External Ba\(^{2+}\) Block of the Two-pore Domain Potassium Channel TREK-1 Defines Conformational Transition in Its Selectivity Filter

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TREK-1 is a member of the two-pore domain potassium channel family that is known as a leak channel and plays a key role in many physiological and pathological processes. The conformational transition of the selectivity filter is considered as an effective strategy for potassium channels to control the course of potassium efflux. It is well known that TREK-1 is regulated by a large volume of extracellular and intracellular signals. However, until now, little was known about the selectivity filter gating mechanism of the channel. In this research, it was found that Ba\(^{2+}\) blocked the TREK-1 channel in a concentration- and time-dependent manner. A mutagenesis analysis showed that overlapped binding of Ba\(^{2+}\) at the assumed K\(^{+}\) binding site 4 (S4) within the selectivity filter was responsible for the inhibitory effects on TREK-1. Then, Ba\(^{2+}\) was used as a probe to explore the conformational transition in the selectivity filter of the channel. It was confirmed that collapsed conformations were induced by extracellular K\(^{+}\)-free and acidification at the selectivity filters, leading to nonconductive to permeable ions. Further detailed characterization demonstrated that the two conformations presented different properties. Additionally, the N-terminal truncated isoform (ΔN41), a product derived from alternative translation initiation, was identified as a constitutively nonconductive variant. Together, these results illustrate the important role of selectivity filter gating in the regulation of TREK-1 by the extracellular K\(^{+}\) and proton.

Potassium channels are ubiquitous pore-forming transmembrane proteins that transport K\(^{+}\) ions selectively and rapidly across the biological membranes. The efflux of K\(^{+}\) ions is controlled not only by the electrochemical gradient, but also by the gating mechanism. Along the ion conduction pathway of potassium channels, three structures are arranged from intercellular to extracellular: the lower activation gate, the selectivity filter (SF), and the upper inactivation gate (also termed the C-type inactivation gate). Correspondingly, there are mainly two kinds of mechanisms controlling K\(^{+}\) ion passage. Manipulation of the lower activation gate controls the transition between the open and close state of the channel. The upper inactivation gate, which is characterized by slow kinetics, controls the transition between conduction and nonconduction of the pore. The selectivity filter of the K\(^{+}\) channels, formed by the highly conserved sequence TV(I)GY(F)G, plays a pivotal role in both mechanisms. Accumulating evidence shows that the selectivity filter itself has the ability to act as the inactivation gate (1–4). High resolution crystallographic analysis has revealed detailed structural changes in the selectivity filter associated with the activation gating and inactivation gating (5, 6). The carbonyl oxygens together with the side chain hydroxyl oxygen of the threonine define four equally spaced ion-binding sites that are commonly termed S1–S4, from the extracellular to the intracellular region (7). The rearrangement of the selectivity filter is well coordinated with the alteration of K\(^{+}\) ion-binding sites, providing a built-in mechanism for adjusting channel gating (5, 8, 9).

The two-pore domain potassium channels (K\(_{2p}\)) family, a branch discovered over a decade ago, has attracted increasing interest in its unique structure and function. In mammals, these channels are divided into six subfamilies on the basis of sequence similarity and function resemblance. All the members of this family are characterized by a distinguishing topology. That is, each subunit contains four transmembrane domains and two pore-forming domains (P1 and P2). Accordingly, K\(_{2p}\) subunits dimerize (in contrast to tetramerization in other potassium subunits) to constitute the functional selectivity filter containing four pore loop domains, a structure common to all known potassium channels (10–12). The current produced by these channels is outwardly rectifying and is insensitive to classic potassium channel blockers such as 4-aminopyridine and tetraethylammonium. K\(_{2p}\) channels are strongly implicated in the background or leak conductance that regulates the resting membrane potential and excitability of many kinds of cells.

