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INSIGHTS INTO THE STIMULATORY MECHANISM OF 2-AMINOETHOXYDIPHENYL BORATE ON TREK-2 POTASSIUM CHANNEL

R.-G. ZHUO,[a,b] X.-Y. LIU,[b] S.-Z. ZHANG,[b] X.-L. WEI,[b] J.-Q. ZHENG,[b] J.-P. XU[a*] AND X.-Y. MA[b*]

[a] *Department of Pharmacology, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, China*

[b] *State Key Laboratory of Toxicology and Medical Countermeasures, Department of Biochemical Pharmacology, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China*

Abstract—2-Aminoethoxydiphenyl borate (2-APB) has been recently identified as a common agonist of TWIK-related K^+ channel (TREK)/TRAAK channels, a subfamily of two-pore domain K^+ (K2P) channels. TREK-2 displays much higher sensitivity to 2-APB compared with TREK-1, despite that these two channels share the highest homology among K2P members. However, the structural basis for their difference in response to 2-APB still remains unknown. Here we identified that the cytosolic C-terminus (Ct) domain plays a dominant role in controlling the stimulatory effects of 2-APB on TREK-2 channel. The distal Ct region negatively regulates the effect of 2-APB, while the proximal Ct is sufficient to evoke the full 2-APB activation of the channel. Further mapping within the proximal Ct revealed that His368 is required for 2-APB activation, and the cooperation of the other non-conserved residues is also necessary. We also identified a secondary active site for 2-APB, which is located at the bottom of the transmembrane segment M2. Finally, we demonstrated that key residues or domains required for 2-APB activation are not involved in the gating mechanism of the selectivity filter. In summary, we reveal a unique modulatory model of TREK-2-Ct that distinguishes it from TREK-1 in high sensitivity to 2-APB. The cooperation of the non-conserved residues within the proximal Ct of TREK-2 plays a dominant role in the 2-APB-induced channel opening, whereas the distal Ct negatively regulates the process.

Key words: TREK-2, TREK-1, 2-APB, C-terminal domain, selectivity filter, gate mechanism.

INTRODUCTION

TREK-2 (TWIK-related K^+ channel 2, also called K2P10.1) is a member of two pore domain K^+ (K2P) channels, which encode background (or leak) K^+ currents that are essential for modulating the resting membrane potential and excitability of many kinds of neurons. Up to now, 15 members of the K2P family have been identified, which are divided into six subfamilies based on their structural and functional properties. Among them, TREK/TRAAK subfamily is comprised of TREK-2, TREK-1 and TRAAK (TWIK-related arachidonic acid-stimulated K^+ channel) (Enyedi and Czirjak, 2010). TREK-2 is regulated by diverse specific stimuli, such as membrane stretch, unsaturated fatty acid, temperature, extra- and intracellular pH and several neurotransmitter or hormone receptors (Bang et al., 2000; Lesage et al., 2000; Deng et al., 2009; Xiao et al., 2009, 2014), which suggests that the channel is coupled with a variety of signaling pathways. Functionally, TREK-2 channels have been found to participate in thermosensation and pain perception (Acosta et al., 2014; Pereira et al., 2014).

Given the contribution of TREK-2 channels to neurophysiology and neuropathology, deciphering the pharmacological properties and its relationship between function and structure is critical. Studies have suggested that the C-terminus (Ct) of TREK-1 modulates the whole gating process, and many key modulation motifs are clustered within the proximal Ct, including phosphorylation sites (Patel et al., 1998; Murbartian et al., 2005), the A-kinase-anchoring protein 150 and microtubule-associated protein 2 binding domains (Sandoz et al., 2006, 2008), sensors of intracellular proton (Honore et al., 2002), phospholipid (Chemin et al., 2005), membrane stretch (Patel et al., 1998) and thermo (Maingret et al., 2000), and general anesthetics acting domain (Patel et al., 1999). In response to these stimuli, the Ct of TREK-1 associates/dissociates from the membrane (Treptow and Klein, 2010; Sandoz et al., 2011) and then transduces the signals to the selectivity filter (SF) to control the gating process (Bagriantsev et al., 2011, 2012). TREK-2 is the closest relative of TREK-1 in K2P family, and they share 63% identity and 78% homology (Lesage et al., 2000). Accordingly, most of the above-mentioned motifs are conserved in TREK-2, which underlines their similar or identical regulation properties. Besides these conserved regions, there also exist non-conserved regions or residues within the Ct of TREK-

*Corresponding authors. Tel: +86-10-66931624; fax: +86-10-68211656 (X.-Y. Ma).
E-mail addresses: jpx@smu.edu.cn (J.-P. Xu), maxyer22@yahoo.com (X.-Y. MA).

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; Ct, C-terminus; K2P, two-pore domain K^+ channel; M2, transmembrane segment 2; Nt, N-terminus; SF, selectivity filter; TRAAK, TWIK-related arachidonic acid-stimulated K^+ channel; TREK, TWIK-related K^+ channel; TRP, transient receptor potential ion channels; WT, wild type.

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2. However, whether and how they function in a specific way remains to be elucidated.

