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Ruthenium Red Inhibits TASK-3 Potassium Channel by Interconnecting Glutamate 70 of the Two Subunits

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ABSTRACT

TASK channels are highly pH-sensitive two-pore-domain background potassium channels expressed in the central nervous system and in some peripheral tissues. Their current can be regulated by receptor-mediated activation of phospholipase C and also by pharmacological means. We have reported previously that the cationic dye, ruthenium red (RR), inhibited homodimeric TASK-3 (kcnk9), whereas TASK-1 (kcnk3) homodimer and TASK-1/TASK-3 heterodimer were not affected by this compound. In the present study, we identify the molecular determinant of the RR-mediated TASK-3 inhibition. Mutation of the negatively charged Glu 70 of TASK-3 to Arg (E70R) or Cys (E70C) abolished the inhibitory action of RR. When two TASK-3 coding sequences were concatenated, and the entire homodimer was expressed as a single polypeptide chain, the resulting tandem channel was also sensitive to RR. Mutation of Glu 70 in either the first (E70R) or the second (E465R) linked subunit prevented the action of the inhibitor. Together with the Hill coefficient of 1.0 for TASK-3 inhibition, these data indicate that simultaneous binding of one polycationic RR molecule to Glu 70 of both subunits is required for the inhibitory action. The pivotal role of this residue in the inhibitory mechanism of RR is confirmed by the gained RR sensitivity of the mutant TASK-1 in which Lys 70 was changed to Glu. Our results indicate that RR inhibits TASK-3 by tethering its two subunits and identify amino acid 70 as a possible target for designing selective inhibitors against the different TASK channels.

The two-pore-domain potassium ($2PK^+$) channels give rise to background (leak) K^+ conductance in resting cells and stabilize the membrane potential at hyperpolarized values, near the potassium equilibrium potential. In addition, they regulate the membrane potential in electrically active cells, in that they remain open during depolarization (in contrast to the inward rectifiers) and conduct robust repolarizing currents. The activity of $2PK^+$ channels is not influenced markedly by the membrane potential, and the majority of $2PK^+$ currents do not exhibit activation and inactivation kinetics. However, the channels can be regulated by various physicochemical alterations (such as membrane tension, extra- or intracellular pH, or temperature changes) and by different signaling pathways [including protein kinases A and C, arachidonic acid, and phospholipase C activation (for review, see Lesage and Lazdunski, 2000)]. These pathways are likely to play a major role in control of $2PK^+$ channel activity and consequently in the excitability of cells.

In concert with the wide spectrum of regulation, $2PK^+$ channels are the most diverse family of potassium channels. Apart from their similar molecular architecture, characterized by intracellular N- and C-terminals, four transmembrane segments (TMS), and two K^+ channel pore-forming domains (one P domain between the first and second TMS and the other P domain between the third and fourth TMS), some members of the family are remarkably different and show only limited homology, which is unusual in other K^+ channel families. This is why the 15 mammalian $2PK^+$ channel genes, cloned until now, have been divided into subfamilies, in which the amino acid sequence of the channels and also their regulatory properties are more similar. At present, five subfamilies of $2PK^+$ channels are known: TWIK (tandem pore domain in a weakly inwardly rectifying K^+ channel), TREK (TWIK related K^+ channel), THIK (tandem pore domain halothane-inhibited K^+ channel), TASK (TWIK-related acid-sensitive K^+ channel), and TALK (TWIK related alkaline pH activated K^+ channel) (for review, see Patel and Honore, 2001).

The TASK subfamily of $2PK^+$ channels comprises TASK-1 (Duprat et al., 1997), TASK-3 (Kim et al., 2000), and TASK-5 (Kim and Gnatenco, 2001). [TASK-2 (Reyes et al., 1998) and TASK-4, also termed TALK-2 (Decher et al., 2001; Girard et al., 2001) belong to the TALK subfamily despite sharing the name with the other TASK channels]. TASK-1 and TASK-3 have been studied in different expression systems, and TASK-1 subunits have been shown to function as homodimers (Lopes et al., 2001). Recently, formation of functional heterodimeric channels by TASK-1 and TASK-3 subunits in *Xenopus laevis* oocytes has also been reported (Czirják and Enyedi, 2002a). In contrast with TASK-1 and

ABBREVIATIONS: $2PK^+$, two-pore-domain K^+; TMS, transmembrane segment; RR, ruthenium red; PCR, polymerase chain reaction; MES, 2-(*N*-morpholino)ethanesulfonic acid.

TASK-3, expression of TASK-5 failed to produce current in heterologous expression systems. In the absence of specific inhibitors and because of the wide and partially overlapping distribution of expression, the relative contribution of the different TASK channels to the background K^+ conductance of different cell types is difficult to determine.