TREK-1 (also called K\(_{2p}\)2.1), one of the best studied members of K\(_{2p}\) family, is expressed at high levels in excitable tissues such as the nervous system (13) and heart (14). Functionally, the channel is involved in many diverse physiological or pathological processes, including neuroprotection, cerebrovascular vasodilatation, regulation of aldosterone production and secretion, tumorigenesis, depression, chemoreception, and pulmonary vasoconstriction (15, 16). The functional versatility of the channel is highly associated with its sensitivity to a large volume of chemical and physical signals. Mechanical stretch, temperature, polyunsaturated fatty acids, intra-and extracellular pH, G-protein coupled receptors, and volatile general anesthetics...
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constitute the large TREK-1 regulatory machine (16). Particular efforts have been made to explain the relationship between these regulations and gating mechanism of TREK-1 (17, 18). It has been confirmed that the regulation of phosphatidylinositol 4,5-biphosphate to TREK-1 involves alteration of gating patterns (19). Extracellular acid could depress the TREK-1 current by facilitating C-type inactivation, in which conformational modification of the pore region could be involved (20). However, up to now, no clues have been found about how these signals interact with the SF in TREK-1. As the Ba\textsuperscript{2+} ion has a similar radius to the K\textsuperscript{+} ion, the divalent ion is able to bind into the SF of several classes of potassium channels (21–23). Because of its greater charge, Ba\textsuperscript{2+} tends to dwell a long time on its binding site, leading to a block of the K\textsuperscript{+} efflux. Thus, Ba\textsuperscript{2+} has been exploited extensively to decipher the SF properties of potassium channels (24–29). However, it has long been believed that the TREK-1 channel is Ba\textsuperscript{2+} insensitive or resistant (13, 30–33). Here, we report for the first time that Ba\textsuperscript{2+} inhibits the TREK-1 current dramatically by binding to the assumed K\textsuperscript{+} binding site 4 (S4). Then, using Ba\textsuperscript{2+} as a probe, we demonstrate that the regulation of extracellular potassium ions and protons involves conformational modification in the SF of TREK-1. Particularly, the N terminus truncated isoform of TREK-1 (ΔN41) possesses a constitutively nonconductive SF.

EXPERIMENTAL PROCEDURES

Molecular Biology—cDNA encoding the 411-amino acid isoform of human TREK-1 used in this work was amplified from a TREK-1 plasmid (a generous gift from Dr. Florian Lesage, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France) using PCR. To investigate whether the isoform was under the regulation of alternative translation initiation (ATI) mechanism, TREK-1 containing the translation initial sequence (UGA UA AGA) preceding the first start codon and the N-terminal truncated isoform (ΔN41) were inserted into pcDNA3.1 vector (Invitrogen). To get a high expression level in Xenopus laevis oocyte, TREK-1 was subcloned into the pGH19 vector as follows: the pGH19-HERG (human ether-à-go-go-related gene) plasmid (a generous gift of Dr. Gail Robertson, University of Wisconsin-Madison Medical School, Madison, WI) was first cut with BamHI and HindIII to get rid of its initial inserted HERG, and then TREK-1 was inserted into the matched sites.

Mutants were generated using the MutanBEST kit (TaKaRa) according to the manufacturer’s manual. All mutations were confirmed by DNA sequencing. cRNA was transcribed in vitro using the RibomAX™ large-scale RNA production systems kit (Promega).

Cell Culture, Protein Expression, and Western Blot Analysis—HEK 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine and held at 37 °C in humidified air with 5% CO\textsubscript{2}. cDNAs encoding the wild-type TREK-1, the mutant M42L, and ΔN41 were transiently transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen). Two days after transfection, the cells were harvested. Protein extracts were prepared by solubilization in buffer X (50 mM Tris (pH 7.4), 270 mM NaCl, 1% Triton X) for 1 h and clarified by centrifugation at 12,000 × g for 30 min. Then the lysates were subjected to SDS-PAGE on 10% gel and wet-transferred onto a PVDF membrane. The anti-myc (ZSGB-BIO, 1:1000) and anti-actin (ZSGB-BIO, 1:5000) primary antibodies were used in Western blotting. Secondary antibodies were used at 1:5000 dilutions.

