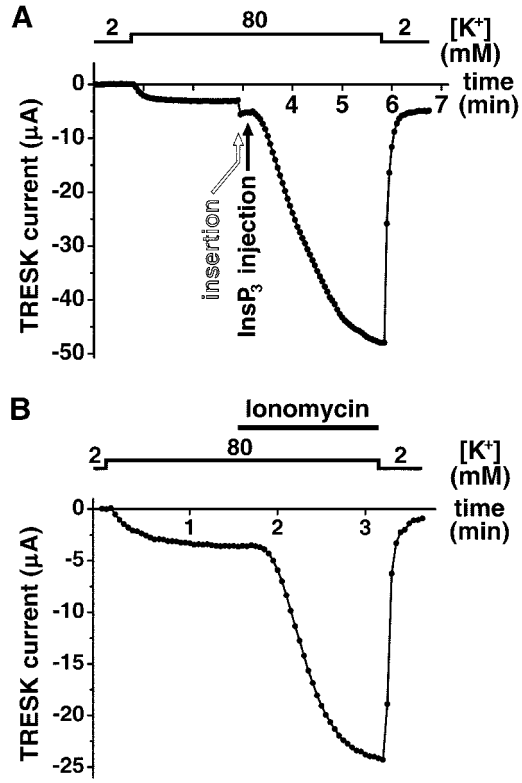

FIG. 3. **Microinjection of $InsP_3$ and extracellular application of ionomycin also activates TRESK.** *A*, $InsP_3$ was microinjected during two-electrode voltage clamp measurement. *Hollow arrow*, insertion of the injection capillary in the oocyte (followed by a small increase of the nonspecific leak); *filled arrow*, injection of 10 ng of $InsP_3$ (resulting in substantial TRESK activation). *B*, an oocyte was perfused with a solution containing 0.5 μM ionomycin (as indicated by the *bar* above the *graph*). The method of measurement and representation was the same as in Fig. 2.

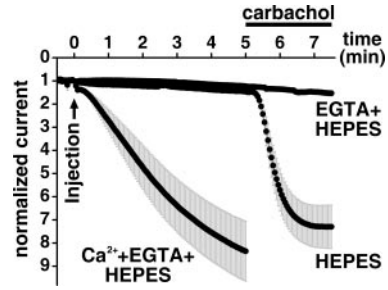

FIG. 4. **Injection of Ca^{2+}-saturated EGTA activates TRESK, whereas EGTA injection prevents the receptor-mediated TRESK activation.** Three groups of oocytes expressing TRESK were injected with Ca^{2+} + EGTA + HEPES (50 mM each), EGTA + HEPES (both 50 mM), and HEPES (50 mM), respectively, at 0 min ($n = 3 \times 4$). Their current was measured in 80 mM extracellular K^+ at −100 mV for 5 min after injection, and then in EGTA + HEPES and HEPES groups cells were stimulated with 1 μM carbachol through their coexpressed M_1 receptor (as indicated by the *bar*). The currents were normalized to the value measured at 0 min, and the averages were plotted for the three groups. The *gray error bars* represent ± S.E.

the high Ca^{2+}-buffering capacity and the low diffusion length of Ca^{2+} in the cytoplasm), a highly Ca^{2+}-saturated Ca^{2+} buffer was injected (Ca^{2+}, EGTA, HEPES, 50 mM each). The microinjection of this solution activated TRESK to a similarly high extent as receptor stimulation (8.4 ± 1.3-fold in 5 min) (Fig. 4). If the injected solution contained only EGTA and HEPES (both 50 mM) and thus the cytoplasmic [Ca^{2+}] was buffered to low levels, TRESK current was not affected by the injection (1.2 ± 0.1-fold increase in 5 min), and the subsequent stimulation of the coexpressed M_1 receptor also failed to activate TRESK (1.2 ± 0.1-fold further increase). This lack of activation by receptor stimulation was not the consequence of the nonspecific effects of microinjection, since the control oocytes injected only with HEPES (50 mM) responded to carbachol stimulation (7.3 ± 0.9-fold activation compared with the 0 min value) (Fig. 4). Thus, TRESK was activated by cytoplasmic calcium irrespective of the source of the ion, and buffering cytoplasmic calcium concentration to low levels prevented the receptor-mediated TRESK activation. These experiments indicate unequivocally that calcium is the key to TRESK activation.

The Ca^{2+}-dependent Activation Is Not Mediated by the Binding of Ca^{2+} to TRESK Channel—The slow reversibility of the whole oocyte TRESK current after the withdrawal of the receptor stimulation suggested that TRESK is not regulated by direct Ca^{2+} binding. To confirm this conclusion, the straightforward experiment was to apply Ca^{2+} to the intracellular side of inside-out membrane patches. Therefore, we investigated the single channel properties of TRESK in excised membrane patches of *Xenopus* oocytes in the inside-out configuration.