Inhibition of channel activity by extracellular acidification was often used to differentiate TASK channels from members of the other $2PK^+$ subfamilies, and also between TASK-1 and TASK-3 (Hartness et al., 2001; Czirják and Enyedi, 2002b; Washburn et al., 2002). TASK-1 is extremely sensitive to pH in the 6.5 to 7.5 range (Duprat et al., 1997), whereas the inhibitory curve of TASK-3 is shifted to more acidic values by about 0.8 pH (Rajan et al., 2000). Discrimination between TASK-1 and TASK-3 currents was also attempted by applying Zn^{2+} and local anesthetics (Buckler et al., 2000; Hartness et al., 2001), based on the observation that the heterologously expressed TASK homodimers were inhibited with slightly different half-maximal inhibitory concentrations. However, in native tissues, these interventions may also have affected the possibly present TASK-1/TASK-3 heterodimers having different inhibitory curve from the homodimers [e.g., the pH dependence of the heterodimer is intermediate between the homodimers (Czirják and Enyedi, 2002a)]. Therefore, the proportion of TASK-1 and TASK-3 homodimers cannot be unequivocally deduced from the intermediate inhibition by pH, Zn^{2+}, or local anesthetics.

Recently, we applied the cationic dye ruthenium red (RR; $[(NH_3)_5Ru\text{-}O\text{-}Ru\text{-}(NH_3)_4\text{-}O\text{-}Ru(NH_3)_5]Cl_6$) to discriminate between TASK channels in *X. laevis* oocytes (Czirják and Enyedi, 2002a) and glomerulosa cells of rat adrenal cortex (Szabadkai et al., 1999; Czirják and Enyedi, 2002b). Although RR is not specific for $2PK^+$ channels, it proved to be remarkably selective within the TASK subfamily. TASK-3 homodimer is inhibited by RR in the range of low micromolar concentrations, whereas the compound does not affect TASK-1 homodimer and the TASK-1/TASK-3 heterodimer (Czirják and Enyedi, 2002a). In the present study, we elucidate the major molecular determinant of RR inhibition of TASK channels and present an explanation for this peculiar asymmetric inhibitory profile.

Materials and Methods

Materials. Enzymes and kits for molecular biological studies were purchased from Ambion (Austin TX), Amersham Biosciences (Little Chalfont, United Kingdom), Fermentas (Vilnius, Lithuania), New England Biolabs (Beverly, MA), Pharmacia (Uppsala, Sweden), Promega (Madison, WI), and Stratagene (La Jolla, CA). Fine chemicals of analytical grade were obtained from Fluka (Buchs, Switzerland), Promega, and Sigma Chemical Co. (St. Louis, MO).

In Vitro Site-Directed Mutagenesis and Construction of Concatenated Channels. The human pEXO-TASK-1 (Duprat et al., 1997) and the rat pEXO-TASK-3 (Czirják and Enyedi, 2002b) plasmids were used in the molecular biological experiments. In vitro site-directed mutagenesis was performed according to the manufacturers 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 forward primers were: 5′-TCCCAGTGCGAACGTTGGAGTTTCTTCCAC-3′ for the TASK-3 D183R mutation; 5′-GTAATCCTCCAGTCTCGGCCCCACCGC-3′ for the TASK-3 E70R mutation, and 5′-CTGCGCCTCGAACCGCACAAGGCAGGCGTGC-3′ for the TASK-1 K70E mutation. The resulting clones were screened for the newly introduced or eliminated restriction enzyme site and sequenced.

The TASK-3/TASK-3 tandem construct coded for a polypeptide chain in which the last amino acid residue (Ile) of the first TASK-3 repeat was followed by the second residue (Lys) of the second TASK-3 repeat. The TASK-3/TASK-3 tandem construct and its mutant versions were created by concatenating appropriately two TASK-3 subunits (wild-type or E70R mutant). The method of concatenation was the same in all cases. The 3′ end of TASK-3 was joined to the 5′ beginning of TASK-3 by the overlapping PCR method. The fragment corresponding to the 3′ the end of TASK-3 was amplified with a TASK-3–specific sense primer (T3s) corresponding to bases 535 to 564, and T33a primer (5′-GTTCTGCCGCTTGATGGACTTGCGACGGATG-3′). The fragment corresponding to the 5′ beginning of TASK-3 was amplified with T33s primer (5′-GCAAGTCCATCAAGCGGCAGAACGTGCGTAC-3′) and a TASK-3–specific antisense primer (T3a) corresponding to bases 113 to 219. The two PCR products were purified, mixed, denatured, allowed to hybridize by their overlapping regions, elongated without oligonucleotides, and finally PCR-amplified with primers T3s and T3a. Using unique restriction enzyme sites, the resulting PCR product (equivalent with the fused coding 3′ and 5′ ends of TASK-3 in frame) was completed with the 5′ part of the first and with the 3′ part of the second TASK-3 coding region, respectively, and the tandem channel was ligated finally into pEXO vector. The mutant TASK-3/TASK-3 tandem constructs were created similarly, except for using the E70R TASK-3 fragment in one of the ligated subunits.

Synthesis of Ion Channel cRNA. The cRNAs coding for TASK-1, TASK-3, and the mutated and/or concatenated $2PK^+$ constructs were synthesized in vitro using the Ambion mMESSAGE mMACHINE T7 in vitro transcription kit. All DNA templates were linearized at the *Xba*I site of pEXO. For increasing the RNA stability in the oocytes, the linear template contained also the 5′ and 3′ untranslated regions of the *X. laevis* globin gene flanking the insert.