Electrophysiology—X. laevis oocytes were isolated and injected with 10 ng of cRNA (46 nl in volume) per cell. Whole-cell currents were measured 1–3 days after injection by the two-electrode voltage clamp technique using an Axoclamp2B amplifier (Axon Instruments, Union City, CA). For two-electrode voltage clamp experiments, the electrodes were filled with 3 M KCl and had a tip resistance of 0.1–1 MΩ. Recordings were performed under constant perfusion at room temperature. Data were sampled at 2 kHz and filtered at 0.5 kHz with the Clampex 10.0 software (Axon Instruments). K\textsuperscript{+} currents through the TREK-1 channel were elicited either by the Ramp protocol (voltage ramps from −120 to +60 mV, with 1 s in duration) or by the Pulse protocol (a 100-ms pulse from −80 to +20 mV from a holding potential of −80 mV, with a 2 s interpulse interval). The normalized current was the average recorded current divided by the original control current. The standard physiological extracellular solution contained the following unless noted otherwise: 5 mM KCl, 93 mM NaCl, 1 mM MgCl\textsubscript{2}, 1.8 mM CaCl\textsubscript{2}, 5 mM HEPES (pH 7.4), adjusted with NaOH (standard solution). HEPES buffer was replaced by Tris in pH 8.5 solutions and MES in pH 6.5 solutions. When required, potassium ions in bath solutions were isotonically replaced by sodium ions. BaCl\textsubscript{2} was diluted from a 1 M stock and added to the various solutions as indicated.

Data Analysis—Concentration-response curves were fitted to the Hill equation according to the following parameters: $I = I_{\text{max}} \frac{[X]_o}{IC_{50}^h}$, where I is the measured current, IC\textsubscript{50} is the concentration of extracellular ions ([X]\textsubscript{o} represents H\textsuperscript{+} or Ba\textsuperscript{2+} in the research) required to achieve 50% inhibition, and h is the Hill coefficient. Time constants were calculated by fitting the data to the following equation: $I = I_0 + A e^{-T/t}$, where I is the measured current, $I_0$ is the blocked current at equilibrium, T is the elapsed time after Ba\textsuperscript{2+} application, and $t$ is the apparent forward time constant in seconds, and A is a constant. The fitting was separately performed for each experiment using Origin 7.5 software (OriginLab). Values were presented as mean ± S.E.

Statistical significance was assessed with Student’s t test using GraphPad Prism 5.0 (GraphPad Software, Inc.) software. Multiple comparisons were performed with one-way analysis of variance followed by Dunnett post-testing. A significant difference was considered for $p < 0.05$. 

RESULTS

Ba\textsuperscript{2+} Blocks TREK-1 in a Concentration- and Time-dependent Manner—It has been reported that the 426 amino acid of TREK-1 (TREK-1\textsubscript{426}) is subjected to the ATI mechanism because of the suboptimal sequence context flanking the first start codon compared with the second one (34). Therefore, a mixture composed of the full-length TREK-1\textsubscript{426} and an N-terminal truncated isoform (termed ΔN41 in this study) will be produced by its mRNA (Fig. 1A). The 411-amino acid of TREK-1 (TREK-1), which may be originated from alternative
splicing, displayed a very different sequence context surrounding the start site compared with TREK-1_{426} (Fig. 1B). To investigate whether TREK-1 was under the control of the AT1 mechanism, a mutant containing the second methionine-to-isoleucine mutation (M42I, no AT1 function) was constructed. Protein expression analysis revealed that only one specific band is the product of M42I. The start codons are N41, the M42I mutant, and wild-type TREK-1 by Western blot analysis with anti-myc antibody. The internal control (Actin) was visualized by an anti-actin antibody.

