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Palmitoylation of STREX domain confers cerebroside sensitivity to the BK_{Ca} channel

Yujiao Zhang [a,1], Li Zhou [a,1], Jun Zou [a], Mingyan Wang [a], Jianhua Qi [b], Zhi Qi [a,*]

[a] Department of Basic Medical Sciences, Medical College of Xiamen University, Xiang'an Nan Lu, Xiamen 361102, China
[b] College of Pharmaceutical Sciences, Zhejiang University, 866 Yuhangtang Rd, Hangzhou 310058, China

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ABSTRACT

Our previous study reported that cerebrosides from traditional Chinese medicine *Baifuzi* directly interact with the STREX domain of BK_{Ca} channels, which in turn results in the therapeutic effect of *Baifuzi* on ischemic stroke. However, it is not known how cerebrosides in the plasma membrane could interact with the STREX domain that is in the cytoplasmic side. Using patch-clamp technique, effects of different cerebrosides on the BK_{Ca} channel were studied by measuring single channel currents in CHO cells expressing wild type or mutated BK_{Ca} channels. Palmitoylation of the STREX domain was removed either by site-directed mutagenesis or pharmacological inhibition. Removal of palmitoylation sites at C646 and C647 by mutating the residues to Ala abolished the ability of cerebrosides to activate the BK_{Ca} channel. In contrast, the mutation neither changed the single channel conductance nor voltage sensitivity of the channel. Both palmitoylation inhibitors tunicamycin and palmitic acid analog 2-bromopalmitate attenuated the activation of the BK_{Ca} channel by cerebrosides. Furthermore, confocal images on STREX-EGFP fragments demonstrated that STREX fragments no longer associated with the plasma membrane when the palmitoylation was removed or blocked. These findings suggest that palmitoylation of the STREX domain is necessary for cerebrosides to activate the BK_{Ca} channel and provide insight into the mechanism of how *Baifuzi* could exert therapeutic effect on ischemic stroke.

1. Introduction

Large-conductance voltage- and Ca^{2+}-activated K^+ (BK_{Ca}, $K_{Ca}1.1$) channels serve numerous cellular functions, and their dysregulation is implicated in various diseases [1,2]. It has been shown that the BK_{Ca} channel is regulated by many kinds of lipid molecules. In rabbit pulmonary artery smooth muscle cells, the BK_{Ca} channel can be activated by arachidonic acid and myristic acid [3]. Fatty acids and negatively charged single-chain lipids increase the BK_{Ca} channel activity, whereas sphingosine and positively charged single-chain lipids suppress their activity [4]. In vascular myocytes, the application of exogenous phosphatidylinositol 4,5-bisphosphate (PIP2) to the cytoplasmic side at a physiological concentration increases the BK_{Ca} channel currents [5]. Recently, it has been demonstrated that long-chain polyunsaturated omega-3 fatty acids such as docosahexaenoic acid (DHA), could directly activate BK_{Ca} channels. The DHA action is observed in cell-free patches and does not require voltage-sensor activation or Ca^{2+} binding but involves destabilization of the closed conformation of the ion conduction gate [6].

We have previously demonstrated that several kinds of cerebrosides, including sulfatides [7], *Baifuzi*-CB (a mixture of cerebrosides obtained from dried tubers of herb *Typhonium giganteum* Engl., Chinese medicine *Baifuzi*) [8] and termitomycesphins A and B (pure cerebrosides from edible mushroom *Termitomyces albuminosus*) [9] could activate BK_{Ca} channels. Besides, we have shown that the STREX domain is necessary for these cerebrosides to activate the BK_{Ca} channel [7–9]. Furthermore, we have reported that *Baifuzi* reduces transient ischemic brain damage by activating BK_{Ca} channels through a direct interaction between *Baifuzi*-CB and the STREX domain of the BK_{Ca} channel [8]. STREX domain is located in the intracellular C terminus within the linker region between the two regulators of potassium conductance (RCK) domains of the BK_{Ca} channel (Fig. 1). It is an important domain in the modulation of the BK_{Ca} channel function, such as calcium and voltage sensitivity [10], regulation by protein phosphorylation [11], hypoxia sensitivity [12] and palmitoylation [13,14]. Intriguingly, how could cerebrosides in the plasma membrane interact with the STREX domain in the cytoplasmic side?

Palmitoylation is a reversible post-translational attachment of a palmitic acid via a hydroxylamine-sensitive thioester linkage onto cysteine residues, which is dependent on a large family of protein palmitoyltransferases (DHHCs) and thioesterases [15]. Protein palmitoylation is known to play roles in signal transduction and in enhancing the hydrophobicity of proteins thus contributing to their membrane association and membrane targeting. Many membrane proteins use palmitoylation to facilitate dynamic membrane interactions. Palmitoylation of SNAP-25 is required for initial membrane targeting of

* Corresponding author. Tel.: +86 592 2181330.
E-mail address: qizhi@xmu.edu.cn (Z. Qi).
[1] These authors contributed equally to this work.

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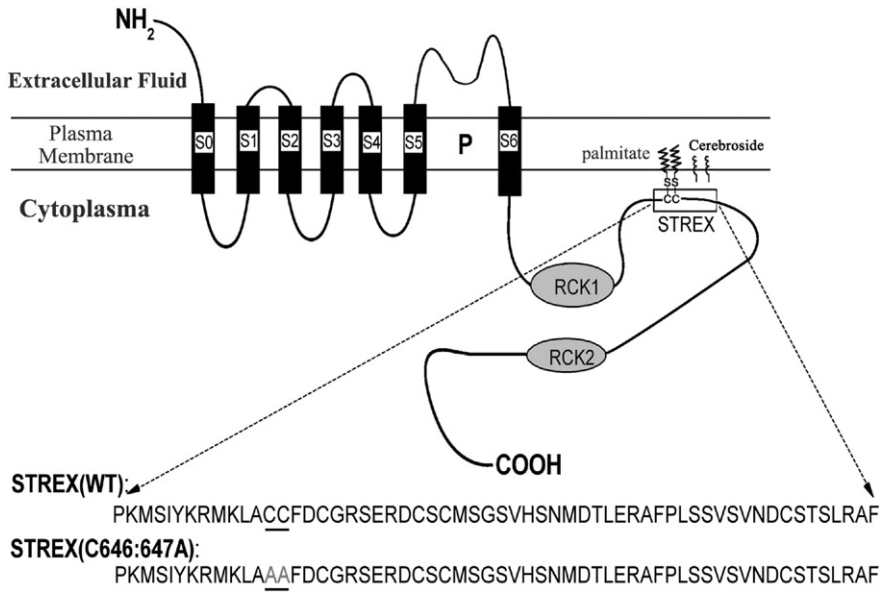

