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# The Inhibition of TREK2 Channel by an Oxidizing Agent, 5,5'-dithio-bis (2-nitrobenzoic acid), via Interaction with the C-terminus Distal to the 353rd Amino Acid

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**TREK (TWIK-RElated $K^+$ channels) and TRAAK (TWIK-Related Arachidonic acid Activated $K^+$ channels) were expressed in COS-7 cells, and the channel activities were recorded from inside-out membrane patches using holding potential of −40 mV in symmetrical 150 mM $K^+$ solution. Intracellular application of an oxidizing agent, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), markedly decreased the activity of the TREK2, and the activity was partially reversed by the reducing agent, dithiothreitol (DTT). In order to examine the possibility that the target sites for the oxidizing agents might be located in the C-terminus of TREK2, two chimeras were constructed: TREK2 (1-383)/TASK3C and TREK2 (1-353)/TASK3C. The channel activity in the TREK2 (1-383)/TASK3C chimera was still inhibited by DTNB, but not in the TREK2 (1-353)/TASK3C chimera. These results indicate that TREK2 is inhibited by oxidation, and that the target site for oxidation is located between the amino acid residues 353 and 383 in the C-terminus of the TREK2 protein.**

**Key Words: TREK2, Oxidizing agent, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), C-terminus, Two-pore domain $K^+$ channel, $K_{2P}$**

## INTRODUCTION

Reactive oxygen species (ROS) cause cell damage by reacting with various cellular constituents, including membrane lipids, proteins, and DNA. This damage may be initiated by alterations in cellular homeostasis by changing the levels of several physiological ions. Changes in the gating of ion channels can initiate the redox signaling system. Regulation of channel activity by ROS has been reported for several ion channels, such as $Ca^{2+}$-activated $K^+$ channel (DiChiara and Reinhart, 1997; Tang et al, 2001), *N*-methyl-D-aspartate receptor, NR1 (Sullivan et al, 1994), voltage-gated $K^+$ channel Kv1.4 (Ruppersberg et al, 1991), ryanodine receptor (Haarmann et al, 1999), inwardly rectifying $K^+$ channel IRK1 (Ruppersberg and Fakler, 1996), G protein-coupled inward rectifying $K^+$ channel GIRK (Zeidner et al, 2001), and two-pore domain $K^+$ ($K_{2P}$) channel, particularly in TREK2 (Kim et al, 2007).

The $K_{2P}$ channels are known to be constitutively active at membrane potentials across the physiological range and are likely to help set the resting membrane potential. $K_{2P}$ channels comprise two pore-forming region and four transmembrane-spanning domains, and the $K_{2P}$ channel family consists of TWIK, TASK, TALK, TRAAK/TREK, THIK, TRESK and KCNK7 sub-families (Honore, 2007; Kim, 2005).

Among these sub-families, TASK1, TASK3 and TREK1 have previously been reported as candidates for oxygen-sensing channels, although the effect of hypoxia on TREK1 still remains controversial (Buckler and Honore, 2005; Caley et al, 2005; Miller et al, 2003; Miller et al, 2005; Miller et al, 2004). Inhibition of TREK1 by hypoxia requires the C-terminus of the channel (Miller et al, 2005), and structural modification of the channel can also lead to hypoxic effects. Indeed, the C-terminal domains of TREK1 and TREK2 may modulate electrophysiological properties that are critical for channel activation, resulting from membrane stretching, temperature changes and intracellular acidosis (Honore et al, 2002; Kim et al, 2001b; Maingret et al, 2000; Maingret et al, 1999; Patel et al, 1998).

We postulated that the C-terminus of TREK2 may be involved in redox-sensing mechanisms in a manner similar to TREK1 and other $K_{2P}$ channels, and that TREK2 can be modulated by DTNB. We, therefore, performed a chimeric analysis to identify the target sites for the action of DTNB in TREK2.

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**ABBREVIATIONS:** TREK, TWIK-related $K^+$ channel; TRAAK, TWIK-related arachidonic acid activated $K^+$ channel; TASK, TWIK-related acid sensitive $K^+$ channel; 5,5'-dithio-bis (2-nitrobenzoic acid), DTNB; dithiothreitol, DTT; Two-pore domain channel, $K_{2P}$ channel.