In oocytes expressing TRESK, a characteristic single channel activity appeared, which was not observed in control cells (Fig. 5, *A* and *B*). In symmetrical 140 mM K^+, this activity consisted of both outward and inward currents of single channel openings at depolarized and hyperpolarized membrane potentials, respectively (Fig. 5*A*, *left panel*). When [K^+] was reduced to 4 mM on the accessible intracellular side, outward currents were diminished, whereas the inward currents of openings were not drastically influenced as is expected for a K^+ channel (Fig. 5*A*, *right panel*). TRESK exhibited an interesting asymmetrical single channel activity also in symmetrical 140 mM K^+. TRESK demonstrated well defined, square wave-like openings at depolarized potentials, but in the case of hyperpolarization it produced bursts of very short openings (<1–2 ms), which could not be resolved as square waves of constant size by our recording apparatus (Fig. 5*B*). Thus, the single channel conductance of TRESK was determined from the outward current of openings in symmetrical 140 mM K^+ (13 ± 1 picosiemens, $n = 4$) (Fig. 5*C*).

High concentration of calcium (10 μM), which activated TRESK severalfold in the whole oocyte microinjection experiments, failed to alter TRESK single channel activity significantly in three examined inside-out patches (for representation, see Fig. 5, *D* and *E*). This result indicates that TRESK is not regulated by direct Ca^{2+} binding; rather, a more complex cytoplasmic signaling mechanism is involved in the regulation.

Pharmacological Inhibition of Calcineurin Eliminates TRESK Activation—In order to gain further insight into the mechanism of TRESK activation, the effect of cyclosporin A (CsA), the specific inhibitor of calcineurin (Ca^{2+}/calmodulin-dependent protein phosphatase, protein phosphatase 2B) was examined. CsA pretreatment (100 nM, 2–3.5 h) did not change base-line TRESK current (3.3 ± 1.3 μA in the CsA-pretreated ($n = 5$) and 2.6 ± 0.7 μA in the control group ($n = 7$)). However, this low concentration of CsA completely eliminated the ionomycin-induced TRESK activation (1.2 ± 0.1-fold activation in 1.5 min in the CsA-pretreated group and 6.9 ± 0.6-fold in the control group) (Fig. 6*A*). The selective effect of CsA on TRESK activation raised the possibility that calcineurin participated in the activation process.

To confirm that calcineurin plays a role in TRESK activation, we applied another calcineurin inhibitor, FK506, which has a different mechanism of action. In the FK506-pretreated (200 nM, 2–3 h) group, the resting TRESK current was smaller (1.7 ± 0.6 μA) than in the control group (6.3 ± 2.6 μA) (an effect that might reflect the extinction of the basal calcineurin activity by FK506). FK506 preincubation also abolished the iono-