Animals and Tissue Preparation and *X. laevis* Oocyte Injection. Mature female *X. laevis* frogs were obtained from Amrep Reptielen (Breda, Netherlands). Frogs were anesthetized by immersing them in benzocaine solution (0.03%). Ovarian lobes were removed, the tissue was dissected and treated with collagenase (1.45 mg/ml, 148 U/mg, type I; Worthington Biochemical Corp. (Freehold, NJ) and continuous mechanical agitation in Ca^{2+}-free OR2 solution containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5) for 1.5 to 2 h. Stage V and VI oocytes were defolliculated manually, and kept at 18°C in modified Barth's saline containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 20 mM HEPES buffered to pH 7.5 with NaOH and supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (4.5 mM), and theophyllin (0.5 mM). Oocytes were injected 1 day after defolliculation. Fifty nanoliters of the appropriate cRNA solution was delivered with Nanoliter Injector (World Precision Instruments, Saratosa, Florida). Electrophysiological experiments were performed 3 or 4 days after the injection.

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

Electrophysiological Recordings. Membrane currents were recorded by two-electrode voltage clamp (OC-725-C; Warner Instrument Corporation, Hamden, CT) using microelectrodes made of borosilicate glass (Clark Electromedical Instruments, Pangbourne, UK) with resistance of 0.3 to 1 MΩ when filled with 3 M KCl. Currents were filtered at 1 kHz, digitally sampled at 1 to 2.5 kHz with a Digidata Interface (Axon Instruments, Union City, CA), and stored on a PC-compatible computer. Recording and data analysis were performed using pClamp software 6.0.4 (Axon Instruments). Experiments were carried out at room temperature, and solutions were applied by a gravity-driven perfusion system. Low $[K^+]$ solution

contained 95.4 mM NaCl, 2 mM KCl, 1.8 $CaCl_2$, and 5 mM HEPES. High $[K^+]$ solution contained 80 mM K^+ (78 mM Na^+ of the low $[K^+]$ solution was replaced with K^+). Unless otherwise stated, the pH of every solution was adjusted to 7.5 with NaOH. Perfusing solutions with pH <6.5 were buffered by including 5 mM MES in addition. RR stock solution (20 mM) was prepared in distilled water, and the inhibitor was diluted further in the perifusing solutions before the measurement. Background K^+ currents were measured in high-extracellular $[K^+]$ at the end of 300-ms voltage steps to −100 mV applied in every 3 s. The holding potential was 0 mV. For estimating the amplitude of the background current, the inward current in high $[K^+]$ was corrected for the small nonspecific leak measured in 2 mM EC $[K^+]$ at −100 mV.

Statistics and Calculations. Data are expressed as means ± S.E.M. Normalized dose-response curves were fitted by least-squares method (Sigmaplot; SPSS Science, Chicago, IL) to a modified Hill equation of the form: $y = \alpha/(1 + (c/K_{1/2})^{nH}) + (1 - \alpha)$, where c is the concentration, $K_{1/2}$ is the concentration at which half-maximal inhibition occurs, ^{nH} is the Hill coefficient, and α is the fraction inhibited by the treatment. Significance between the pH sensitivity of different TASK-3 constructs was calculated by two-way repeated measures analysis of variance and Scheffé's F test.

Results

Mutation of Glutamate 70 to Arginine in TASK-3 Abolishes the Ruthenium Red Sensitivity. TASK-3 K^+ channel was expressed in *X. laevis* oocytes, and its currents were measured at −100 mV by two-electrode voltage clamp as the difference of inward currents in high (80 mM) and low (2 mM) $[K^+]$, as described previously (Czirják et al., 2001). The high level of expression resulted in background potassium currents in the microampere range at −100 mV (9.0 ± 1.8 μA, n = 13), exceeding the endogenous oocyte currents by at least 1 order of magnitude. This allowed reliable estimation of the effects of pharmacological manipulations on TASK-3 currents (Czirják and Enyedi, 2002a,b). Application of RR into the perifusing medium of the oocyte inhibited TASK-3 current in a concentration-dependent manner (Fig. 1.). The half-maximal effect was achieved by 0.7 μM RR. The Hill coefficient of the inhibition was found to be 1.0 (Fig. 2B, ●), indicating the binding of one RR molecule to one functional TASK-3 homodimer. The onset of the inhibition was rapid, suggesting an action on the extracellular side, and the inhibition was reversible by extensive washout (not shown). We reported previously that the inhibition of TASK-3 by the positively charged RR was not voltage-dependent (Czirják and Enyedi, 2002a), indicating that the RR binding site on the channel is not in the transmembrane electrical field.

Considering the positive charge of RR, the rapid onset, and voltage independence of RR inhibition, we looked for negatively charged amino acids in the extracellular loops of TASK-3 as potential RR targets. In a selection of the potential target residues, we also compared the sequences of the extracellular loops of TASK-3 to RR-resistant TASK-1 (Czirják and Enyedi, 2002a). Mutation of aspartate 183 to arginine (D183R) in the second extracellular loop gave functional channel and did not reduce the RR sensitivity (not shown). In contrast, mutation of glutamate 70 to arginine in the first extracellular loop (E70R) resulted in functional K^+ channels (3.5 ± 0.5 μA, n = 10) that were resistant to RR (Fig. 2B.)

Binding of RR to Glu 70 in Both Subunits of Homodimeric TASK-3 Is Required for Inhibition. Given the fact that $2PK^+$ channels work as dimers, next we addressed the question of whether Glu 70 at both subunits was necessary for the high-affinity RR binding. If RR required both glutamates at position 70 for interacting with the channel, converting only one of them to a positively charged amino acid should be sufficient to prevent the binding of the inhibitor and consequently render the mutant channel RR insensitive.