FIGURE 1. Characterization of the TREK-1 isoform. A, comparison of protein sequences of ΔN41, TREK-1, and TREK-1_{426}. The second methionine in TREK-1 and TREK-1_{426} are boxed. B, comparison of mRNA sequences flanking the first (M1) and second (M2) start codon of the TREK-1, the optimal Kozak sequence (Optimal), and the first start codon (AT1) of TREK-1_{426}. The start codons are boxed. C, protein expression analysis of ΔN41, the M42I mutant, and wild-type TREK-1 by Western blot with anti-actin antibody.

Currents recorded from a representative Xenopus oocyte expressing TREK-1 were shown in Fig. 2A in the absence and presence of different concentrations of extracellular Ba^{2+} ([Ba^{2+}]_o). When [Ba^{2+}]_o was up to 4 mM, a drastic current reduction was observed compared with the control current. For currents recorded at +20 mV, the inhibition was concentration-dependent (Fig. 2B). The fitting to the Hill equation revealed a half block (IC_{50}) at 0.56 ± 0.03 mM with an apparent Hill coefficient of 1.48 ± 0.18 (n = 7).

Then the time-dependence of the Ba^{2+} inhibition was investigated. In the presence of 1 mM Ba^{2+}, the currents evoked by depolarizing from the holding potential of −80 to +20 mV were decreased gradually as the time elapsed until the steady-state blockade was obtained (Fig. 2C). The process was plotted in Fig. 2D, and the current recovery by immediately following Ba^{2+}-free solution exchange was also included. The time courses for the block and complete recovery were well fitted by a single exponential equation, with a \( \tau_{\text{block}} \) of 20.28 ± 1.95 s and a \( \tau_{\text{unblock}} \) of 45.68 ± 4.80 s (n = 6), respectively.

External K^{+} Impedes the Blocking Effect of Ba^{2+} on TREK-1 Currents—Ba^{2+} inhibition of some potassium channels resulted from its docking properties in the SF (21–23). If that is also the case in TREK-1, competitive binding between Ba^{2+} and K^{+} would occur. To demonstrate the situation, the access rate of Ba^{2+} to the channel was firstly assessed. It was found that the inhibition rate (\( \tau_{\text{block}} \)) was accelerated as [Ba^{2+}]_o increased. The \( \tau \) value declined from 54.32 ± 3.00 s at the 0.25 mM level to 13.18 ± 1.12 s at the 4 mM level (Fig. 3, A and B, n = 6). Then the effects of extracellular K^{+} on Ba^{2+} access were evaluated. When [K^{+}]_o was increased from 0 mM to 10 mM, the block time constant at 1 mM [Ba^{2+}]_o was prolonged from 14.63 ± 3.88 s to 68.69 ± 10.34 s (p < 0.001, n = 6). Very little access of Ba^{2+} was observed in the presence of 20 mM [K^{+}]_o (Fig. 3, C and D). Accordingly, the blocking effects of Ba^{2+} were also alleviated as [K^{+}]_o increased. The inhibition was decreased from 93.91 ± 7.41% at the 0 mM [K^{+}]_o level to 3.80 ± 0.97% at 20 mM [K^{+}]_o level (Fig. 3E, n = 6, p < 0.001). These results reveal the competitive binding between Ba^{2+} and K^{+}, which may result from closing binding sites within the SF of TREK-1.