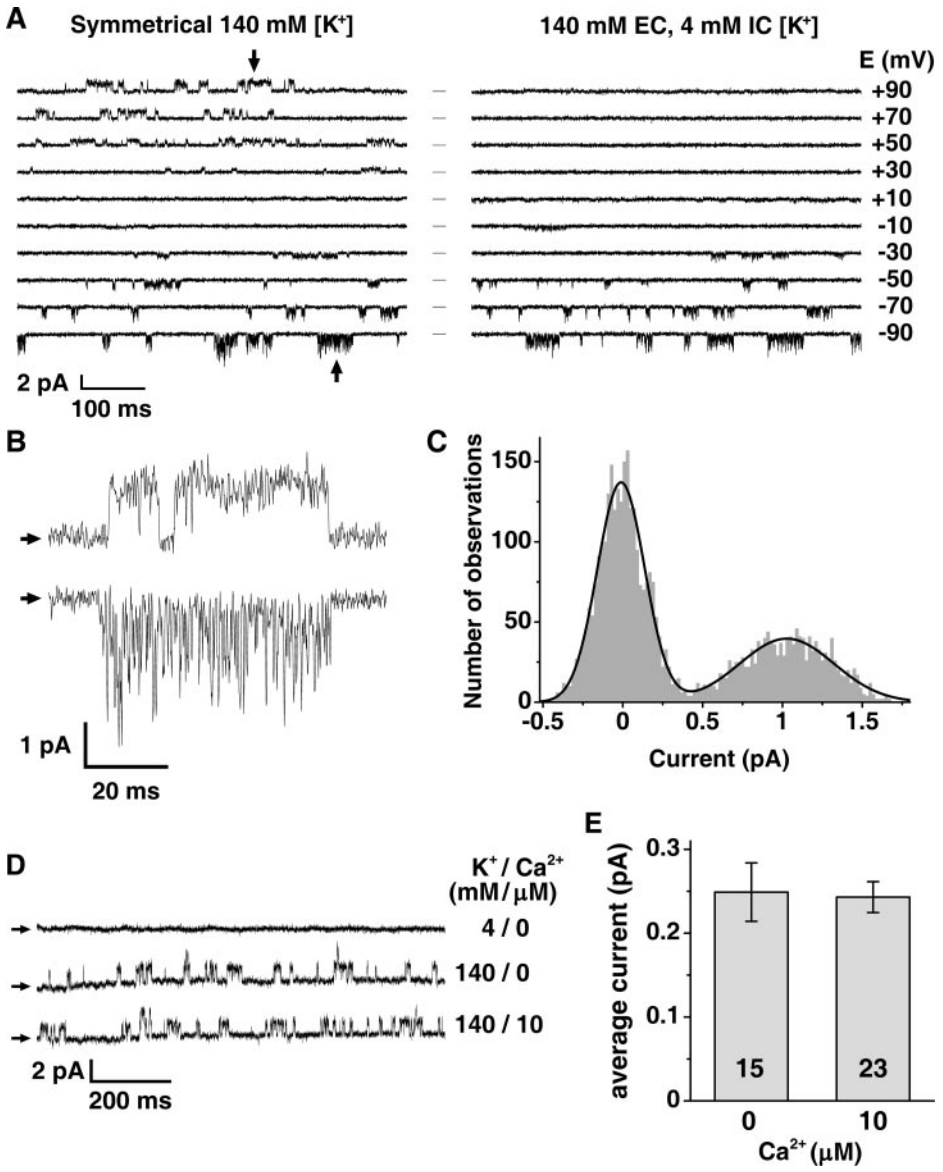

FIG. 5. **Direct application of high [Ca^{2+}] to TRESK in inside-out membrane patches does not alter the single channel activity.** *A–C*, characterization of TRESK single channel properties. *A*, representative single channel openings of TRESK were recorded in an inside-out *Xenopus* oocyte membrane patch at different command potentials (as indicated on the *right*) in symmetrical 140 mM K^+ (*left*) and in the same patch after decreasing [K^+] to 4 mM on the intracellular side (*right*). Zero current levels are indicated with *horizontal lines* in the *middle*. *B*, channel openings at +90 and −90 mV, indicated with *vertical arrows* in *A*, were magnified. Note the square wave-like openings of outward current and the train of very short openings of inward current. Zero current levels are indicated with *horizontal arrows*. *C*, *histogram* of the current recording in symmetrical 140 mM K^+ at +90 mV was fitted with the sum of two normal distributions. The unitary current of TRESK is represented by the distance of peaks along the *abscissa*. *D*, representative TRESK single channel activity of an inside-out patch is shown in intracellular solutions of different [K^+] and [Ca^{2+}], as indicated on the *right*. In every 4 s, a segment (1-s duration) of single channel activity was recorded at +90 mV. Zero current levels are indicated by *horizontal arrows*. *E*, the average current was calculated from the 1-s segments of the same patch as in *D*. The *numbers* in the *bars* represent the number of analyzed segments in the absence and presence of 10 μM Ca^{2+} in symmetrical 140 mM K^+.

mycin-induced TRESK activation (1.5 ± 0.1-fold activation in 1.5 min in the FK506-pretreated group and 6.3 ± 1.5-fold in the control group (n = 5 for both groups)) (Fig. 6*B*). This massive inhibition of the response to ionomycin confirmed that calcineurin is a key element in TRESK activation.

Coexpression of a Constitutively Active Form of Calcineurin with TRESK Increases the K^+ Current and Attenuates the Response of the Channel to the Calcium Signal—In addition to the pharmacology, the role of calcineurin in the regulation of TRESK was also demonstrated by another approach. A constitutively active form of the phosphatase (CnA 1–441), lacking the C-terminal autoinhibitory domain, was coexpressed with the K^+ channel. Coexpression of the truncated calcineurin A and the regulatory calcineurin B subunits with TRESK (TRESK + CnA 1–441 + CnB, triple coexpression) induced significantly higher basal background K^+ currents than TRESK expression alone (Fig. 7*A*). Moreover, the calcium signal (evoked by ionomycin application) did not stimulate this higher basal current further as much as in cells expressing TRESK only (Fig. 7*B*). These results indicate that the constitutively active calcineurin converted TRESK to its activated state, which could not be further stimulated by the calcium signal. The regulatory B subunit of calcineurin was also required for efficient TRESK activation, since the coexpression of only CnA 1–441 with TRESK (without calcineurin B, *TRESK + CnA 1–441*) failed to affect the base-line K^+ current and only slightly reduced the response to the calcium signal (Fig. 7). Only the constitutively active form of calcineurin could stimulate TRESK permanently; coexpression of wild type calcineurin A and B subunits with TRESK (*TRESK + wtCnA + CnB*) influenced neither the basal channel activity nor the response to ionomycin (Fig. 7).