Fig. 1. Schematic model of the role of STREX palmitoylation for the interaction between cerebrosides and STREX domain of the channel. The STREX domain is localized in the linker between the two RCK domains in the intracellular C terminus. The 59 amino acids of the STREX(WT) and the STREX(C646:647A) mutant are shown between the two arrows, in which the palmitoylation sites have been underlined. Attachment of palmitates to Cys646 and 647 of the STREX domain results in the integration of STREX to the plasma membrane to facilitate its interaction with cerebrosides in the plasma membrane.

the protein [16]. Palmitoylation of PSD-95 regulates association with cell membranes and interaction with Kv1.4 channel. Palmitoylated PSD-95 partitions exclusively with cell membranes, while palmitoylation-deficient mutants of PSD-95 do not partition as integral membrane proteins [17]. Palmitoylation can regulate the gating and pharmacological properties of sodium channels [18]. Palmitoylation of the Kv1.1 potassium channel at residue C243 modulates its voltage sensing through protein-membrane interactions [19]. Palmitoylation of the KChIP auxiliary subunits controls plasma membrane localization of their associated channels [20]. Palmitoylation of the P2X7 receptor controls its expression and association with lipid microdomains of the plasma membrane [21]. Palmitoylation is also needed for Rac1 function in actin cytoskeleton remodeling by controlling its membrane partitioning, which in turn regulates membrane organization [22]. Interestingly, for the BK_{Ca} channel, it has been demonstrated that the STREX domain could target to the plasma membrane by palmitoylation of its two continuous cysteine residues C646 and C647 [11]. Therefore, we infer that cerebrosides in the plasma membrane could only interact with the palmitoylated STREX domain associated with the membrane, which in turn activates the BK_{Ca} channel.

In the present study, we applied multiple techniques involving electrophysiology, fluorescent imaging and biochemical assays to test the above inference. Our results demonstrated that the activation of the BK_{Ca} channel by cerebrosides could be abolished by mutation of the palmitoylation sites within the STREX domain. And palmitoylation inhibitors not only attenuated the activation of the BK_{Ca} channel by the cerebrosides but also reduced the association of the STREX fragment with the plasma membrane. These results suggest that palmitoylation induced membrane association of the STREX domain plays a significant role in the BK_{Ca} channel activation by cerebrosides.

2. Materials and methods

2.1. Channel constructs

The site directed mutagenesis and subcloning were performed on the chick BK_{Ca} channel α-subunit (GenBank accession number AB072618). The chick BK_{Ca} channel gene has been subcloned into the plasmid vector pcDNA3.1(-). To make the mutant BK_{Ca}(C646:647A), the generation of site directed mutagenesis was performed using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene).

To generate the STREX-EGFP fusion proteins, a forward primer containing a XhoI restriction site and a reverse primer with a HindIII site were used to amplify the STREX(WT) or STREX(C646:647A) genes. The PCR amplicons were digested and ligated into pEGFP-N1 Vector (Clontech, Palo Alto, CA, USA) so that EGFP is an N-terminal in-frame fusion with the respective STREX(WT) and STREX(C646:647A). All clones were confirmed by sequencing.

2.2. Cell culture and transfection

Chinese hamster ovary (CHO) cells were cultured in Ham's F-12 nutrient mixture (Gibco) supplemented with 10% fetal bovine serum (Gibco). Cells were grown in a 37 °C incubator with 5% CO_2 humidified environment and passaged twice weekly through exposure to 0.05% trypsin (Gibco) in Dulbecco's phosphate-buffered saline (DPBS; Gibco) solution. For gene transfection, cells were transferred to sterile glass coverslips in 35-mm Petri dishes. After cell density reached 60–80% confluence, they were transiently cotransfected with the channel and EGFP genes. Transfection was performed with Lipofectamine and Plus reagents (Invitrogen) following the manufacturer's instructions. Cells were used for electrophysiological recordings 24–72 h post transfection. Human Embryonic Kidney 293 (HEK 293) cells were grown as monolayers on 35-mm Petri dishes in high glucose Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum. For immunofluorescence assays, cells were plated onto gelatin coated glass coverslips. After cell density reached 60–80% confluence, they were transiently transfected with STREX(WT)-EGFP or STREX(C646:647A)-EGFP genes. Cells were used for immunofluorescence assay 12–24 h post transfection.

2.3. Electrophysiology

Experiments were performed as described earlier [7]. Recording electrodes were pulled from thin walled borosilicate capillary tubes using a Micropipette puller (model P-10, Narishige) with resistance in a range of 2–5 MΩ. The pipette solution was Hanks' balanced salt solution (HBSS; Gibco, in mM): 1.26 $CaCl_2$, 0.49 $MgCl_2$, 0.41 $MgSO_4$, 5.33 KCl, 0.44 KH_2PO_4, 4.17 $NaHCO_3$, 137.9 NaCl, 0.34 Na_2HPO_4 and 5.56