Ba^{2+} Blocks TREK-1 by Overlapped Binding at the S4 Site within the Selectivity Filter—Structural insight into the Strep-tomyces lividans K^{+} channel, KcsA (potassium channels from S. lividans), provided by crystallographic studies has demonstrated that the binding sites of Ba^{2+} is located in the innermost SF, very close to the K^{+} S4 (9, 35). S4 is composed of four main chain carbonyl oxygen atoms and four threonine side chain hydroxyl oxygen atoms in most potassium channels. The threonine positions 142 (T142S) and 251 (T251S) were constructed. In the presence of Ba^{2+}, the T142S and T251S mutants displayed very little blockade (5.97 ± 0.64% and 11.93 ± 2.46% inhibition, respectively, n = 6), whereas the current of the wild-type TREK-1 was decreased dramatically (72.90 ± 6.43% inhibition, n = 7) (Fig. 4B). As the concentration-response curve shows in Fig. 4C, the IC_{50} of externally applied Ba^{2+} to wild-type TREK-1 was 0.56 ± 0.03 mM, whereas to T142S and T251S it was 3.60 ± 0.19 mM and 3.28 ± 1.20 mM, respectively (n = 6). Obviously, the threonine mutants increase the IC_{50} for Ba^{2+} by approximately 6 fold, suggesting that Ba^{2+} depresses the TREK-1 current by occupying the K^{+} binding site at the SF.

The Ba^{2+} Exit Rate Uncovers Conformational Alterations within the Selectivity Filter Induced by Different Levels of [K^{+}]_o—The SF of KcsA has two distinct conformations associated with low and high concentrations of K^{+}. In low-K^{+} solutions, the filter adopts a nonconductive conformation, which is pinched...
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FIGURE 2. TREK-1 channels are inhibited by externally applied Ba$^{2+}$ in a concentration- and time-dependent manner. A, representative current-voltage relationship for TREK-1 expressed in Xenopus oocytes in the absence and presence of different [Ba$^{2+}$]o. Currents were elicited by the ramp protocol. The concentration of K$^+$ in extracellular solution is 5 mM. B, concentration-response for Ba$^{2+}$ inhibition (%) of TREK-1 channels at 20 mV with the protocol in A. Data points were mean ± S.E. of seven cells. The solid line is a fit of the data to the Hill equation. C, Ba$^{2+}$ block of representative TREK-1 current amplitude during application 1 mM Ba$^{2+}$. Currents were recorded with the pulse protocol. $I_o$ is the original current before application of Ba$^{2+}$. D, plot of current-time recorded from the currents recorded in C. The following withdrawal of Ba$^{2+}$ was also included.

closed at S2 and S3, and K$^+$ binds at the ends of the filter (S1 and S4) (36). In high K$^+$ solutions, the existence of four fully dehydrated K$^+$ ion sites endows the filter in a conductive conformation. According to the well established rule, the alteration of coordinated K$^+$ sites would influence the exit of barium from its binding site (35). Therefore, the change of the Ba$^{2+}$ exit pattern would correspond to the conformational alteration of the SF. In our experiments, it was found that the macroscopic currents of TREK-1 were enhanced by increasing [K$^+$]o from 0 to 20 mM (Fig. 5A), implying a conformational transition of the SF from nonconductive to conductive. Then, after the currents were depressed and reached a stable blockade in the presence of 1 mM Ba$^{2+}$, different levels of [K$^+$]o were used to facilitate the exit of Ba$^{2+}$ from its binding site. As shown in Fig. 5, B and C, the exit rate of Ba$^{2+}$ was strongly speeded up as [K$^+$]o increased. The decreased $\tau$ values were $45.68 \pm 4.80$ s, $24.13 \pm 1.33$ s, and $19.09 \pm 5.20$ s at 5 mM, 10 mM, and 20 mM [K$^+$]o, respectively ($n = 6, p < 0.001$). Particularly, the incomplete exit of Ba$^{2+}$ was observed in the K$^+$-free external solution (Fig. 5B). Therefore, alteration of [K$^+$]o evokes conformational transitions in the SF of TREK-1, and the off rate of Ba$^{2+}$ is highly associated with the transitions.