S276A Mutation in the Intracellular Loop of TRESK Confers High Basal Activity to the Channel and Abolishes Its Response to the Calcium Signal—Mutagenesis experiments were performed in order to identify the regions of TRESK that were important in the regulation evoked by the calcium signal. The initial approaches (deletion of a large part of the intracellular loop or replacement of this region with the corresponding (significantly shorter) loop of another 2PK$^+$ channel, TASK3) failed to result in functional channels (data not shown). Therefore, more subtle changes were introduced by *in vitro* site-directed mutagenesis.

Disruption of the amphiphysin-type Src homology 3-binding site motif in the intracellular loop (P255G/R257G double mutant) did not influence the response to the calcium signal (not shown). To examine whether the channel itself might be the substrate of calcineurin, we focused an alanine-scanning mu-

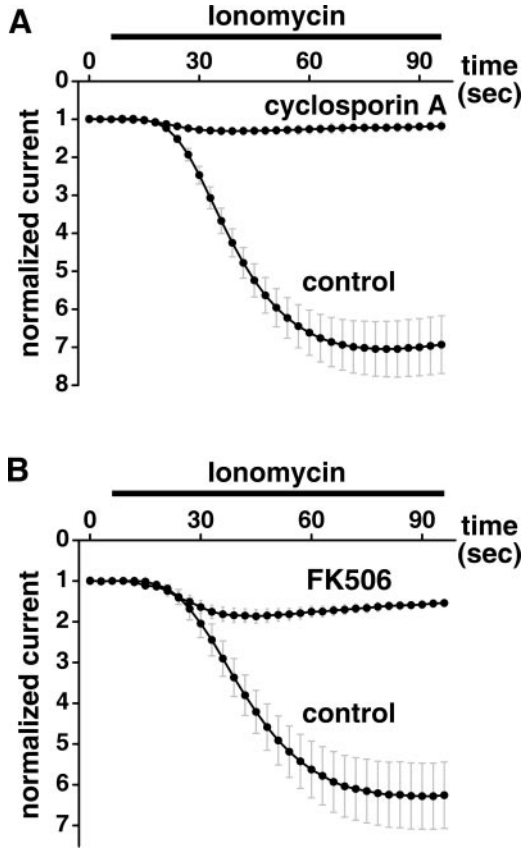

FIG. 6. **Cyclosoprin A and FK506 eliminate the Ca^{2+}-dependent TRESK activation.** *A*, cyclosporin A-pretreated (100 nM, 2–3.5 h, $n = 5$) and control ($n = 7$) oocytes were stimulated with ionomycin (0.5 μM) in 80 mM extracellular K^+. TRESK currents were measured at −100 mV as in Fig. 2, normalized to the value at the beginning of the stimulation, and averaged. The *gray error bars* represent ± S.E. *B*, the same experiment as in *A* was performed with FK506 (200 nM, 2–3-h preincubation) instead of cyclosporin.