D-glucose. The bath solution was (in mM): 145 KCl, 10 EGTA, 10 HEPES (pH 7.3 with KOH), and $CaCl_2$. For a desired intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$), the necessary amount of $CaCl_2$ was calculated using the online MaxChelator software (http://maxchelator.stanford.edu/CaEGTA-NIST.htm). Unless stated otherwise, free intracellular calcium concentration was 0.34 μM with 10 mM Ethylene glycol bis(2-aminoethyl) tetraacetic acid (EGTA). Chloride-coated silver wires connected the pipette to the probe input. The probe of the patch amplifier was an EPC-10 amplifier (HEKA Elektronic, Lambrecht, Germany). The output signals were filtered at 2–5 kHz and low-pass filtered at 1 kHz. Patchmaster software was used for data acquisition and analysis. All experiments were performed at room temperature (20–25 °C). The value of the single channel open probability (Po) in a patch with multiple channels was calculated by using TAC 4.1 (HEKA, Germany), based on the equation: $Po = (1 - Pc^{1/N})$, where Pc is the probability when all of the channels are in the closed state, N is the number of channels in the patch, which was estimated from the maximum number of channels observed over a voltage of +50 mV. The single channel currents were plotted against a series of voltage and fitted to the GHK (Goldman–Hodgkin–Katz) current equation [23]:

$$I_K = P_K Z^2 \left(EF^2/RT\right)\left\{\left[K_i - K_o e^{(-ZsFE/RT)}\right]/\left[1 - e^{(-ZsFE/RT)}\right]\right\},$$

where I_K is the K^+ current, P_K is membrane permeability to K^+, Z = 1, and E is the transmembrane potential. The reversal potential, Erev, is obtained when $I_K = 0$. Then the single channel conductance, G, is calculated as: $G = I_K / (E - Erev)$. Cerebrosides were dissolved in DMSO and stored as a stock solution at −20 °C, which were diluted with bath solution upon use. The final concentration of DMSO in the recording solution did not exceed 0.1% (v/v), which did not affect BK_{Ca} channel's activity [9].

2.4. Imaging assays

For immunofluorescence assays, HEK 293 cells transfected with STREX(WT)-EGFP or STREX(C646:647A)-EGFP genes were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 at room temperature for 15 min. After blocking with 5% goat serum at room temperature for 30 min, cells were incubated sequentially with vimentin antibody (1:100, Sigma Aldrich) for 1 h at room temperature, and with goat anti-mouse IgG (GAM5492, 1:500, Multisciences) for 1 h plus 4′,6-diamidino-2-phenylindole (DAPI; C1005, 1:10, Beyotime) in darkness at room temperature for 10 min. Coverslips were then sealed onto the microscope slides using antifade mounting medium. The endoplasmic reticulum was labeled by ER-Tracker Red dye (C1041, 1:1000, Beyotime). Fluorescence images were observed by confocal microscopy (Olympus IX-81; Olympus FV1000, Tokyo, Japan).

2.5. Statistical analysis

Data analysis was carried out using SPSS software. Statistical analysis was performed using paired-sample Wilcoxon signed rank test. Averaged results were expressed as mean ± SEM from at least 6 independent experiments. The differences between groups were considered significant when $P < 0.05$.

3. Results

3.1. Removal of palmitoylation sites in the STREX domain abolished the ability of cerebrosides to activate the BK_{Ca} channel

To test whether palmitoylation of the STREX domain is necessary for cerebrosides to activate the BK_{Ca} channel, we compared the effects of cerebrosides on both wild type BK_{Ca}(WT) channels and palmitoylation sites removed BK_{Ca}(C646:647A) channels. Cerebroside concentration was chosen as 10 μM in the experiment, because our previous studies [7–9,24] have shown that most cerebrosides tested could significantly activate the channel at this concentration. Application of 10 μM *Baifuzi*-CB to the intracellular face of the membrane increased the BK_{Ca}(WT) channel activity in the most patch membranes. In contrast, *Baifuzi*-CB no longer or only weakly activated the BK_{Ca}(C646:647A) channel (Fig. 2A–C). Statistical summary demonstrated that the Po of the BK_{Ca}(WT) channel was increased from 1.6 ± 0.3% of control to 4.8 ± 1.1% (n = 19) upon application of 10 μM *Baifuzi*-CB at +20 mV, while 10 μM *Baifuzi*-CB only slightly increased Po of BK_{Ca}(C646:647A) channels from 1.7 ± 0.5% to 2.5 ± 0.9% (n = 10) (Fig. 2C). *Baifuzi*-CB is a mixture of cerebrosides, which differ in length of fatty acid, saturation, location and configuration of double bonds on long chain base. Therefore, we examined the effect of 1-*O*-β-D-glucopyranosyl-(2*S*, 3*R*, 4*E*, 8*Z*)-2-[(2′ *R*)-hydroxydocosanoyl-amino]-4, 8-octadecadiene-1,3-diol, a single pure cerebroside (single CB), isolated from mixture of *Baifuzi*-CB, on BK_{Ca}(WT) and BK_{Ca}(C646:647A) channels. As illustrated in Fig. 2D and E, 10 μM single CB significantly increased the Po of the BK_{Ca}(WT) channel without significant effect on the BK_{Ca}(C646:647A) channel. The Po of BK_{Ca}(WT) channels was increased from 1.1 ± 0.3% of control to 3.6 ± 1.0% (n = 17) in the presence of 10 μM single CB, while 10 μM single CB only increased Po of the BK_{Ca}(C646:647A) channels from 0.7 ± 0.1% to 0.8 ± 0.1% (n = 8) (Fig. 2F), indicating that the effect of cerebrosides to activate the BK_{Ca} channel was abolished by the removal of the palmitoylation sites in the STREX domain. To further confirm the importance of palmitoylation on the BK_{Ca} channel activation by cerebrosides, we selected sulfatides, consisting primarily of galactocerebroside sulfate, for the assay. In contrast to significant increase in the Po of the BK_{Ca}(WT) channel, sulfatides had no significant effect on the BK_{Ca}(C646:647A) channel (Fig. 2G and H). The Po of the BK_{Ca}(WT) channel was increased from 2.1 ± 0.5% of control to 5.0 ± 1.2% (n = 8) upon application of 10 μM sulfatides, while the same concentration of sulfatides almost had no effect on BK_{Ca}(C646:647A) channels: Po only changed from 1.2 ± 0.3% to 1.5 ± 0.4% (n = 6) (Fig. 2I). These results suggest that palmitoylation of the STREX domain is necessary for the cerebrosides to activate the BK_{Ca} channel.