External H$^+$ Traps Ba$^{2+}$ Inside the Selectivity Filter of TREK-1—TREK-1 is very sensitive to external protons, and acidification strongly inhibits the channel via a mechanism resembling C-type inactivation in voltage-dependent potassium channels (20, 37). The key property of this mechanism is conformational transition of the SF to the nonconductive state. Because structural transitions of the SF occurred between low-K$^+$ and high-K$^+$ extracellular solution in TREK-1 (Fig. 5), we speculated that the alteration of extracellular pH values (pHo) might also influence the behavior of Ba$^{2+}$ in response to conformational modifications. In our experiment, the currents of TREK-1 fell quickly after the extracellular solution was switched from pH 8.5 to pH 6.0. Subsequent application of 1 mM Ba$^{2+}$ at pH 6.0 caused a further decrease. However, the washout with pH 6.0 solution achieved only a limited recover of the current (Fig. 6A). High pHo accelerated Ba$^{2+}$ dissociation with decreased $\tau_{unclock}$ values from $45.68 \pm 4.80$ s at pH 7.4 to $22.59 \pm 3.80$ s at pH 8.5 (Fig. 6, B and C, $n = 6, p < 0.001$). H126A, a pH-insensitive mutant containing a histidine-to-alanine transition (20, 37), was used to eliminate the pH sensitivity of TREK-1. External application of 1 mM Ba$^{2+}$ still blocked the currents quickly, but the currents of H126A recovered completely from Ba$^{2+}$ blockade in pH 6.0 external solutions (Fig. 6D). These data suggest that the conformational transition in TREK-1 occurs in the course of the pHo switch and that the SF structure evoked by acid tends to trap Ba$^{2+}$ because of its collapsed state.

The Conformational Transitions Induced by Extracellular H$^+$ and K$^+$-free Are Different in TREK-1—Most recently, it has been demonstrated that the SF conformation caused by low pH is very similar to that caused by low K$^+$ in KcsA, of which S2 and S3 are lost (5). In TREK-1, incomplete recovery from Ba$^{2+}$ inhibition was observed in the two conformation evoked by low-pH (acid conformation) and K$^+$-free (K$^+$-free conformation) conditions. We asked whether the SF structures of TREK-1 caused by K$^+$-free and acid conditions are also similar. Our results demonstrate that they are different from each other. Firstly, although the K$^+$-free conformation promoted
the access of Ba\(^{2+}\) (Fig. 3, C and D), the acid conformation tended to impede the process (Fig. 7, A and B). The \(\tau_{\text{block}}\) was prolonged from 19.14 ± 4.52 s at pH 8.5 to 25.48 ± 1.69 s at pH 6.0 (\(n = 6\), \(p < 0.01\)). Next, the interaction between the two conformations was investigated. As shown in Fig. 7C, currents recorded from a representative *Xenopus* oocyte expressing TREK-1 were subjected to a pH drop from 8.5 to 6.0 in the absence and in the presence of K\(^+\). Unequivocally, the K\(^+\)-free
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conformation was more sensitive to the pH drop (I_{6.0}/I_{8.5} of 0.22 ± 0.11 at 20 mV) than the conformation in standard solution (I_{6.0}/I_{8.5} of 0.45 ± 0.12 at 20 mV) (Fig. 7E, n = 5, p < 0.01). Furthermore, the pH-insensitive mutant H126A also showed inhibition by acid in K\(^+\)-free solution (I_{6.0}/I_{8.5} is 0.44 ± 0.10 at 20 mV), compared with that in standard solution (I_{6.0}/I_{8.5} is 0.90 ± 0.10 at 20 mV) (Fig. 7, D and E, n = 6, p < 0.001). Therefore, it seems that the K\(^+\)-free conformation potentiates the formation of acid conformation.