To test this hypothesis we expressed two TASK-3 subunits as one continuous polypeptide (Fig. 3A). This arrangement was expected to highly favor the assembly of the functional channel from the two linked subunits. Expression of the TASK-3/TASK-3 tandem construct yielded K^+ currents in *Xenopus* oocytes (7.1 ± 1.5 μA, n = 10). The concatenation did not have a major impact on the functional properties of the channel; the expressed K^+ current was RR sensitive (half-maximal effect at 0.35 μM, Hill coefficient = 1.4, Fig. 3B) and pH-dependent (the curve was shifted only slightly

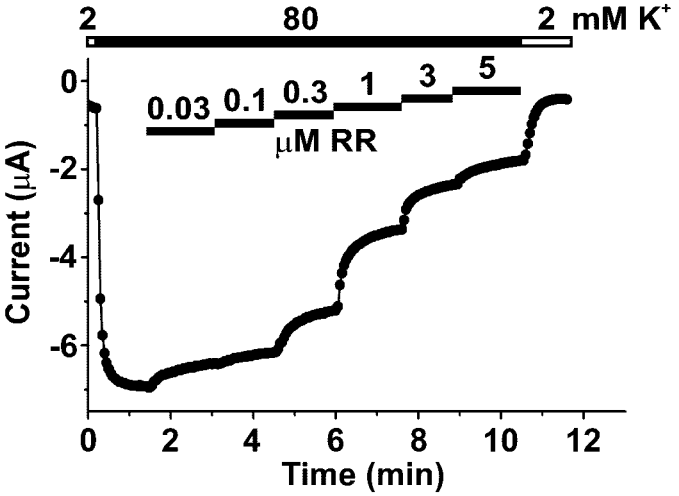

Fig. 1. Concentration-dependent inhibition of TASK-3 homodimer by ruthenium red. Currents of a representative *Xenopus* oocyte expressing TASK-3 in extracellular solutions of different K^+ and ruthenium red concentrations (as indicated above the curve). The plotted inward currents were measured at the end of 300-ms voltage steps to −100 mV applied every 3 s from a holding potential of 0 mV.

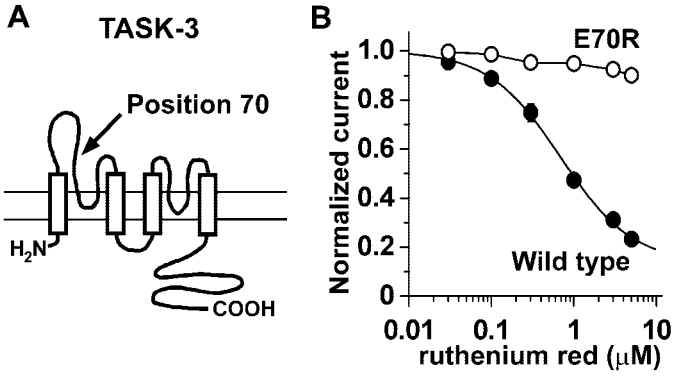

Fig. 2. Mutation of glutamate 70 to arginine (E70R) in TASK-3 eliminates the ruthenium red sensitivity. A, position of amino acid residue 70 in the transmembrane topology of TASK-3. B, the effect of ruthenium red on wild-type and E70R mutant TASK-3 homodimeric K^+ channels. Inhibition by RR was measured as described in Fig. 1. The currents were corrected for the small nonspecific leak in 2 mM extracellular $[K^+]$ at −100 mV and normalized to the value without RR. Each data point represents the average of normalized currents of five to eight oocytes. Error bars are smaller than symbols in most cases. Wild-type TASK-3 points were fitted by a modified Hill equation (see *Materials and Methods*). E70R points were connected with straight lines.

toward more alkaline values (the difference was statistically significant $p < 0.001$, Fig. 4).

Because the TASK-3/TASK-3 tandem construct formed the whole background K^+ channel as a single protein, we could change single amino acid residues of the RR binding site by creating mutants of this TASK-3/TASK-3 tandem. Glutamate 70 and 465 of the TASK-3/TASK-3 tandem channel corresponded to Glu 70 of the two subunits in the TASK-3 homodimer. For mutating glutamate 70 or 465, an E70R mutant TASK-3 subunit was linked before or after a wild-type TASK-3 subunit, respectively. Expression of the E70R and E465R mutant TASK-3/TASK-3 tandem constructs resulted in K^+ currents in *X. laevis* oocytes [2.1 ± 0.3 μA (n = 11) and 2.1 ± 0.4 μA (n = 11), respectively]. These E70R and E465R mutants were also regulated by pH in the appropriate range [similar to the wild-type TASK-3, the TASK-3/TASK-3 tandem construct, and the E70R mutant TASK-3 (Fig. 4)]. However, RR inhibited neither the E70R nor the E465R mutant TASK-3/TASK-3 tandem (Fig. 3B). (The RR insensitivity was not related to the somewhat lower expression of the mutant tandem constructs, RR (1 μM) was not effective in oocytes expressing higher current amplitudes [9.3 ± 5.3 μA, with 6.2 ± 6.1% inhibition for E70R (n = 3) and 7.3 ± 2.6 μA, with 3.3 ± 0.9% inhibition for E465R (n = 3) mutant TASK-3 tandems].