3.2. Removal of palmitoylation sites in the STREX domain had no effect on single channel conductance and voltage-dependence of the BK_{Ca} channel

Next, we tested whether the removal of palmitoylation sites in the STREX domain influences the intrinsic properties of the BK_{Ca} channel. As shown in Fig. 3A and B, there was no significant difference in the single channel unitary current amplitude between the wild-type and C646:647A mutant BK_{Ca} channels. The single channel conductance was 134.2 pS for the wild type channel, while it was 135.4 pS for the BK_{Ca}(C646:647A) channel at +20 mV (Fig. 3C), indicating that the removal of palmitoylation sites in the STREX domain does not change the single channel conductance of the BK_{Ca} channel. To determine whether the voltage-dependence of the channel was altered, the voltage dependence of activation was studied. The values of Po were fitted to the Boltzmann equation: $Po = 1 / \{1 + e^{[(V1/2 - V) / k]}\}$, where V is voltage, $V_{1/2}$ is the voltage producing the half maximal activity and k is the slope factor. Values of 68.1 ± 0.2 mV for $V_{1/2}$ and 11.1 ± 0.1 mV for k were obtained in the wild type BK_{Ca} channels (n = 25), and they were 70.3 ± 0.2 mV and 11.1 ± 0.2 mV, respectively, for the C646:647A mutant BK_{Ca} channel (n = 25) (Fig. 3D), indicating that the voltage-sensitivity of the channel was not markedly altered by the removal of palmitoylation in the STREX domain.

3.3. Palmitoylation was required for STREX fragment to associate with the plasma membrane

Previous reports have demonstrated that palmitoylation could make STREX containing fragments (STREX C-terminus or cysteine-rich domain)

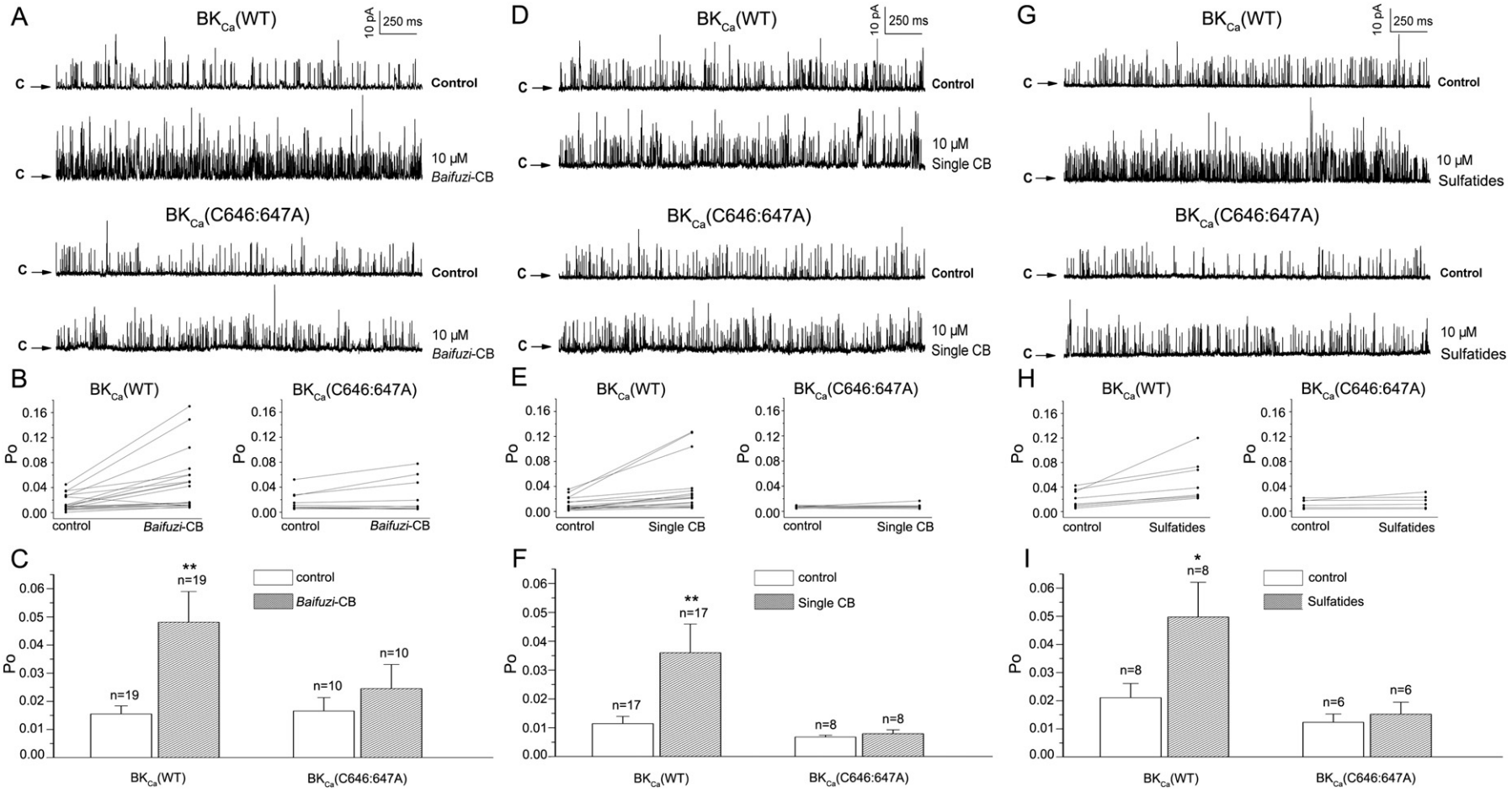

Fig. 2. Removal of palmitoylation sites in STREX domain abolished the ability of cerebrosides to activate the BK_{Ca} channel. (A, D and G) Representative single channel current traces for BK_{Ca}(WT) and BK_{Ca}(C646:647A) channels in the absence (control) and presence of *Baifuzi*-CB (A), single CB (D) or sulfatides (G) at +20 mV. Arrows indicate the level that all channels in the patch membrane are in the closed state. (B, E and H) The two data points on each end of the line represent Po of the BK_{Ca} channel before and after application of *Baifuzi*-CB (B), single CB (E) or sulfatides (H) from the same patch membrane. (C, F and I) Statistical summary for the Po of the BK_{Ca}(WT) and BK_{Ca}(C646:647A) channels before and after application of *Baifuzi*-CB (C), single CB (F) or sulfatides (I), respectively. *: $P < 0.05$, **: $P < 0.01$ compared to the control.