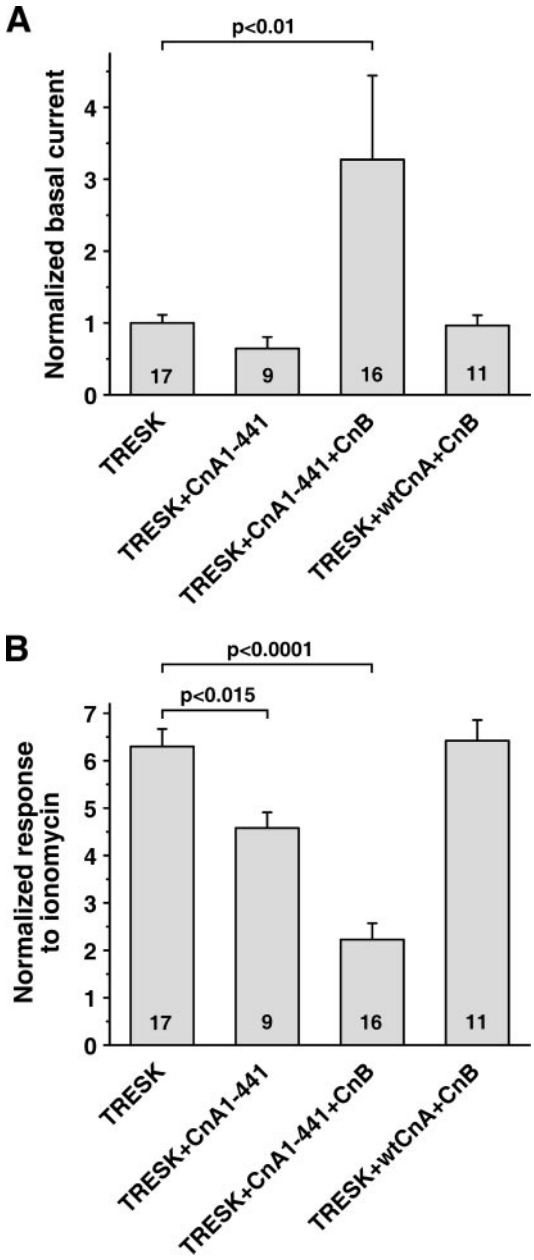

FIG. 7. **The constitutively active calcineurin increases TRESK base-line currents and reduces the response to the Ca^{2+} signal.** *A*, background K^+ currents were measured in oocytes expressing TRESK (*TRESK*); coexpressing TRESK and the deleted, constitutively active, calcineurin A (*TRESK + CnA1–441*); coexpressing TRESK, the deleted calcineurin A, and wild type calcineurin B (*TRESK + CnA1–441 + CnB*); or coexpressing TRESK plus wild type calcineurin A and B (*TRESK + wtCnA + CnB*). The currents were normalized to the average of the control (*TRESK*) group of the same oocyte preparation. *B*, the cells, (co)expressing the same protein (combinations) as in *A*, were stimulated with ionomycin as illustrated on Fig. 3*B*. The current emerging after the stimulation was normalized to the value measured before the application of ionomycin in each oocyte. The *numbers* in the *bars* represent the number of measured oocytes. The *brackets above* the *graph* indicate the significant differences determined by the pairwise comparisons.

tagenesis study on serine and threonine amino acids of TRESK. In the initial attempts to find the potential target(s) of the phosphatase, we modified known motifs and/or amino acids corresponding to important regulatory sites of other $2PK^+$ channels. First we tried to disrupt two consensus sites for the phosphorylation-dependent binding of 14-3-3 protein in the loop of the channel. One of these mutations, S264A, reduced the responsiveness of TRESK to the calcium signal (evoked by carbachol through the M_1 receptor in this case) (Fig. 8*A*), suggesting that serine 264 is one of the potential targets of calcineurin in TRESK. On the other hand, the mutant of the other 14-3-3 binding site, S192A, responded to M_1 receptor stimulation similarly to the wild type channel (and the response of the triple mutant, S175A/S192A/S264A, did not differ from that of S264A). The significantly reduced, but not abolished (3-fold), response of the S264A mutant to carbachol suggested that another (perhaps more important) residue may also be involved in the regulation. Since the phosphorylation of a serine close to the C terminus was suggested to have an impact on the regulation in other $2PK^+$ channels (23), we mutated serine 391 to alanine; however, the S391A mutant responded well to the calcium signal (Fig. 8*A*).

In the following more detailed screening, we tested not only the responsiveness of the mutant channels to the Ca^{2+} signal but compared also their basal activity to that of the wild type channel, with the assumption that a mutant TRESK, containing alanine instead of serine/threonine residue in a location of regulatory importance, corresponds to the dephosphorylated form of the channel, and thus it is expected to have higher basal activity and lower response to the calcium signal. Eight further serine/threonine residues were tested by creating three double/triple mutants. Whereas the basal currents and responses to the calcium signal (evoked by ionomycin) were not affected in the cases of the S227A/S232A/S234A triple and the T256A/S262A double mutants (Fig. 8, *B* and *C*), the response of the