## METHODS

### *Cell culture and transfection*

COS-7 cells were plated on 35 mm culture dishes one day before transfection. Rat TREK2 and its mutant forms were cloned into the pcDNA3.1 vector. The plasmids were then co-transfected with green fluorescent protein (GFP) into COS-7 cells using the LipoFectamine reagent (Invitrogen, Calsbad, CA). GFP expression in the transfected cells was detected using a Nikon microscope equipped with a mercury lamp light source. Cells were examined 2-3 days after transfection.

### *Solutions*

For single channel recording, the pipette and the bath solution contained (mM) 150 KCl, 1 $MgCl_2$, 10 HEPES and 5 EGTA titrated to pH 7.2 with HCl. All chemicals were from Sigma. To ensure a rapid turnover of the solution, the rate of perfusion was kept above 5 ml $min^{-1}$, which corresponded to 50 times the bath volume (100 $\mu$l) per minute. The reference electrode was a 3M KCl-agar bridge electrode.

### *Point mutation and chimeric constructs*

PCR was used to generate deletions and chimeras in TREK2 and TASK3, as described previously (Kim et al, 2001b). The point mutation was performed using a site-directed mutation kit (Stratagene, La Jolla, CA). All constructs were sequenced to confirm the correct reading frames and amino acid sequences present, including the C-terminus of TREK2.

### *Electrophysiological studies (voltage clamp recording and analysis)*

Single channel currents were recorded from single COS-7 cells in the outside-out patches or inside-out patches configuration. Voltage clamping was performed using an Axopatch-200 (Axon Instruments, Union city, CA). All recordings were performed at room temperature (22~24°C). The recorded signal was filtered at 1 kHz using an 8-pole bessel filter (−3dB: Frequency Devices) and transferred to a computer using the digidata 1,200 interface (Axon Instruments, Union City, CA) at a sampling rate of 5 kHz. Single-channel currents were analyzed with the pClamp program (Version 6.03, Axon Instruments, Union City, CA). The current tracings, shown in the figures, were filtered at 1 kHz. When using patches that contained many channel openings (>5 channel levels), currents were integrated over time to determine the relative channel activity. *n* represented the number of channels in the patch, and Po represented the probability of a channel being open.

### *Statistics*

The data are shown as means±SEM. *n* represents the number of cells tested. The significance of the differences between the means was established using a Student's t-test. $p<0.05$ was regarded as significant. All statistical analyses were conducted with the Origin program (Microcal, Northampton, MA).

## RESULTS

### *The effect of chemical oxidizing agents on $K_{2P}$ channels*

To confirm whether two-pore domain $K^+$ channels can be regulated by chemical oxidizing agents, we tested the effect of DTNB on TREK1, TRAAK, and TREK2. Representative single-channel openings were recorded in a symmetrical 150 mM KCl solution before and after intracellular application of DTNB, following the formation of inside-out