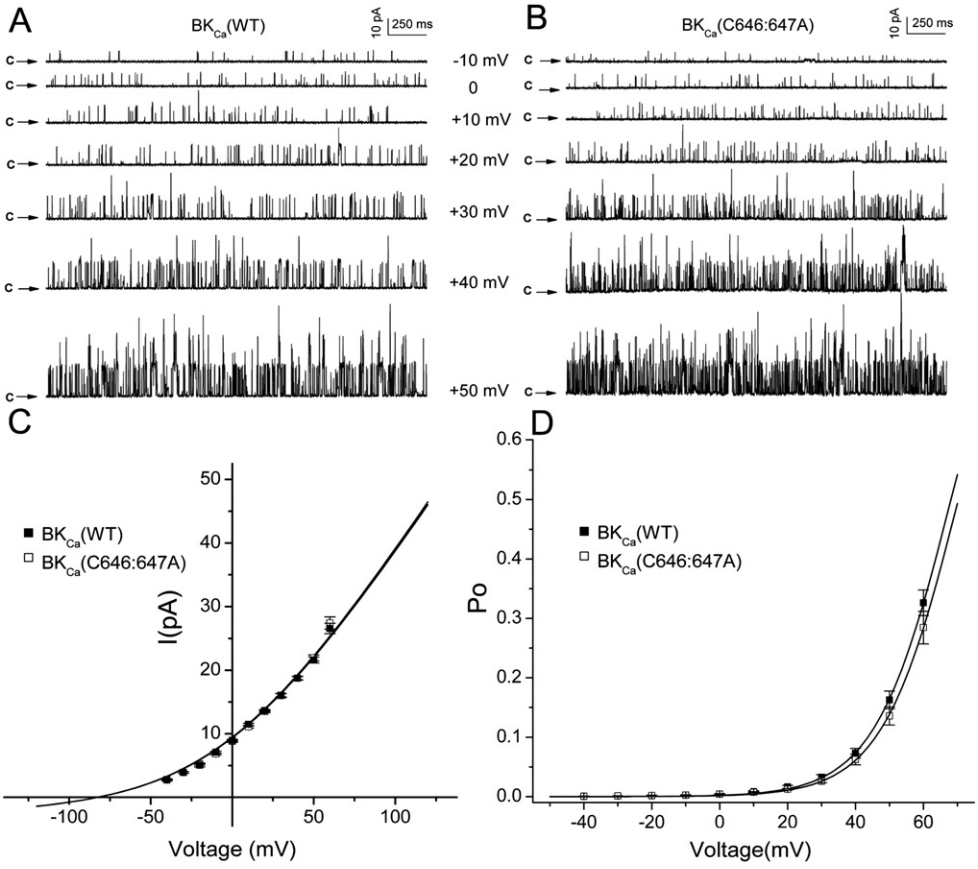

Fig. 3. Effect of the removal of palmitoylation sites in the STREX domain on single channel conductance and voltage-dependence of the BK_{Ca} channel. (A and B) Representative single channel current traces for BK_{Ca}(WT) and BK_{Ca}(C646:647A) channels from inside-out patches at a voltage range of −10 mV to +50 mV. (C) Single channel current–voltage relationship for BK_{Ca}(WT) (n = 23) and BK_{Ca}(C646:647A) (n = 21) channels. The solid lines were obtained by the best fit to data according to GHK current equation with E_{rev} = −82.8 mV. (D) The Po for BK_{Ca}(WT) (n = 25) and BK_{Ca}(C646:647A) (n = 25) channels at membrane potentials ranged from −40 to +60 mV.

target to the plasma membrane [11,25]. To examine whether the STREX fragment alone could target to the plasma membrane by palmitoylation, we generated STREX(WT)-EGFP and STREX(C646:647A)-EGFP constructs. Transient expression of the STREX(WT)-EGFP in CHO cells resulted in robust fluorescence around the plasma membrane (Figs. 4A and 5A). In the C646A:C647A mutant, even though the fusion protein was robustly expressed, it only showed cytoplasmic distribution (Figs. 4B and 5B). Furthermore, the plasma membrane localization of the STREX(WT) domain was abolished by preincubation of cells with palmitic acid analog 2-bromopalmitate (2-BP) or palmitoylation inhibitor tunicamycin (TM) in CHO cells (Figs. 4C, D and 5C). To determine the location of the STREX domain more accurately, we used an anti-vimentin for immunofluorescence assay on HEK 293 cells. Vimentin is a type III intermediate filament, along with tubulin-based microtubules and actin-based microfilaments, comprising the cytoskeleton. The merged images in HEK 293 cells showed that the wide type STREX fragment could locate at the outside of vimentin (white arrows in Fig. 6A), while the STREX(C646:647A) fragment could overlap but not be able to locate to the outside of vimentin (white arrows in Fig. 6B). These results indicate that palmitoylation could make the STREX fragment alone target to the plasma membrane.

3.4. Prevention of palmitoylation abolished the ability of cerebrosides to activate the BK_{Ca} channel

To further validate that palmitoylation is necessary for cerebrosides to activate the BK_{Ca} channel, we compared channel susceptibility to cerebrosides in the presence of 2-BP or TM. The single channel current traces showed that the application of 10 μM *Baifuzi*-CB, single CB or sulfatides respectively to the intracellular face of the inside-out patches could only slightly or no longer activate the BK_{Ca} channel in cells

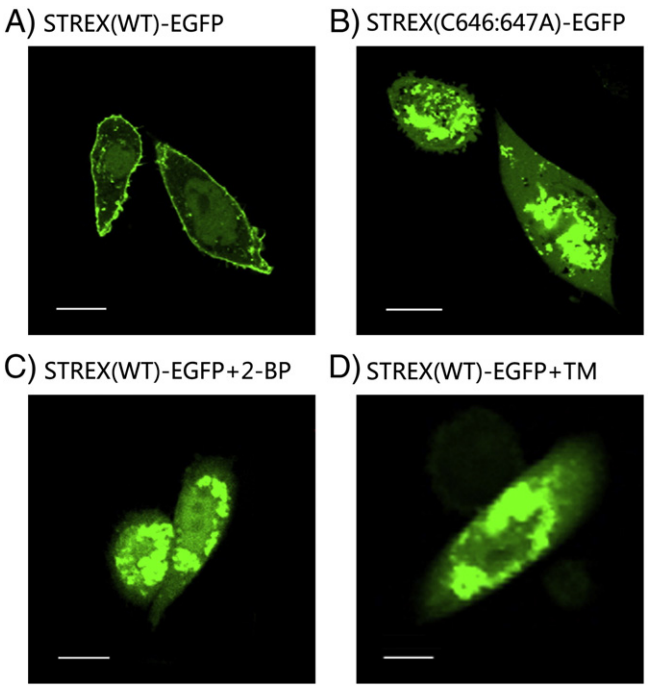

Fig. 4. Palmitoylation is required for STREX fragment to associate with the plasma membrane. (A and B) Representative single confocal sections from CHO cells expressing STREX(WT) and STREX(C646:647A) constructs. (C and D) Representative single confocal sections from STREX(WT) expressed in CHO cells that were pretreated with 100 μM 2-BP overnight (C) or 6 μM TM for 2 h at 37 °C (D). Scale bar = 10 μm.