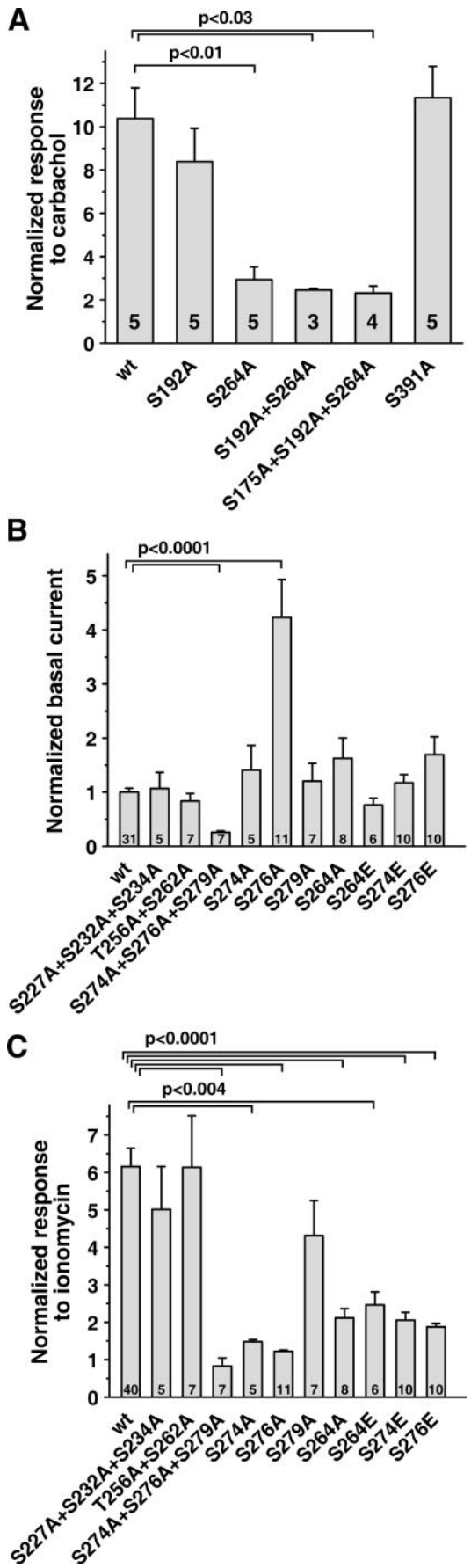

FIG. 8. **Basal currents of TRESK mutants and their responses to the Ca^{2+} signal.** *A*, the oocytes, coexpressing wild type TRESK (*wt*) or one of the mutant versions of the channel (as indicated *below* the *columns*) with M_1 muscarinic receptor, were stimulated with carbachol (1 μM). The current emerging after the stimulation was normalized to the value measured before the application of carbachol in each oocyte. *B*, basal background K^+ currents were measured in oocytes expressing TRESK (wild type) or one of the different mutant versions of the channel. The currents of mutant TRESK channels were normalized to the average of the control (wild type) group of the same oocyte preparation. *C*, the response of the same mutants as in *B* to ionomycin was measured and represented in the same way as in Fig. 7*B*.

S274A/S276A/S279A mutant to the Ca^{2+} signal was completely eliminated (Fig. 8*C*). However, in contrast to the expectations, this mutant expressed lower basal current than the wild type channel (Fig. 8*B*). To dissociate the effects of the three point mutations of this triple mutant, we examined S274A, S276A, and S279A separately. Whereas the S279A mutant did not differ significantly from the wild type channel, S276A expressed extremely high basal currents (4.2-fold compared with the wild type) (Fig. 8*B*) and was minimally (1.2-fold) stimulated further by the Ca^{2+} signal (Fig. 8*C*). Therefore, according to the premises, the serine 276 residue seems to be the major functionally important target of calcineurin in TRESK. The S274A mutation also reduced the response to the Ca^{2+} signal to very low levels; however, the basal current of this mutant was not different from that of the wild type (Fig. 8*B*), suggesting that the alanine in position 274 may rather interfere with the dephosphorylation mechanism and does not replace a phosphoserine residue of regulatory importance. The reduced responsiveness of the S264A mutant to the Ca^{2+} signal was also reproduced with the ionomycin stimulus (Fig. 8*C*), suggesting again that the potential phosphorylation of serine 264 has a more limited functional consequence than that of serine 276.

A TRESK mutant with a negatively charged glutamate residue replacing serine in a location of regulatory importance mimics the phosphorylated form of the channel; accordingly, this mutant may be expected to have normal/low basal activity and lower response to the calcium signal compared with the wild type. To test this assumption on the locations identified by alanine-scanning mutagenesis, three glutamate mutants (S264E, S274E, and S276E) were examined. In good accordance with the results obtained from the alanine scanning, the basal current of these glutamate mutants was not significantly different from that of the wild type TRESK (Fig. 8*B*); however, their response to the calcium signal was significantly reduced (Fig. 8*C*).

DISCUSSION

Cellular activation is often mediated by membrane depolarization and/or by the cytoplasmic Ca^{2+} signal. The two phenomena may be interconnected in either a positive or a negative way in different cells. The most direct link from membrane potential to Ca^{2+} signal is established through voltage-dependent calcium channels (24) and electrogenic Ca^{2+} carriers (*e.g.* Na^+/Ca^{2+} exchangers (25)). In contrast, the membrane potential can be regulated by the cytoplasmic Ca^{2+} level through various Ca^{2+}-regulated ion channels (*e.g.* SK and BK K^+ channels (26)). In addition, many cells possess more complex signaling systems to coordinate their membrane potential and cytoplasmic [Ca^{2+}], including Ca^{2+}-dependent phosphorylation of ion channels (*e.g.* see Ref. 27) or regulation by spatially arranged protein complexes (*e.g.* DHP receptor/ryanodine receptor interaction (28)). Our present study reveals a novel mechanism, whereby cytoplasmic [Ca^{2+}] can regulate the membrane potential. We show that the Ca^{2+} signal activates TRESK, a recently cloned background $2PK^+$ channel.