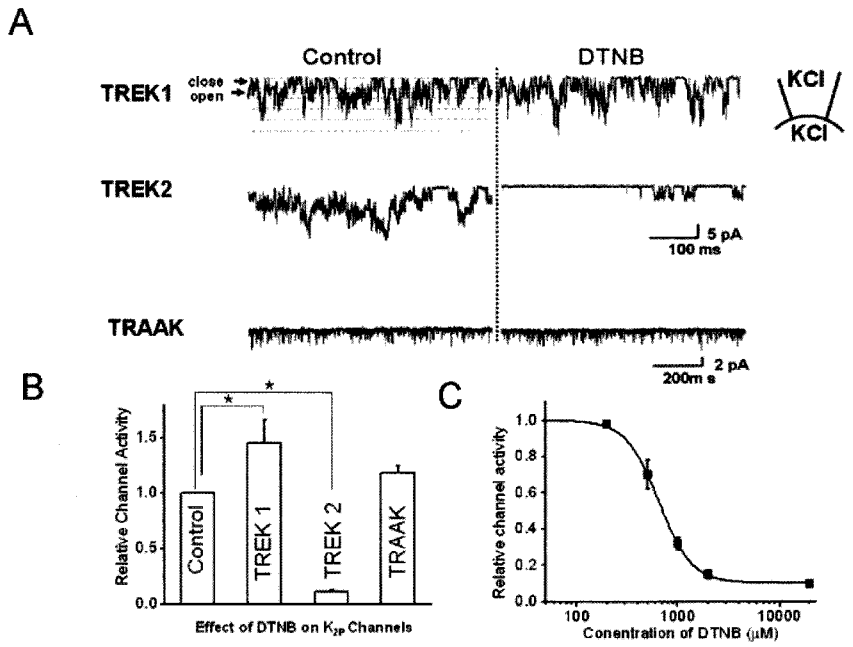


**Fig. 1.** The effects of oxidizing chemical agent, DTNB, on TRAAK, TREK1, and TREK2 in inside-out patch configurations. (A) The left panel shows the general single channel activity without DTNB, and the right panel shows channel activity with DTNB (2 mM). DTNB was applied to the bath solution, and the holding potential was held at −40 mV. "Close" represents the closed-channel level, and "open" represents the open-channel level. (B) The relative channel activity with DTNB on each channel. (C) Dose response curve of DTNB on TREK2. The pipette and bath solution contained 150 mM KCl, 5 mM EGTA, 10 mM HEPES, and 1 mM $MgCl_2$. The asterisk indicates a significant difference from the respective control ($p<0.05$).

patches (Fig. 1). DTNB solution (2 mM) markedly inhibited the activity of TREK2, in the excised inside-out patch configurations compared to the bath solution (89±5%, n=30). However, the activities of TREK1 and TRAAK channels were not decreased or even slightly increased by the addition of DTNB (Fig. 1). These DTNB-based effects on TREK1 and TRAAK were different from those of TREK2, and therefore, we focused our further efforts on TREK2. TASK1 and TASK3 were also unaffected by DTNB (data not shown). Extensive washout of DTNB for at least 5 min did not reverse its inhibitory effects on TREK2, and the channels remained as inactive as they were in the presence of the oxidizing agent. The inhibition of TREK2 by DTNB was partially reversed by the addition of reducing agent, dithiothreitol (DTT), in most of the patches tested (Fig. 1). The inhibition of TREK2 by DTNB was dose-dependent, and the half inhibitory concentration ($IC_{50}$) was 650.2±12.0 $\mu$M, with a Hill coefficient of 0.94±0.32 (Fig. 1C, n=8). To test the extracellular response of the membrane impermeable DTNB on TREK2, we analyzed upon outside-out patch configurations in TREK2-expressing COS-7 cells (Fig. 2, n=5). DTNB had little effect on TREK2 when it was present on the extracellular side.

These results suggest that the active site of TREK2, which is modulated by redox agents, is located on the intracellular side and is membrane-delimited. However, the reversible effects of DTNB may depend on intracellular factor(s) that may be absent in the inside-out patch configuration.

### *The effect of DTNB on the TREK2 C-terminus*

Amongst the amino acid residues, methionine and cysteine are most easily oxidized and susceptible to oxidation by $OH^-$. It is, therefore, highly possible that the two cysteine residues ($Cys^{494}$ and $Cys^{507}$) and the seven methionine residues ($Met^{375}$, $Met^{391}$, $Met^{493}$, $Met^{503}$, $Met^{514}$, $Met^{518}$, and $Met^{521}$) in the C-terminus of TREK2 may be possible targets for oxidation (Fig. 3 and Fig. 5). To address this possibility, the two cysteine residues at positions 494 and 507 were