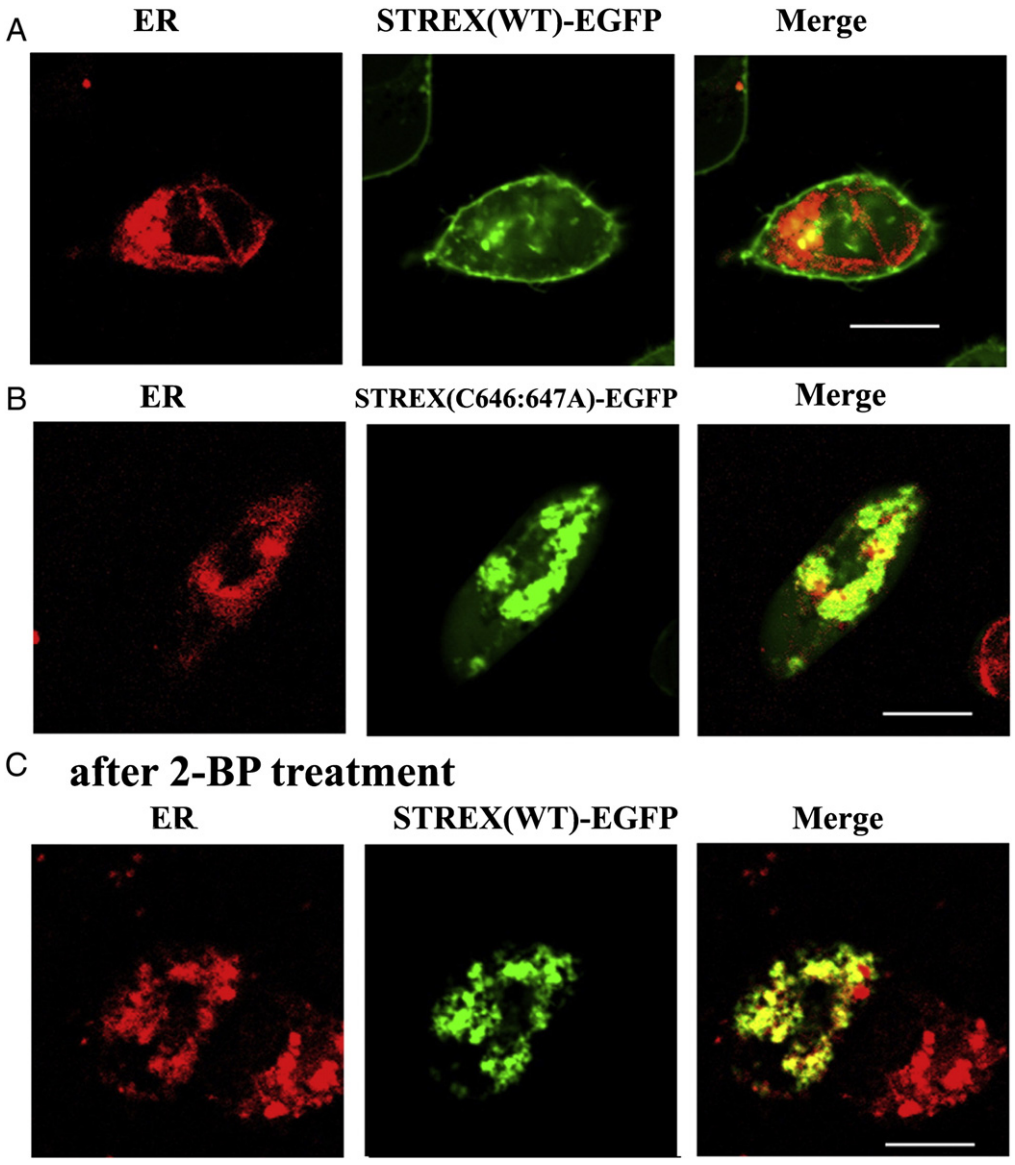

Fig. 5. Localization of the mutant STREX-C646:647A and 2-BP treated STREX domain. (A and B) Representative confocal images from CHO cells expressing STREX(WT)-EGFP or STREX(C646:647A)-EGFP. (C) Representative confocal images from STREX(WT)-EGFP expressed in CHO cells that were pretreated with 100 μM 2-BP. Twenty-four hours following transfection cells were stained with 1 μM ER Tracker red and live cells analyzed by confocal microscopy. Scale bar = 10 μm.

pretreated with 2-BP or TM (Fig. 7). For example, in 2-BP treated cells, the Po of the BK_{Ca} channel was 1.6 ± 0.5% in the absence of sulfatides, while it only increased to 1.9 ± 0.5% (n = 6) after the application of 10 μM sulfatides (Fig. 7B). Previously, we have shown that the therapeutic effect of the traditional Chinese medicine *Baifuzi* on rat MCAO model of ischemic stroke is due to the ability of its cerebrosides to activate the BK_{Ca} channel [8]. Therefore, we checked the effects of 2-BP on the rat BK_{Ca} channels (rBK). We found that the rat BK_{Ca} channel could not be activated by the single cerebroside that was purified from *Baifuzi*-CB after the application of 2-BP (Fig. 8). These results indicate that palmitoylation is necessary for cerebrosides to activate the BK_{Ca} channel.