It has been reported previously that certain $2PK^+$ channels can be inhibited by Ca^{2+}-mobilizing agonists. For example, we have shown that TASK-1 is inhibited by stimulation of AT_{1a}

angiotensin and M_1 muscarinic receptors (16, 29), and similar effects of the thyrotropin-releasing hormone, metabotrop Glu type I, and several other Ca^{2+}-mobilizing receptors were found by others in different expression systems or in native tissues (30–35). We have also demonstrated that the receptor-mediated TASK-1 inhibition is related to the activation of phospholipase C, but it is independent of DAG and $InsP_3$ generation (and of the concomitant calcium signal) (16). Apparently, the inhibitory effect in the case of TASK-1 is a direct consequence of the breakdown of phosphatidylinositol 4,5-bisphosphate, suggesting that the phospholipid environment in the plasma membrane affects channel activity (16, 36). This mechanism of inhibition applies also to another member of the TASK subfamily; Ca^{2+}-mobilizing agonists exert a weaker inhibitory effect also on TASK-3 (37), whereas the heterodimer of TASK-1 and TASK-3 subunits shows intermediate inhibition (22). TREK channels that belong to another $2PK^+$ subfamily may also be affected by Ca^{2+}-mobilizing receptors. They were found to be inhibited by type I metabotrop Glu receptor stimulation (38), an effect attributed to the receptor-mediated accumulation of diacylglycerol and phosphatidic acid in the membrane (36). The common theme in all of these cases is that the receptor-mediated regulation is independent of the elevated $[Ca^{2+}]$.

A potential direct relationship between a cloned $2PK^+$ channel and the cytoplasmic $[Ca^{2+}]$ has been suggested in the case of KCNK6, since this channel has a Ca^{2+}-binding EF-hand motif (39). However, KCNK6 has never been expressed functionally, and thus the functional relevance of its Ca^{2+}-binding region remained elusive. In the present study, we found that the recently described TRESK channel is also a target of receptor-mediated regulation. However, unlike the TASK and TREK channels, TRESK is not inhibited but rather activated by Ca^{2+}-mobilizing agonists. The activation of TRESK by $InsP_3$ microinjection or ionomycin application and the inhibition of the response to M_1 receptor stimulation by thapsigargin pretreatment all suggested a Ca^{2+}-dependent activation mechanism of the channel. To provide direct evidence for the regulatory role of the ion, exogenous Ca^{2+} was administered by injecting a saturated Ca^{2+} buffer. Considering the high Ca^{2+} buffering capacity of the cytoplasm and the continuous pumping of Ca^{2+}-ATPases, the applied microinjection resulted in low micromolar (later high submicromolar) free $[Ca^{2+}]$ (for details, see "Experimental Procedures"), which corresponds well to the values attained during the physiological activation of Ca^{2+}-mobilizing receptors, voltage-dependent Ca^{2+} channels, and other Ca^{2+} release and influx mechanisms. This microinjection of exogenous Ca^{2+} also strongly activated TRESK, confirming that the calcium signal itself mediates the effect.

The receptor-mediated activation of TRESK lasted several minutes after the withdrawal of the receptor agonist and outlived the decaying Ca^{2+} signal. This slow reversibility already indicated that TRESK activation by the Ca^{2+} signal might be indirect, and, indeed, application of high $[Ca^{2+}]$ (10 μM) to the intracellular side of inside-out membrane patches failed to influence the TRESK single channel activity, supporting the conclusion that the effect of Ca^{2+} does not depend on a direct interaction between the channel protein and Ca^{2+} ions.

Pretreatment of the oocytes with low concentration of cyclosporin A (CsA; 100 nM) interfered with the Ca^{2+}-induced TRESK activation without altering the basal channel activity. CsA binds to cyclophilins and consequently inhibits calcineurin (protein phosphatase 2B (40)); 50% inhibition of this Ca^{2+}/calmodulin-dependent phosphatase was reported to occur in the 10–100 nM CsA concentration range (41). The effect of CsA suggested that calcineurin was involved in TRESK activation. The involvement of calcineurin in the process was addressed also by FK506. This other drug inhibits the phosphatase through a distinct pathway, by forming an inhibitory complex with FKBP protein. Pretreatment of the oocytes with FK506 also eliminated the Ca^{2+}-dependent TRESK activation, confirming that calcineurin is required for the regulation of TRESK. As cyclosporin A and FK506 (tacrolimus) are the two most extensively used immunosuppressive agents today, the effect of these drugs on TRESK activation may have pharmacological significance in patients who undergo organ transplantation.

In order to obtain further evidence for the role of calcineurin in TRESK regulation, a constitutively active form of the phosphatase was coexpressed with the channel. This constitutively active form was constructed by deleting the C-terminal autoinhibitory domain of the catalytic calcineurin A subunit after amino acid 441. It has been reported previously that this deletion does not remove the calcineurin B and calmodulin binding domains but renders the enzyme constitutively active, independently from the cytoplasmic $[Ca^{2+}]$ (42). Coexpression of the constitutively active calcineurin activated TRESK, which was reflected by the enhanced base-line K^+ current. Simultaneously with the higher basal current, the response to the calcium signal was reduced. This indicated that the constitutively active calcineurin activated TRESK via the same mechanism as the calcium signal, and thus it diminished the subsequent action of the latter.