2-Aminoethoxydiphenyl borate (2-APB) is a synthetic compound that was originally introduced as a membrane-permeable inhibitor of intracellular inositol 1,4,5 trisphosphate receptors (Bootman et al., 2002), and has been used extensively as a modulator of many kinds of ion transport proteins (Bilmen et al., 2002; Lemonnier et al., 2004; Bai et al., 2006; Clapham, 2007; Chokshi et al., 2012; Amcheslavsky et al., 2014; Takahashi et al., 2014). Recently, 2-APB has been identified as a universal agonist of TREK/TRAAK subfamily members. Interestingly, despite that TREK-1 and TREK-2 share the highest homology among K2P members, TREK-2 displays much higher sensitivity compared with TREK-1 (Beltran et al., 2013). However, both the underlying stimulatory mechanism and the structural bases for this difference remain unclear. Herein, we intend to explore the gating mechanism of TREK-2 activated by 2-APB, clarify the role of the cytosolic Ct in 2-APB function, and identify the key structural elements for the different sensitivity to 2-APB between TREK-1 and TREK-2 channels.

EXPERIMENTAL PROCEDURES

Molecular biology

cDNA encoding the 543-amino acid isoform of human TREK-2 (NM_138318) was amplified from a TREK-2 plasmid (Origene) using PCR. TREK-2 was subcloned into pGH19 vector using *Bam*H I and *Xba* I sites. Mutants were generated using the MutanBEST kit (TaKaRa) according to the manufacturer's manual. Chimeras were produced by PCR. All mutations were confirmed by DNA sequencing.

Electrophysiological measurement

Plasmids were linearized by *X*ho I restriction enzyme (TAKARA) before *in vitro* transcription. cRNA was transcribed using the RiboMAX™ Large Scale RNA Production Systems (Promega) kit. *Xenopus laevis* oocytes were isolated and injected with 0.1∼10 ng cRNA (46 nl in volume) per cell. Cells were kept at 18 °C in ND96 medium containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM pyruvate, 100 mg/ml gentamycin (pH 7.2).

Currents in oocytes were measured on 1–3 days after cRNA injection by using the two-electrode voltage-clamp technique with an Axoclamp2B amplifier (Axon Instruments, Union City, CA, USA). 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 Clampex 10.0 software (Axon Instruments). K^+ currents through TREK-2 channel and all the mutants were elicited by continuous voltage-ramps from −120 to +60 mV from a holding potential of −80 mV, with a 2-s duration. The normalized current was the average recorded current divided by the initial current at 0 mV. Unless otherwise indicated, the standard extracellular solutions contained (in mM): 5 KCl, 93 NaCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, pH 7.4, adjusted with NaOH. 2-APB (Promega) was freshly diluted with the standard extracellular solution. Because TREK channels undergo run up in oocytes, the recorded current was stabilized for about 20 min before applying 2-APB or switching extracellular pH. The activity of all the channels (wild type (WT) or mutated) at each concentration of 2-APB and at a specific pH was tested using at least two independent oocyte batches.

Data analysis

Data were analyzed with origin 8.0 (OriginLab Corporation) and GraphPad Prism version 5.0 (GraphPad Software, Inc.). Concentration–response curves were fitted to the Hill equation. The fitting was separately performed for each experiment using Origin 8.0 software. For all the concentration dependence of 2-APB activation or extracellular pH (pH_o) inhibition curves, the relative I/I_o was measured at 0 mV and was plotted as a function of extracellular concentration of 2-APB ($[2\text{-APB}]_o$) or pH_o. All values were presented as mean ± SEM. The overall statistical significance was tested for individual groups by using an unpaired Student's *t* test if necessary (GraphPad Prism). Differences were considered statistically significant if $p < 0.05$.

RESULTS

Intracellular C-terminus of TREK-2 is essential for 2-APB-induced activation

2-APB activates TREK-2 in a much higher degree when compared with TREK-1 (Beltran et al., 2013). Therefore, there should be difference (s) in domains between the two channels responsible for the 2-APB response. We thus performed sequence alignment analysis, and have found that the intracellular N-terminus (Nt) and Ct exhibit the primary difference between the two channels (the positions of the two domains were depicted in Fig. 1A). To characterize the role of these fragments in the channel gating induced by 2-APB, we constructed two chimeras with the Cts swapped between TREK-2 and TREK-1 channels (TK2TK1Ct and TK1TK2Ct, Fig. 1B, top panel) and one deletant of TREK-2 with Nt omitted (ΔNt). As a negative control, currents from oocytes uninjected with channel cRNA were measured. Only small currents (∼20 nA) could be recorded and the application of 2-APB did not present any significant effect on the currents (data not shown).

Upon the external application of 100 μM 2-APB, exchange of TREK-1's Ct to TREK-2 (TK2TK1Ct) strongly interfered with the stimulatory response of TREK-2 to 2-APB, while swapping of TREK-2's Ct to TREK-1 (TK1TK2Ct) prominently promoted the sensitivity of TREK-1 to 2-APB, as shown in the current–voltage relationships (*I–V* curves, Fig. 1B, bottom panel) and current–time relationships (*I–T* curves, Fig. 1C, D). The response of these channels at

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