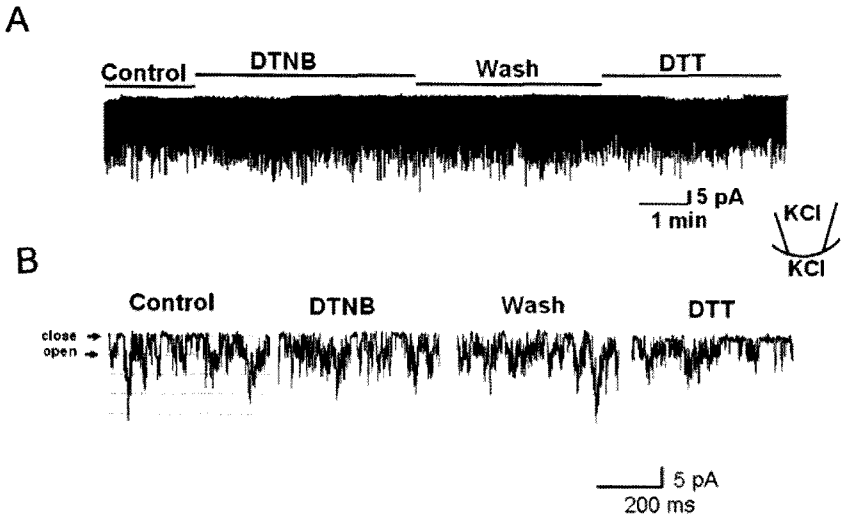


**Fig. 2.** The effect of extracellular application of DTNB (2 mM) and DTT (5 mM) on TREK2 using outside-out patch configurations. The holding potential was held at −40 mV. (A) The channel activity was not changed by DTNB and DTT. (B) Expanded scale trace of channel activity from A.

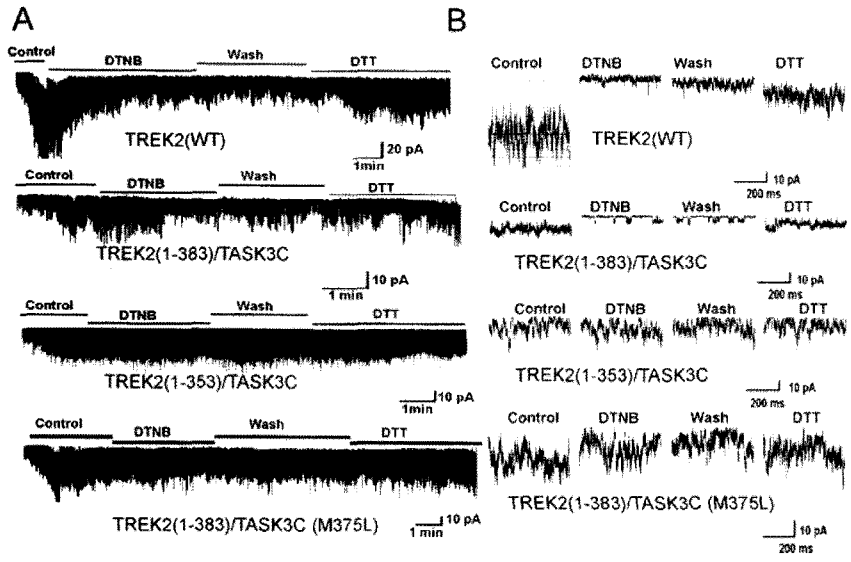


**Fig. 3.** The intracellular C-terminus of TREK2 modulates DTNB effects upon channel activity. (A) The first and second panels show the effect of DTNB (2 mM) on wild type TREK2 (1-538) and TREK2 chimera [TREK2 (1383)/TASK3C], respectively. The third panel shows the effect of DTNB on TREK2 chimera [TREK2 (1-353)/TASK3C]. The fourth panel shows the effect of DTNB on mutant TREK2 (1-383, M375L)/TASK3C (methionine-to-leucine substitution at position 375). (B) Expanded scale trace of channel activity from the A.

mutated to test the inhibitory actions of DTNB. These mutants were still inhibited by DTNB, even in mutants with double mutations (Cys494Gly and Cys507Gly, 80±9%, n=7, Fig. 5). Furthermore, we constructed a deletion series of the TREK2 C-terminus, and we also replaced a portion of the TREK2 C-terminus with the TASK3 C-terminus (Fig. 3).

We observed a decreased effect of DTNB in the chimera that had 155 amino acids of the TREK2 C-terminal end replaced with the C-terminus of TASK3 [TREK2(1-383)/TASK3C, 85±7%, n=11] (second panel of Fig. 3). The other chimera, where the 185 amino acids of the TREK2 C-terminal end were replaced with the C-terminus of TASK3 [TREK2(1-353)/TASK3C], was not inhibited by the oxidizing agent (n=7) (third panel of Fig. 3). These results suggest that the target site of DTNB may be located between the amino acids between positions 358 and 383 in the TREK2 C-terminus. Based on these results, we assumed that the methionine residue at position 375 is likely to be a redox