The high level of expression and the unaltered regulation by pH verified that the structure of the TASK-3/TASK-3 tandem channel was maintained in the E70R and E465R mutants and only the RR sensitivity was lost, suggesting that high-affinity binding of the inhibitor to the channel requires the presence of glutamate 70 in both subunits.

The E70C Mutant of TASK-3 Is Also RR-Insensitive. To test whether the absence of the negatively charged residue in position 70 or the introduction of the positively charged arginine is responsible for the loss of RR-sensitivity, Glu 70 was mutated to cysteine. The E70C mutant also became RR-insensitive (similarly to the E70R mutant), indicating that the presence of negative charges is required for the inhibition (Fig. 5.). To cross-link the two E70C TASK-3 subunits we perfused the oocytes with the sulfhydryl reagent phenylarsine oxide (100 μM for 3 min); however, this treatment failed to affect the channel activity.

K70E Is a Gain-of-RR-Sensitivity Mutation in TASK-1. As discussed above, TASK-1, the close relative of TASK-3, is resistant to RR inhibition (Fig. 6). Because TASK-1 has positively charged Lys 70, we replaced this basic residue with the acidic glutamate. The K70E mutant was functional and the amplitude of its expressed current (3.7 ± 0.5 μA, n = 7) was similar to that of wild-type TASK-1 (3.7 ± 0.3 μA, n = 4). However, as opposed to the wild-type channel, RR inhibited the K70E mutant of TASK-1 (Fig. 6.), indicating that Glu 70 is sufficient to create a RR binding site even in the molecular context of TASK-1. Presumably, the amino acid environment surrounding position 70 (being different in TASK-1 and TASK-3) contributes to the formation of the RR binding site, or the distance of amino acids in position 70 are different in TASK-1 and TASK-3, because higher concentrations of RR (half-maximal effect at 10.6 μM, Hill coefficient = 0.6) were required to reduce the currents of the K70E mutant of TASK-1 compared with TASK-3. For excluding the possibility that RR inhibits TASK channels by two distinct inhibitory mechanisms in different concentration ranges, a high concentration of RR (required for TASK-1 K70E inhibition) was also tested on TASK-3 E70R. A high concentration of RR (80 μM) failed to inhibit the E70R mutant of TASK-3 (−0.9 ± 1.4% inhibition, n = 4). Thus, RR sensitivity of the K70E TASK-1 mutant together with the abolished RR sensitivity of TASK-3 mutants lacking glutamates in position 70 in one or

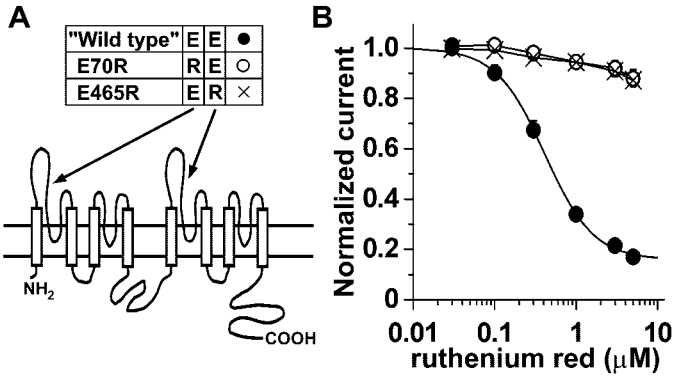

Fig. 3. Mutation of glutamate 70 to arginine either in the first or in the second linked subunit renders the TASK-3/TASK-3 tandem construct ruthenium red resistant. A, transmembrane topology of the TASK-3/TASK-3 tandem construct. Amino acid residues 70 and 465 (the latter analogous to location 70 in the second TASK-3 repeat) are indicated with arrows. B, concentration-dependent inhibition of the "wild-type" (●), E70R (○), and E465R mutant (×) TASK-3/TASK-3 constructs by RR. The method of measurement and representation was the same as in Fig. 2B. "Wild-type" data points were fitted by a modified Hill equation; data points of the mutants were connected with straight lines.