ΔN41 Possesses a Constitutively Collapsed Selectivity Filter—ΔN41, the product of ATI, is permeable to sodium under physiological conditions leading to membrane depolarization (34). The decreased macroscopic current, reduced open probability, along with the alteration of permeability of the isoform reminded us that conformational alteration to nonconductive state at its SF may happen. According to our results, Ba\(^2+\) ions were trapped in the nonconductive SF induced by low pH\(_o\) and K\(^+\)-free conditions in TREK-1. We then employed the blocker to test the possibility. No significant difference was found in the blockade ratio and access rate of Ba\(^2+\) to wild-type TREK-1 and ΔN41 (Fig. 8, A and C), but the behavior of Ba\(^2+\) exit displayed a big divergence. Ba\(^2+\) could not dissociate completely from
In alkali solution (pH 8.5), the time constant of Ba\(^{2+}\) access to TREK-1 was 73.9 ± 10.74 s (n = 7), much bigger than that of the wild type (22.59 ± 3.80 s, n = 6, p < 0.001, Fig. 8, B and C).

To further learn about the conformational changes of ΔN41, the pH sensitivity of wild-type TREK-1 and ΔN41 was examined next. As shown in Fig. 8D, for the steady-state currents recorded at +20 mV, the IC\(_{50}\) of acid inhibition was 7.29 ± 0.07 and 7.02 ± 0.51 (pH) for wild-type TREK-1 and ΔN41 (n = 5, respectively). No significant difference was found between them (p > 0.05), indicating that the location of conformational alteration induced by acid was similar in wild-type TREK-1 and ΔN41. Meanwhile, the pinched location in ΔN41 was not accordance with that in the wild type induced by acid.

**DISCUSSION**

Over the past decade, great progress has been made in the study of function and regulation in TREK-1. However, relatively little is known in the field of its gate mechanism, which may be due to the complex structure in the pore region compared with its voltage-gated brethren. The alkaline metal Ba\(^{2+}\) has a similar radius as K\(^{+}\) and is able to prevent the rapid flow of K\(^{+}\) effectively in several types of potassium channels through fitting into the SF by binding at S4 (21, 35, 38). Because of the inhibitory properties, Ba\(^{2+}\) has been used as a probe to learn about the pore structure of potassium channels (21–23). However, several studies have reported that TREK-1 is a Ba\(^{2+}\)-insensitive or resistant channel (13, 30–33). In the current study, it was firstly demonstrated that Ba\(^{2+}\) could inhibit TREK-1 channel expressed in *Xenopus* oocytes in a concentration- and time-dependent manner (Fig. 2). Moreover, from the competitive binding between Ba\(^{2+}\) and K\(^{+}\) (Fig. 3), we could speculate that the docking site for the blocker may be very close to K\(^{+}\) coordinated sites in the SF of TREK-1. Through further mutagenesis study, it was elucidated that Ba\(^{2+}\) inhibits TREK-1 current via binding at the location occupied by T142 and T251, the assumed S4 site of the channel (Fig. 4). These results indicated that the interactive pattern between Ba\(^{2+}\) and TREK-1...
was very similar to other studied potassium channels. This is less surprising because the SF is highly conserved among all the potassium channels. Only methyl groups in Thr-142 and Thr-251 were deleted in the Ba^{2+}/H11001 insensitive mutants, and therefore the methyl groups were responsible for the Ba^{2+} binding site in the SF of TREK-1. This phenomenon is also found in Kcv and Kir2.1 potassium channels (21, 23). On the other hand, the similarly retained Ba^{2+} sensitivity in the two mutants implies that in the SF of TREK-1 either another low affinity binding site exists, as in the case of KCNQ1 (39), or that Thr-142 and Thr-251 are only partly composed of the binding site, and the neighbored threonines carry the retained binding ability, as in the case of Kir2.1 (21, 23).

