Molecular Membrane Biology, January–February 2011; 28(1–2): 145–154

The effect of single cerebroside compounds on activation of BK_{Ca} channels

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(Received 1 June 2010; and in revised form 19 October 2010)

Abstract

We have previously shown that a mixture of cerebrosides obtained from dried tubers of herb *Typhonium giganteum* Engl. plays a neuroprotective role in the ischemic brain through its effect on activation of BK_{Ca} channels. It is very curious to know whether a single pure cerebroside compound could activate the BK_{Ca} channel as well. This study explored the possible effects of pure cerebroside compounds, termitomycesphins A and B, on the BK_{Ca} channel activation. Both termitomycesphins A and B activated the BK_{Ca} channels at micromole concentration without significant difference. Termitomycesphin A increased the single channel open probability of the BK_{Ca} channels in a dose-dependent manner without modifying the single channel conductance. Termitomycesphin A activated BK_{Ca} channel more efficiently when it was applied to the cytoplasmic face of the membrane, suggesting that binding site for termitomycesphin A is located at the cytoplasmic side. Termitomycesphin A shifted the voltage-dependent activation curve to less positive membrane potentials and the Ca^{2+}-dependent activation curve of the channel upwards, suggesting that termitomycesphin A could activate the channels even without intracellular free Ca^{2+}. Furthermore, STREX-deleted BK_{Ca} channels were completely insensitive to termitomycesphin A, indicating that STREX domain is required for the activation of the BK_{Ca} channel. These data provide evidence that termitomycesphins are potent in stimulating the activity of the BK_{Ca} channels. As BK_{Ca} channels are associated with pathology of many diseases, termitomycesphins might be used as therapeutic agents for treating these diseases through its regulatory effect on the BK_{Ca} channels.

Keywords: *BK_{Ca} channel, cerebroside, STREX domain, termitomycesphin*

Introduction

The large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels are widely distributed in many tissues such as neurons (Lancaster and Nicoll 1987), chromaffin cells (Lingle et al. 1996), inner hair cells of cochlea (Issa and Hudspeth 1994), chick cardiac myocytes (Ruknudin et al. 1993, Kawakubo et al. 1999), and skeletal (Pallotta et al. 1981) and smooth muscles (Toro et al. 1991). Functional BK_{Ca} channels are formed by tetramerization of pore-forming α subunits and an auxiliary β subunit (Shen et al. 1994, Wang and Sigworth 2009). The pore-forming α subunit is encoded by *KCNMA1* gene (Butler et al. 1993), which is subject to vast tissue-specific (Tseng-Crank et al. 1994) and cell-specific alternative pre-mRNA splicing (Zarei et al. 2004). Molecular structure of the α subunit consists of seven membrane-spanning segments (S0–S6) at the N-terminus and a large intracellular domain at the C-terminus (Magleby 2003). The C-terminus contains multiple regulatory sites, e.g., regulator of conductance for potassium (RCK) domain (Jiang et al. 2001), 'calcium bowl' region (Schreiber and Salkoff 1997), and multiple phosphorylation sites for cAMP- and cGMP-dependent protein kinases (Zhou et al. 2001). BK_{Ca} channels are activated by membrane depolarization and elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Horrigan and Aldrich 2002). These channels have been implicated in many pathological processes. For example, malfunction of BK_{Ca} channels can lead to hypertension (Brenner et al. 2000, Sausbier et al. 2005), epilepsy (Brenner et al. 2005, Du et al. 2005), cerebellar ataxia (Sausbier et al. 2004), incontinence (Meredith et al. 2004), and ischemic stroke (Gribkoff et al. 2001). They were also involved in diverse physiological processes,

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ISSN 0968-7688 print/ISSN 1464-5203 online © 2011 Informa UK, Ltd.
DOI: 10.3109/09687688.2010.538731

including regulation of blood pressure (Brenner et al. 2000, Sausbier et al. 2005), neurotransmitter release (Raffaelli et al. 2004), micturition (Meredith et al. 2004), epithelial transport (Sausbier et al. 2006), and cerebrovascular circulation (Filosa et al. 2006).