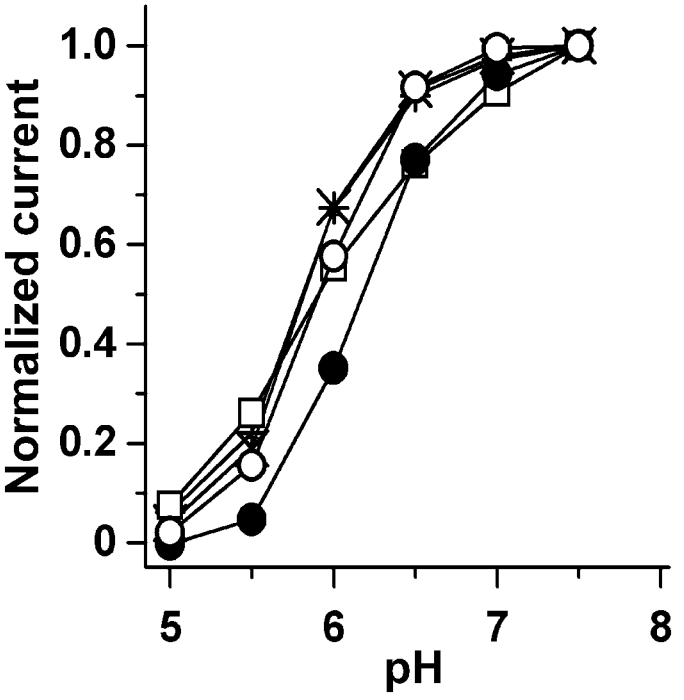

Fig. 4. pH-sensitivity of the TASK-3 constructs. Currents of oocytes expressing TASK-3 (○), E70R mutant TASK-3 (□), the TASK-3/TASK-3 tandem construct (●), the E70R mutant TASK-3/TASK-3 tandem (+), and the E465R mutant TASK-3/TASK-3 tandem (×) were measured at different pH values in high (80 mM) extracellular $[K^+]$ at −100 mV. The currents were corrected for the small nonspecific leak in 2 mM extracellular $[K^+]$ at −100 mV and normalized to the maximum at pH 7.5. Each point represents the average of normalized currents of five to six oocytes. In most cases, error bars are smaller than symbols. Data points are connected with straight lines.

both subunits indicate the crucial role of this amino acid residue in RR binding and inhibition.

Discussion

Ruthenium red, the polycationic trinuclear ruthenium ammine, was originally used as a dye for histological staining. It also became well known to interfere with transmembrane Ca^{2+} or Ca^{2+}-related ion transports at intracellular organelles and the plasma membrane. It has been shown that application of RR to subcellular fractions or in the cytosol (the large, positively charged molecule cannot penetrate the membrane easily) inhibits the mitochondrial Ca^{2+}-uniporter (Moore, 1971), the Ca^{2+}-release channel (ryanodine receptor) (Volpe et al., 1986), and the Ca^{2+}-ATPase of the sarcoplasmic reticulum (Vale and Carvalho, 1973). RR also inhibits the plasma membrane Ca^{2+}-pump (Missiaen et al., 1990), voltage-dependent Ca^{2+} channels (Cibulsky and Sather, 1999), the highly Ca^{2+}-permeable nonspecific cation channel vanilloid (capsaicin) receptors (Garcia-Martinez et al., 2000), and members of the recently described epithelial Ca^{2+}-selective channel family, ECaCh (Hoenderop et al., 2001). RR binds to and may also affect the function of several Ca^{2+}-binding proteins, including calsequestrin, calreticulin, troponin C, and calmodulin (Charuk et al., 1990). With respect to potassium channels, RR was found to inhibit the high conductance Ca^{2+}-activated K^+ channel (maxi, BK) from the intracellular side, and it was assumed that it interacts with the Ca^{2+}-binding region of the channel (Wann and Richards, 1994; Wu et al., 1999).

We have reported previously a calcium-independent effect of RR. Extracellular application of the dye inhibited the background K^+ conductance of rat adrenal glomerulosa cells, in addition to the reduction of L-type voltage-dependent Ca^{2+} current (Szabadkai et al., 1999). Because the glomerulosa background K^+ conductance has been assigned mainly to the TASK subfamily of the two-pore-domain K^+ channels (Czirják et al., 2000), it was feasible to examine the effect of RR on different 2P channels. TASK-3 and TRAAK [TWIK-related arachidonic acid-stimulated K^+ channel (Fink et al., 1998), TREK subfamily] were found to be sensitive to RR, whereas their closely related subfamily members, TASK-1 and TREK-1, respectively, were resistant (Czirják and Enyedi, 2002a). By taking advantage of the RR sensitivity of TASK-3 versus TASK-1, and using also competitive PCR, TASK-3 was found to dominate the background K^+ conductance of rat adrenal glomerulosa cells (Czirják and Enyedi, 2002b). RR was also used to demonstrate TASK-1/TASK-3 heterodimer formation in *Xenopus laevis* oocytes (Czirják and Enyedi, 2002a).

In addition to adrenal glomerulosa cells, TASK-1 and TASK-3 are expressed at many other locations and show partially overlapping distribution. TASK-1 and TASK-3 expression (and often also the corresponding pH-sensitive background K^+ currents) were detected in the central nervous system in cerebellar granule neurons (Millar et al., 2000;