4. Discussion

In the present study we have demonstrated that palmitoylation of the STREX domain could make the domain associate with the plasma membrane so that cerebrosides in the plasma membrane could interact with the STREX domain in the membrane interface to activate the BK_{Ca} channel. Firstly, we found that the cerebrosides could not activate the BK_{Ca} channel with mutation at the palmitoylation sites (C646:647A) within the STREX domain, indicating that mutation of palmitoylation sites in STREX domain abolished the BK_{Ca} channel activation by the cerebrosides. Secondly, confocal images of STREX-EGFP fragments demonstrated that palmitoylation of C646 and C647 targeted STREX domain to the plasma membrane, while the STREX(C646:647A)-EGFP mutant resulted in cytoplasmic distribution, indicating that palmitoylation of the STREX domain is required for its association with the membrane. Thirdly, palmitoylation inhibitor or palmitic acid analog not only attenuated the activation of the BK_{Ca} channel by the cerebrosides but also reduced the distribution of STREX fragment around the plasma membrane. Taken together, these results suggest that palmitoylation of the STREX domain is necessary for cerebrosides to activate the BK_{Ca} channel.

It is noticed that Po of the BK_{Ca} channel is variable from patch to patch even in the control condition. To reliably calculate Po of the channel in a patch, it is critical to know the number of channels in the patch. In practice, the number is estimated from the maximum number of channels observed, which is usually underestimated even though it is judge by the favorite condition for channel activation. In our experiment, we did not figure out the reason, but found that the base lines were un-stable

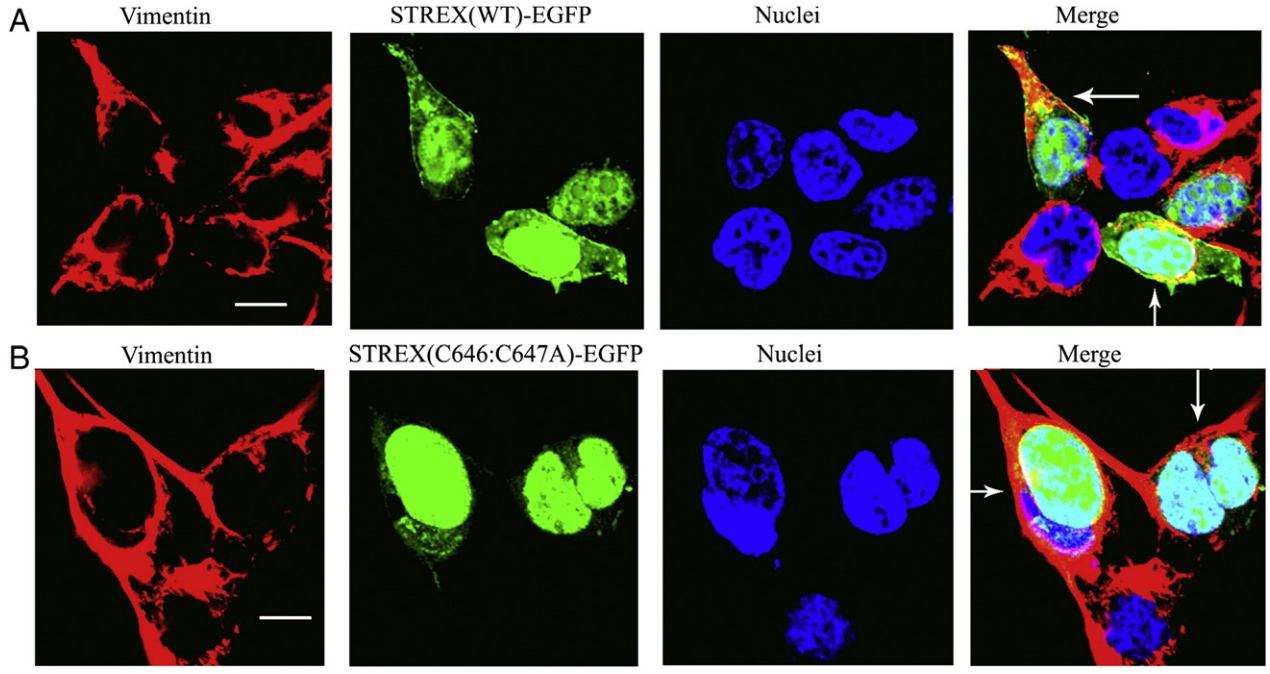

Fig. 6. Palmitoylation leads the STREX fragment to associate with the plasma membrane in HEK 293 cells. (A and B) Representative confocal sections of HEK 293 cells expressing STREX(WT)-EGFP (A) and STREX(C646:647A)-EGFP (B) constructs. Cells were transiently transfected into STREX(WT)-EGFP or STREX(C646:647A)-EGFP constructs. Red: Vimentin. Green: STREX-EGFP. Blue: nuclei stained with DAPI. Scale bars = 10 μm.

in the presence of cerebrosides, especially at higher depolarized potentials. This prevented us to estimate the number of channel in the patch at the favorite condition for the BK_{Ca} channel activation. To reduce the influence as less as possible, the control Po for each set of experiment was obtained only from that set of experiment. Furthermore, we compared the Po of the BK_{Ca} channel before and after application of different cerebrosides from the same patch membrane (Fig. 2B, E and H). The data set from the same patch membrane clearly indicated that cerebrosides increased the BK_{Ca}(WT) channel activity in the most patch membranes, while no longer or only weakly activated the BK_{Ca}(C646:647A) channel.