We have previously reported that *Baifuzi* cerebroside (*Baifuzi*-CB) obtained from dried tubers of herb *Typhonium giganteum* Engl. (Chinese name: *Baifuzi*) could potently activate BK_{Ca} channels. And this channel activation might be caused by the direct interaction between STREX domain (a 59-amino acid splice insert of BK_{Ca} channel) and *Baifuzi*-CB (Chi et al. 2010). However, as we showed in the supplementary figure, *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, it is crucial to know whether a single pure cerebroside compound could activate the BK_{Ca} channel as well. In the present study, taking termitomycesphins A (Ter A) and B (Ter B) as pure cerebroside compounds, we examined the effect of Ter A and Ter B on BK_{Ca} channels. Our results indicated that both Ter A and Ter B activated the BK_{Ca} channels at micromole concentration, suggesting that this type of chemicals needs to be studied more extensively to find better BK_{Ca} channel openers.

Materials and methods

Cell culture and transfection

Chinese hamster ovary (CHO-K1) cells were cultured in Ham's F-12 nutrient mixture supplemented with 10% FBS at 37°C in a 5% CO_2 incubator. Cells were plated onto poly-L-lysine coated coverslips in 35 mm dishes at 60–80% confluence. Then they were transiently cotransfected with the full length chick BK_{Ca} channel gene (GenBank accession number AB072618) and GFP (Clontech, Palo Alto, CA, USA), or the STREX-deleted BK_{Ca} channel and GFP. The full length chick BK_{Ca} channel is used throughout unless where indicated that the STREX-deleted form is used. Transfection was performed with LipofectAMINE Plus reagent (Invitrogen) following the manufacturer's instructions. Cells were used for electrophysiological recordings 1–2 days after the transfection.

Electrophysiology

All the single-channel recordings were sampled at inside-out patch-clamp configuration using an EPC-10 amplifier (HEKA Elektronic, Lambrecht, Germany). Data were acquired at 2–5 kHz and low-pass filtered at 1 kHz. Patch electrodes were pulled from thin walled borosilicate capillary tubes using a Sutter programmable puller (model P-97, Sutter Instrument, Novato, CA, USA) and fire-polished to achieve an electrical resistance in a range of 3–7 MΩ. An Ag/AgCl wire bath electrode was used as ground electrode. The pipette solution was Hanks' balanced salt solution (HBSS, Sigma, in mM): 1.3 $CaCl_2$, 0.5 $MgCl_2$, 0.4 $MgSO_4$, 5.4 KCl, 0.4 KH_2PO_4, 136.9 NaCl, 0.3 Na_2HPO_4, 5.6 D-glucose and 4.2 $NaHCO_3$. The bath solution consisted of (in mM): 145 KCl, 10 EGTA, 2 Mg-ATP, 10 HEPES (pH 7.3 with KOH), and $CaCl_2$ adjusted to the desired $[Ca^{2+}]_i$ using a free software CALCON3. The $[Ca^{2+}]_i$ was 0.1 μM, if not stated otherwise. Continuous recordings of at least 7500 ms were used to determine the single channel open probability (Po). The value of Po in a patch with multiple channels was calculated by using TAC 4.1 (HEKA, Germany), based on the equation: $Po = (1 - P_C^{1/N})$, where P_C is the probability that all of the channel is in 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 +60 mV and relatively higher concentration of Ter A when it was consecutively applied. Ter A and Ter B were dissolved in DMSO and stored as a stock solution at −20°C, which was diluted with bath or pipette 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 activity. Unless otherwise stated, all chemicals and reagents were obtained from Sigma. All experiments were done at room temperature (20–25°C).

Extraction and isolation

Termitomyces albuminosus, an edible Chinese mushroom *T. albuminosus* (Berk.) Heim. (Chinese name: *Jizong*), were collected in Yanyuan County (Sichuan Province, China). The dried fruiting bodies of the mushroom were powdered and immersed in EtOH with occasional stirring. The EtOH extract of *T. albuminosus* was washed with hexane, and then partitioned between BuOH and H_2O. The active BuOH fraction was chromatographed on octadecylsilyl (ODS), then on silica gel to give a mixture of cerebrosides. Ter A and Ter B (Figure 1A) were purified from the mixture by reversed-phase HPLC. The detailed process was described in the previous paper (Qi et al. 2000).

Statistics

The results were presented as mean ± SEM. The Origin 7.0 (Originlab, Northampton, MA, USA) was used for statistical analyses and plotting graphs.