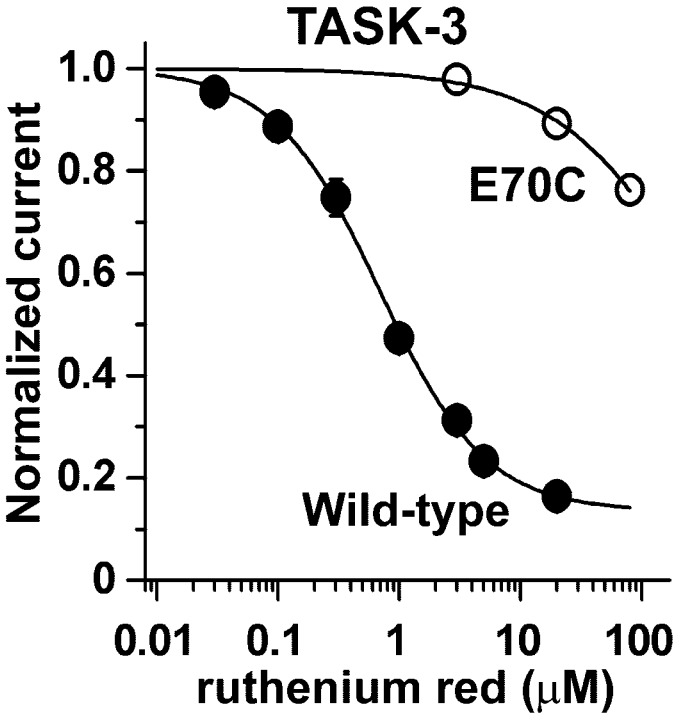

Fig. 5. Mutation of glutamate 70 to cysteine renders TASK-3 RR-resistant. Effect of ruthenium red on wild-type and E70C mutant TASK-3 homodimeric K^+ channels is shown. The protocol and calculations were identical to those of Fig. 2B. However, higher concentrations of ruthenium red (3, 20, and 80 μM) were used for the E70C mutant. For comparison, the data points of wild-type TASK-3 are repeated in this figure.

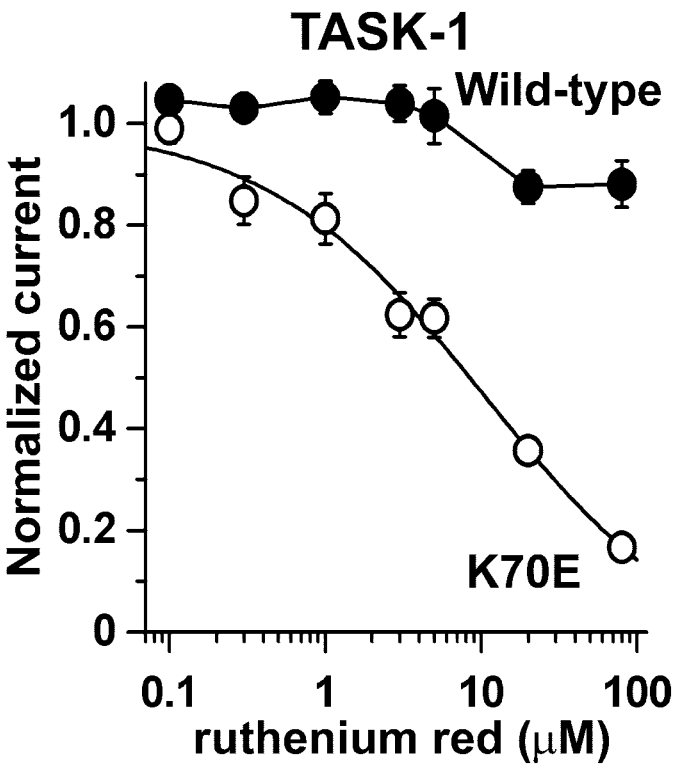

Fig. 6. Mutation of lysine 70 to glutamate renders TASK-1 RR-sensitive. Concentration-dependent inhibition of wild-type and K70E mutant TASK-1 homodimeric K^+ channels by RR. The method of measurement and representation was the same as in Fig. 2B; however, higher concentrations of RR were applied (0.1, 0.3, 1, 3, 5, 20, and 80 μM). Wild-type TASK-1 points were connected with straight lines; data points of the K70E mutant were fitted by a modified Hill equation.

Medhurst et al., 2001), somatic motoneurons of the brainstem and spinal cord (Talley et al., 2000), neurons of the locus ceruleus (Bayliss et al., 2001), serotoninergic raphe neurons (Washburn et al., 2002), formatio reticularis, and nuclei of the hypothalamus (Talley et al., 2001). In peripheral tissues, TASK expression and TASK-like currents were demonstrated in type I chemosensitive cells of the rat carotid body (Buckler et al., 2000) and in H-146 cells, an established model of the lung oxygen sensor neuroepithelial bodies (O'Kelly et al., 1999).

TASK currents of native cells were discriminated by their moderately different pH, Zn^{2+}, and local anesthetic sensitivity (Bayliss et al., 2001; Hartness et al., 2001; Czirják and Enyedi, 2002b; Washburn et al., 2002). RR seems to be a more powerful tool to facilitate the separation of TASK currents in native cells. In position 70, TASK-1 and TASK-3 subunits possess a positive lysine and a negative glutamate, respectively. Binding of and inhibition by RR requires glutamates to be present in both subunits at this location. Replacing glutamate 70 by a positive amino acid in even one of the subunits extinguishes RR inhibition. This, together with a Hill coefficient of 1.0 for the inhibition, suggest that RR interconnects two glutamates 70 (for schematic model, see Fig. 7.) All of our present and previous experimental data are consistent with this model. As we have reported recently (Czirják and Enyedi, 2002a), TASK-3 homodimeric K^+ channel (having glutamates 70 in both subunits) is inhibited by RR. On the contrary, the TASK-1 homodimer (lysines 70) and the TASK-1/TASK-3 heterodimer [lysine 70 (K70) and glutamate 70 (E70), respectively] are not affected. In the present study, we demonstrate that the TASK-3/TASK-3 tandem construct (E70, E465) is inhibited by RR, whereas the homodimer of E70R or E70C mutant TASK-3 subunits [having positive arginines 70 (R70) or neutral cysteines (C70), respectively] and the E70R and E465R mutants of the TASK-3/TASK-3 tandem channel (both of them containing one glutamate and one arginine in the key positions) are RR resistant. Furthermore, even the TASK-1 homodimer became RR sensitive when its lysines 70 were replaced by glutamates. The half-maximal inhibitory concentration of RR on the mutated TASK-1 is higher if compared with the wild-type TASK-3, indicating that the environment around the introduced glutamate may also influence the binding. Still, the most plausible interpretation of these observations is that glutamate 70 residues are cornerstones of the RR binding site formed by the channel subunits, and RR binds to both of these glutamates simultaneously. Binding of RR to the outer surface of the channel is consistent with the voltage-independence of the inhibition (Czirják and Enyedi, 2002a). RR does not enter the transmembrane electrical field, because in this case, the positively charged drug would be concentrated around its binding site and induce stronger inhibition at negative membrane potentials. Instead, RR may bind to both glutamates 70 and may repel K^+ from the outer aperture of the channel by its positive charge, cause steric hindrance or may fix glutamates 70 at a given distance and stabilize the closed conformation.