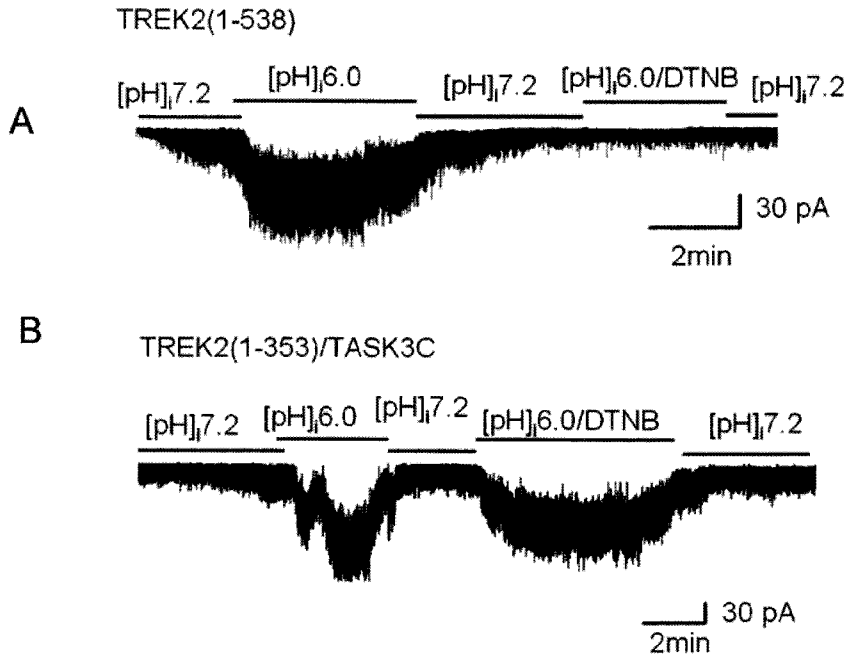


**Fig. 4.** The effect of intracellular acidic pH on wild type TREK2 and a chimera [TREK2 (1-353)/TASK3C] in the presence of DTNB. (A) An intracellular acidic pH activated wild type TREK2 in the absence of DTNB, but intracellular acidic pH did not activate wild type TREK2 in the presence of DTNB. (B) An intracellular acidic pH activated the chimera [TREK2 (1-353)/TASK3C] regardless of the presence of DTNB.

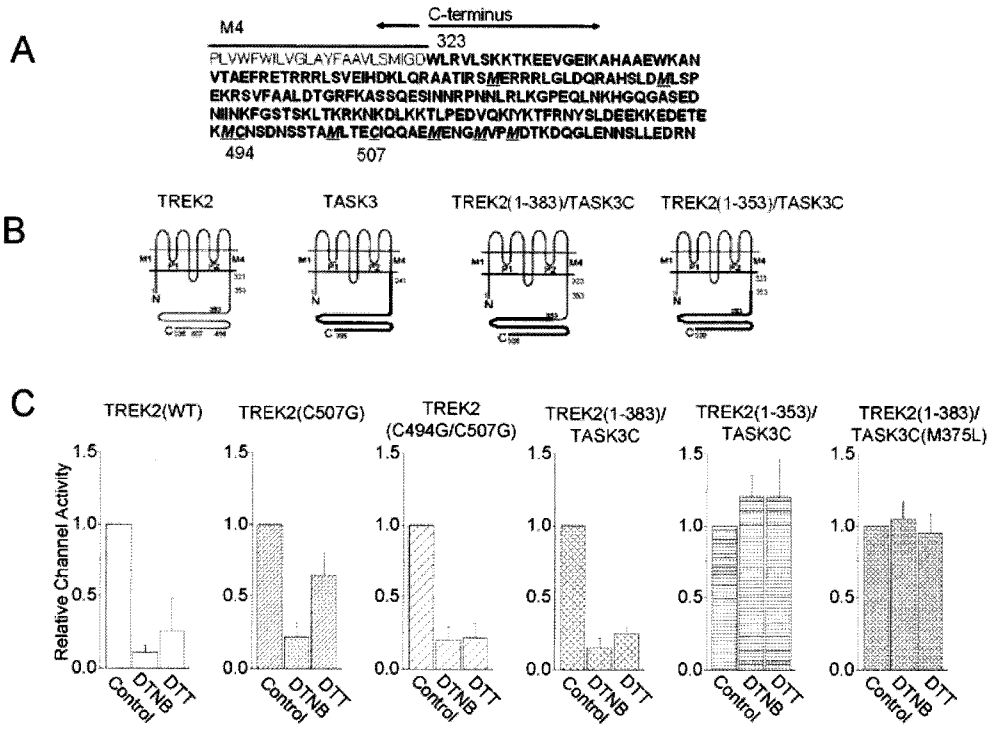


**Fig. 5.** Summary of the effects of DTNB on wild type TREK2 and mutants. (A) The amino acid sequence of the C-terminus of TREK2. (B) Membrane topologies of TREK2 and mutants show two pore-forming domains and four transmembrane segments (The membrane topology has been modified from Kim et al, 2001b). The amino acid positions indicate where the C-terminal replacements were made. The portion of the TREK2 C-terminus that was replaced with the C-terminus of TASK3. (C) The bar graph shows a summary of the effects of DTNB on wild type TREK2 and mutants.