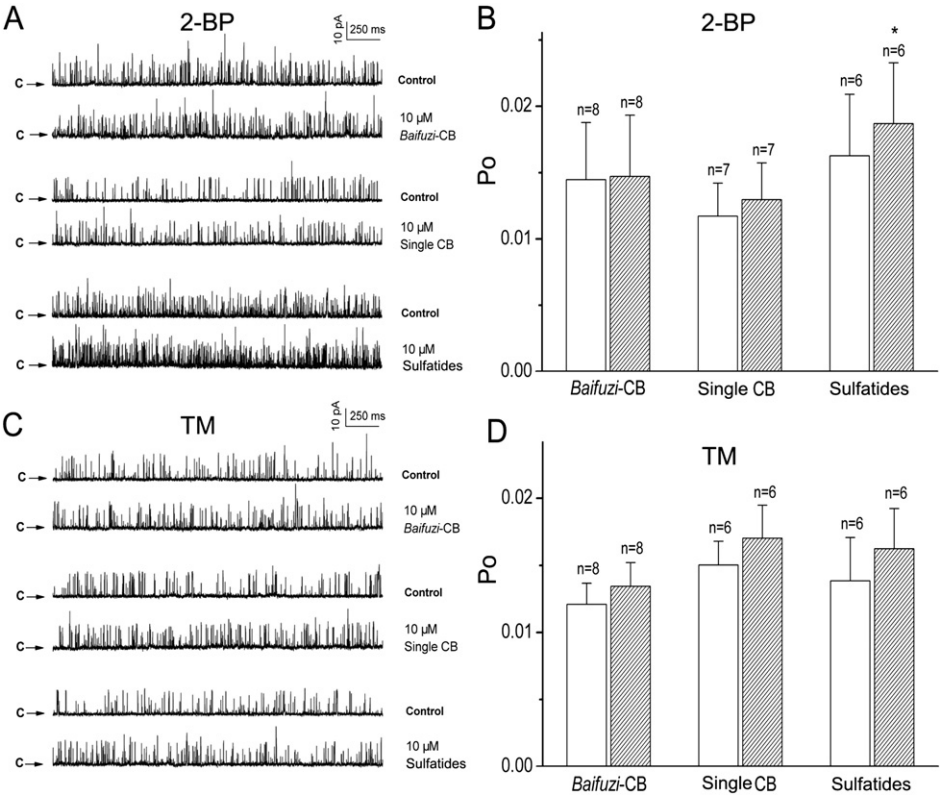

Fig. 7. Prevention of palmitoylation abolished the ability of cerebrosides to activate the BK_{Ca}(WT) channel. (A and C) Representative single BK_{Ca}(WT) channel current traces in CHO cells pretreated with 100 μM 2-BP overnight (A) or 6 μM TM for 2 h (C) before and after application of 10 μM *Baifuzi*-CB, single CB and sulfatides at +20 mV. (B and D) Statistical summary for Po of the BK_{Ca}(WT) channel under the same conditions as that in A and C, respectively. *: $P < 0.05$ compared to the control.

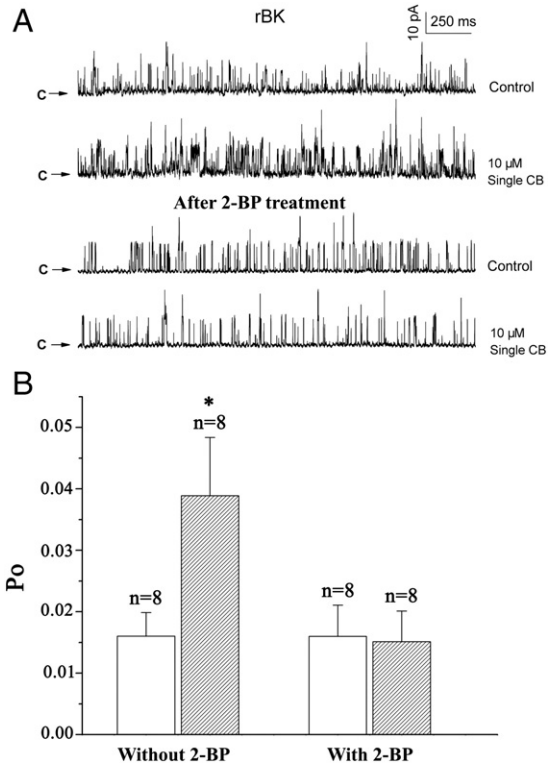

Fig. 8. 2-BP abolished the ability of the *Baifuzi* cerebroside to activate the rat BK_{Ca}(WT) (rBK) channel. (A) Representative single BK_{Ca}(WT) channel current traces in CHO cells pretreated with 100 μM 2-BP before and after application of 10 μM single CB that was purified from *Baifuzi*-CB. (B) Statistical summary for Po of the BK_{Ca}(WT) channel in the absence and presence of 2-BP. *: $P < 0.05$ compared to the control.

Therefore, the variation in the Po of the channel should not affect our main conclusion that palmitoylation of the STREX domain is necessary for cerebrosides to activate the BK_{Ca} channel. Interestingly, by comparing the Po of the BK_{Ca}(WT) channel from the same patch membrane in the absence and presence of cerebrosides, we found that all kinds of cerebrosides tested could not or only weakly activate the BK_{Ca}(WT) channel in some of the patch membranes (Fig. 2B, E and H). As palmitoylation is a dynamic process, it is impossible that all BK_{Ca} channels are palmitoylated at the same time. If no or only few channels in a patch membrane were palmitoylated, the STREX domain of these channels could not associate with the membrane and not be able to interact with cerebrosides in the membrane. This might be the reason why cerebrosides could not activate the channels in some of the patch membranes. In contrast, cerebrosides, without exception, could not activate the BK_{Ca} channel when palmitoylation of STREX was abolished by site-directed mutagenesis or pharmacological inhibition of palmitoylation (Figs. 2 and 7). These observations are in good consistence with the dynamic nature of protein palmitoylation.

Many kinds of lipid molecules, including fatty acids [3,26,27], phospholipids [5,28] and cholesterol [29,30], could modulate the function of BK_{Ca} channels. For fatty acids, attributing to differences in surface charges, negatively or positively charged head group with sufficiently long ($C > 8$) carbon chain, they increase or decrease the BK_{Ca} channel open probability, respectively, while neutral and short-chain lipids have no effect on the channel activation [27]. For cholesterol, it has been suggested that cholesterol induces structural stress in the membrane which enhances the transition from the open to the close state of the channel, and therefore results in the reduction in the Po of the BK_{Ca} channel [29]. In addition, biophysical properties of the membrane, such as membrane deformation [31,32] and membrane thickness [33,34] can affect function of the BK_{Ca} channel as well. In our previous studies, we have demonstrated that the ability of cerebrosides to activate the BK_{Ca} channel is due to their direct interaction with the STREX domain [8]. In the present study, we show that it is palmitoylation that makes the STREX domain to associate with the plasma membrane, therefore provides a platform for cerebrosides in the membrane to interact with the STREX domain to activate the BK_{Ca} channel. Active components of traditional Chinese medicines often have not been specified and measured precisely, which subsequently hinders the understanding of their mechanism of action on a well-defined molecular target and the evaluation of their therapeutic efficacy. This work illustrated the molecular mechanism of how cerebrosides activated the BK_{Ca} channel and provided a rational for the use of *Baifuzi* for treating stroke at the molecular level.

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