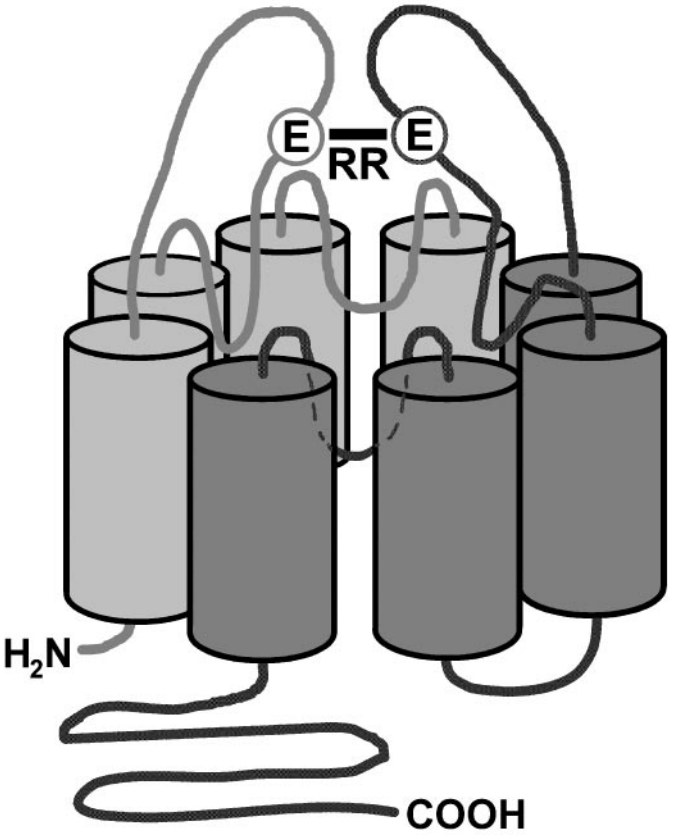

Fig. 7. Model of TASK inhibition by ruthenium red. RR interconnects glutamates 70 (E) located in the first extracellular loop of the channel subunits. The two subunits are drawn in different shades of gray.

RR is far too nonspecific to be used as a TASK-3 inhibitor in vivo. Intraperitoneal administration of RR to mice, rat, or cat caused flaccid paralysis, an effect ascribed to the inhibition of the Ca^{2+}-dependent release of acetylcholine in neuromuscular junctions (Tapia and Velasco, 1997). On the other hand, injection of the dye into the cerebrospinal fluid (mice, rat, and cat) induced intense generalized convulsions accompanied by continuous electroencephalographic discharges characteristic of status epilepticus. Stereotaxic microinjection of RR into the substantia nigra pars reticulata produced long-lasting intense circling movements and head orientation, whereas intrahippocampal microinjection resulted in a well-defined motor abnormality known as "wet-dog shakes". Initially, these abnormal motor activities were explained by inhibition of the release of inhibitory neurotransmitters and by the neurotoxic effect of RR (Tapia and Velasco, 1997). Later, susceptibility of different neuron populations to RR was related to the inhibition of K^+ currents. Supporting this assumption, the membrane potential of *X. laevis* oocytes injected with mRNA extracted from cultured cortical and cerebellar granule neurons was depolarized by RR (Velasco et al., 1999), but the molecular target of the drug remained unknown. We propose that the inhibitory effect of RR on the neuronal background K^+ channels, TASK-3 and TRAAK, contributes to the neuronal excitation and the consequent motor activity induced by intracerebral or intracerebroventricular injection of RR.

Development of specific two-pore-domain K^+ channel inhibitors would highly facilitate the evaluation of the functional significance of these background K^+ channels in native tissues and in vivo. In turn, specific two-pore-domain channel agonists might also be clinically useful. Accumulating evidence indicates that activation of $2PK^+$ channels (mainly members of the TASK and TREK subfamilies) by volatile

anesthetics contributes to the general central depressive effect of these compounds (Patel et al., 1999; Sirois et al., 2000; Talley and Bayliss, 2002). Regarding the distinct expression pattern of different $2PK^+$ channels in the central nervous system, selective $2PK^+$ channel agonists might have more targeted clinical effects (e.g., hypnotic, analgesic, immobilizing, or neuroprotective) than those of volatile anesthetics. Within the closely related members of the TASK $2PK^+$ channel family, a single amino acid in position 70 profoundly modified the susceptibility of the channel to RR, suggesting that this site may be a possible target for designing selective TASK antagonists of higher overall specificity.

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