The interaction between K^{+} and Ba^{2+} follows the rule established previously: The presence of K^{+} at the external lock-in site (S1 and/or S2) would impede the outward movement of barium, and occupancy of both the lock-in site and the enhanced site (S3) by K^{+} would destabilize Ba^{2+} and promote its exit (35). Interestingly, conformational transition occurs in the switch of low [K^{+}]_{o} to high [K^{+}]_{o}. According to the data from the crystallography analysis, the low K^{+} conformation of KcsA represents a nonconductive state in which only S1 and S4 are retained. That is, the external lock-in site is present. The high K^{+} conformation of KcsA represents a conductive state in which all four sites are present that means the lock-in site and enhance site are retained. Our presented results reveal that a low [K^{+}]_{o} prevented Ba^{2+} exit from the pore and that a higher [K^{+}]_{o} enhanced its exit (Fig. 5). Therefore, we can speculate that the conformational transition in a different [K^{+}]_{o} may be similar to that of KcsA. In a K^{+}-free structure, the external lock-in site is occupied by K^{+} (probably S1) whereas the enhanced site is vacant (probably S2 and/or S3). According to the data from a large-conductance Ca^{2+}-activated channel, this kind of conformation may lead to a longer dock time of Ba^{2+} (27). In the high-K^{+} conformation, both the external lock-in site and the enhanced site are occupied by K^{+} (S1-S3), and Ba^{2+} ions are repelled mainly by electrostatic repulsion.

In KcsA, the structure of C-type inactivation is very similar to that resulting from low concentration of K^{+}, of which the SF is collapsed at S2 and S3 (5). However, data from some other potassium channels seem not to agree with the view. C-type inactivation conformation evoked by a low pH_{o} in TASK1 (TWIK-related acid-sensitive), another member of the K_{2p} family, displayed S0 lost (40). TASK2, a close relative of TASK1, shows another pattern of C-type inactivation in which the permeating ions are trapped at S1 and/or S2 (41). In addition, it was believed that conformational alterations happened at the outer pore of KCNK0 during C-type inactivation (42). In the acid conformation of TREK-1, Ba^{2+} was not able to dissociate completely from its binding site, indicating that collapse occurs in the SF. What about the collapsed location in the C-type inactivation (acid conformation) in TREK-1? Our results support the view that the position is located at the outer pore (S0 and/or S1) rather than at the middle location corresponding to S2 and S3. Three reasons may explain the situation. 1) As discussed above, it seems that evidence from the K_{2p} channels supports this
hypothesis. The pore structure of TREK-1 is more homologous with K$_{2p}$ members than with KcsA. The composition of the pore structure in K$_{2p}$ channels is heterogeneous, whereas KcsA is homogeneous. 2) From our data, the access rate of Ba$^{2+}$ to TREK-1 is probably associated with two factors: the concentration of itself and the SF conformation of TREK-1. If [Ba$^{2+}$]$_{o}$ is fixed, the change of the access rate would reflect the conformational alteration. As shown in Figs. 3 and 7, the K$^+$-free conformation caused an increased access rate, whereas the acid conformation caused a decreased tendency, indicating that the two conformations are different in the collapsed location. 3) The decreased access rate in acid conditions may reflect constriction in the outer pore. This phenomenon was also observed in Kv 1.5 (24). The interaction between K$^+$-free and acid conformations reveals that the K$^+$-free structure can facilitate the formation of the low-pH$_o$ structure. It has been revealed previously that an increased [K$^+$]$_{o}$ could alleviate C-type inactivation resulting from acid in TREK-1 (20). Clearly, the alleviation originated from the conformation transition to the conductive state, according to our results.

In light of the above results, we also identified ΔN41 as a constitutively nonconductive variant in the SF. Compared with wild-type TREK-1, ΔN41 showed a similar blocking rate of Ba$^{2+}$ but a slower unblocking rate. These results imply that the large difference in current density between the two isoforms may originate from their conformational divergence of SF rather than trafficking properties of the channels. TREK2, the closest relative of TREK-1 in K$_{2p}$ family, also uses the ATI rather than trafficking properties of the channels. TREK2, the similar response to pH$_o$ alteration, which implied that the col-lumous modulators. We also found that TREK-1 isoforms showed plays a large conductance and sodium permeability (43).

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