The efficient activation of TRESK required the coexpression of the regulatory B subunit with the truncated calcineurin A. Calcineurin B was found to influence the enzyme activity *in vivo* (43) and also to decrease the K_m and increase the V_{max} value of the wild type and the truncated calcineurin A *in vitro* (44). These activating effects may explain the requirement of calcineurin B for TRESK regulation; however, the structural implication of the B subunit in the mechanism (*e.g.* by the localization of calcineurin A) cannot be entirely excluded. Coexpression of the wild type calcineurin A and B with TRESK did not alter the basal K^+ current and the response to the calcium signal, reflecting that Ca^{2+} was also required for the activation of the expressed wild type enzyme.

We performed extensive mutagenesis experiments to analyze the mechanism of the calcineurin-mediated activation. During the pilot experiments, groups of adjacent serine residues were mutated simultaneously. Neither the basal current nor the degree of stimulation of the T256A/S262A double and S227A/S232A/S234A triple mutants was different from the wild type channel. Both the basal and the stimulated currents were significantly reduced, however, at the S274A/S276A/S279A triple mutant. When the effect of this combined mutation was dissected by individual mutations of the respective amino acid residues, the serine residue at position 276 of TRESK was identified as the potential major target for the calcineurin effect. Replacing serine 276 with alanine, thus mimicking the dephosphorylated (constitutively active) state of the channel, resulted in a large basal K^+ current, which could not be stimulated further by the Ca^{2+} signal. In turn, imitation of the phosphorylated (permanently resting) state of the channel (by replacing serine 276 with glutamate) did not alter the basal K^+ current but strongly reduced the activation evoked by the Ca^{2+} signal. The effects of the two mutations, both on the basal and the stimulated currents, are unequivocally compatible with the calcineurin-mediated activation mechanism. The S276A mutant still exhibited a small activation (about 20%) in response to the Ca^{2+} signal, and even higher responsiveness (about 2-fold) was maintained in the case of S276E. This residual activation may reflect the presence of another potential regulatory site(s) of the channel.

The 279 position turned out to be indifferent for the channel

function. (S279A basal and stimulated currents were not significantly different from the control). The responses of both S274A and S274E mutants to the Ca^{2+} signal were smaller than that of the wild type channel; however, their basal currents were nearly equal to the wild type (the mean basal current of both mutants was somewhat higher). These characteristics do not suggest that serine 274 is a potential target of the calcineurin-mediated dephosphorylation. In addition, the lower basal current of the S274A/S276A/S279A triple mutant (if compared with the normal or elevated values of the individual mutants) cannot be explained by the direct calcineurin-mediated effect on serine 274. Perhaps the reduced expression or the substantial distortion of the channel structure by two additional mutations close to a regulation-sensitive location (serine 276) caused the low basal activity.

As an alternative for the possible phosphorylation of serine 274, the close proximity of this residue to serine 276 offers a more plausible explanation for the reduced responsiveness. Serine 274 may be an essential part of the molecular context for the dephosphorylation of serine 276, and its mutation may destroy this molecular context, thereby preventing the dephosphorylation of serine 276. It was reported that calcineurin did not have a well defined consensus amino acid sequence for substrate recognition, and its specificity depended rather on the more complex structure of the target protein (45). It has been shown that a basic residue about 3 amino acids upstream from the phosphorylated residue plays an important role in the dephosphorylation (45). This requirement is met by serines 264, 274, and 276 (and also by several other serine residues) of TRESK. Changing a residue (serine 274) between arginine 272 and the phosphorylated serine 276 may have had deleterious effects on the structure recognized by calcineurin.

The mutation of serine 264 to alanine or glutamate suggests that this amino acid is a likely candidate for an auxiliary calcineurin-mediated dephosphorylation. Both S264A and S264E mutants were less responsive to the Ca^{2+} signal than the wild type channel. In addition, the order of the mean basal currents was S264E $<$ wild type $<$ S264A, which also supports the assumption that the conversion of the negative charge to neutral at this position (*i.e.* dephosphorylation) confers higher activity to the channel. (Although these differences were not significant, estimation of the basal currents may have been less sensitive to minor alterations, since in this case the currents were normalized to an external reference, the average value of the control (wild type) oocytes.) These results suggest that serine 264 may also be a target residue of the dephosphorylation mediated by calcineurin; however, the impact of this dephosphorylation on the channel activity is more limited than that of the main regulator, serine 276.

In summary, we have described a novel mechanism, by which changes in the cytoplasmic Ca^{2+} concentration can regulate the membrane potential. The novel, calcineurin-mediated regulatory mechanism of TRESK channels is able to connect cytoplasmic [Ca^{2+}] changes to the membrane potential negatively. When cellular activation results in the rise of cytoplasmic [Ca^{2+}], the activation of TRESK hyperpolarizes the cell and stabilizes the resting membrane potential at negative values. Also, in a depolarized excitable cell, the outwardly rectifying TRESK may provide a substantial repolarizing current. Activated TRESK, therefore, may counteract the cellular activation via a negative feedback mechanism (especially if the calcium signal originated in a depolarization-dependent manner), or it may decrease the responsiveness of the cell to further stimuli.

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