regulatory target, and therefore, we tested mutant TREK2 (1-383, M375L)/TASK3C (methionine- to-leucine substitution at position 375). The channel activity was not inhibited by the oxidizing agent (n=3, fourth panel of Fig. 3). However, it was not entirely clear whether the methionine residue at position 375 was, in fact, a redox regulatory target of TREK2, because we did not test the mutant of the wild typeTREK2.

The cytoplasmic C-terminus of TREK2 (215 C-terminal amino acids long, out of a total of the 538 amino acids in TREK2) is longer than that of the other mechanosensitive $K_{2P}$ channels (TREK1 and TRAAK). It has earlier been suggested that truncation of the TREK2 C-terminal end can reduce mechanosensitivity, arachidonic acid sensitivity, and intracellular hydrogen sensitivity (Bang et al, 2000; Kim et al, 2001b). Therefore, we compared the effects of intracellular acidic pH on wild type TREK2 and TREK2 (1-353)/TASK3C chimera in the presence or absence of DTNB (Fig. 4). As expected, wild type TREK2 and TREK2 (1-353)/TASK3C chimera were activated by acidic pH in the absence of DTNB. The activation of wild type TREK2 by acidic pH was blocked by DTNB. However, the activation of TREK2 (1-353)/TASK3C chimera by acidic pH was not affected by DTNB (n=6). These results confirmed that the C-terminus of TREK2 was involved in the DTNB-mediated inhibition, and suggested that the redox regulatory sites of TREK2 that interact with oxidizing agents are located between amino acids 353 and 383.

## DISCUSSION

In this study, we found that TREK2 channel activity in a TREK2 (1-383)/TASK3C chimera was modulated by oxidizing agents, but not in another chimera, TREK2 (1-353)/TASK3C. However, TREK1 and TRAAK activities were not decreased by oxidizing agents, suggesting that the C-terminus of TREK2 contains a crucial regulatory site.

The C-terminal domain has been known to be critical for TREK1 and TREK2 activation, resulting from membrane stretching, temperature and intracellular acidosis (Honore et al, 2002; Kim et al, 2001b; Maingret et al, 2000; Maingret et al, 1999; Patel et al, 1998). On the other hand, the C-terminus of TRAAK is not important for mediating fatty acid sensitivity (Kim et al, 2001a). The differential effect of redox agents on $K_{2P}$ channels such as TRAAK and TREKs may explain various degrees of redox modulation, due to different reactivities of the sulfhydryl or sulfur group of cysteine or methionine residues on the channel protein itself or the presence of distinct redox regulation proteins. Activation of wild type TREK2 by acidic pH was blocked by DTNB, however, activation of the TREK2 (1-353)/TASK3C chimera by acidic pH was not affected by DTNB, suggesting that the target sites for channel activation by acidic pHs or oxidizing agents may be located in different positions.

Although we confirmed that the effects of DTNB are targeted towards C-terminal cysteine residues, we could not exclude the possibility that a cysteine residue at position 28 in the N-terminus is a redox target because we did not test the effect of DTNB on N-terminal TREK2 mutants. In addition, we could not exclude the possibility that the oxidizing agent interacted with other transmembrane regions in addition to the C-terminus. Therefore, further studies are needed to identify the interactions between oxidizing agents and other amino acid residues, such as $Cys^{494}$, $Cys^{507}$, $Met^{375}$, $Met^{391}$, $Met^{493}$, $Met^{503}$, $Met^{514}$, $Met^{518}$, and $Met^{521}$ in TREK2.

In conclusion, TREK2 channels expressed in COS-7 cells can be modulated by intracellular oxidants, and possible target site of the oxidant is located between the amino acid residues 353-383 in the C-terminus of the TREK2 channel protein.

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