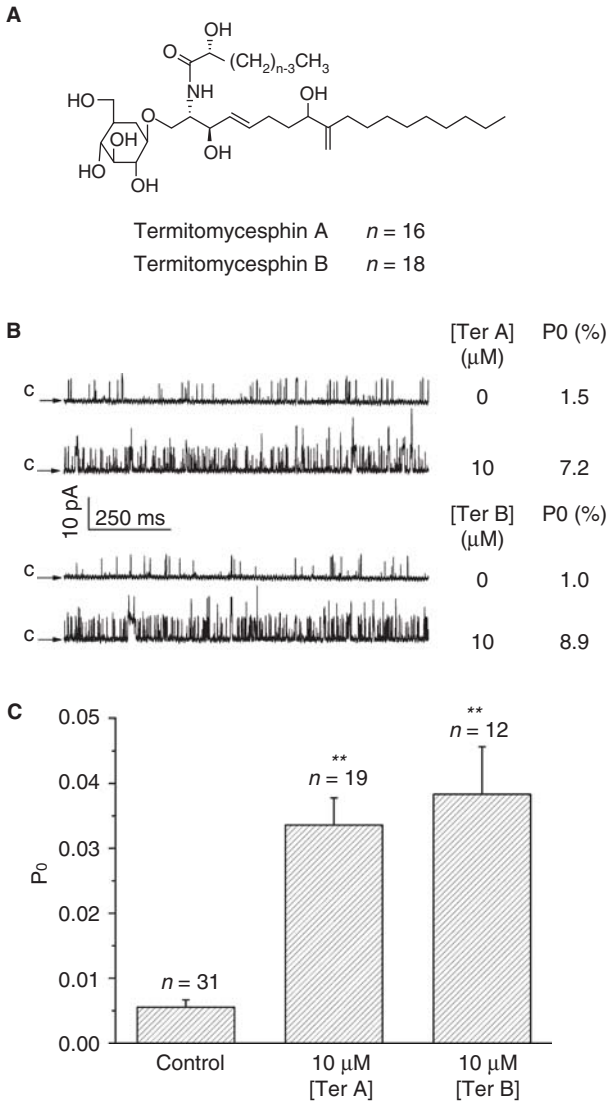

Figure 1. Effect of intracellular Ter A and Ter B on the BK_{Ca} channels. (A) Chemical structures of Ter A and Ter B. (B) Representative single channel recordings in the absence and presence of Ter A or Ter B at +20 mV. Arrows indicate the closed state of the channel. The Po values for each trace are indicated at the right. (C) Statistical data for mean Po of the BK_{Ca} channels in the presence of Ter A (10 μM) or Ter B (10 μM). $^{**}p < 0.005$ compared to the control.

Statistical comparisons were made by using one-way ANOVA, followed by Bonferroni's *post hoc* test. The significance was set at $p < 0.05$.

Results

Effect of intracellular Ter A and B on the BK_{Ca} channels

We first compared the effect of Ter A and Ter B on the activation of the BK_{Ca} channels. Application of 10 μM Ter A or Ter B to the intracellular face of the membrane significantly increased the BK_{Ca} channel activity (Figure 1B). In contrast, there was no significant difference in the single channel current amplitude, indicating that the unitary conductance of the channel was not altered (Figure 2C). Statistical summary in Figure 1C demonstrated that 10 μM Ter A increased Po of the BK_{Ca} channels from 0.6 ± 0.1% (n = 31) of control to 3.4 ± 0.4% (n = 19), while the same concentration of Ter B increased the Po to 3.8 ± 0.7% (n = 12), indicating that there were no significant difference between them. As more amount of Ter A than Ter B in hand, we examined the effect of Ter A on the BK_{Ca} channels in the following studies. The single channel recordings usually got stable after 5–10 min upon formation of inside-out patches. In the control experiment, 0.1% DMSO was subsequently applied, which did not change the Po of the channel (Figure 2A). To judge the effect of Ter A on the channel activation, Ter A was added only after the recordings getting stable within a 3-min period. Usually, the Po of the channel gradually increased upon intracellular application of Ter A. And the increase reached a plateau in less than 5 min (Figure 2B).

Dose-dependent effect of Ter A on the BK_{Ca} channels

To study the dose-dependent response of the channel, different concentrations of Ter A were applied to the cytosolic side of inside-out patch membrane at +20 mV. As illustrated in Figure 3A, the activity of the channel in the same patch was gradually increased with the increase in [Ter A] from 0–10 μM. Figure 3B showed that the mean Po of the channel was 0.4 ± 0.06% (n = 72) before application of Ter A, and it increased to 1.2 ± 0.2% (n = 15, $p < 0.01$), 2.0 ± 0.3% (n = 20, $p < 0.01$), 2.7 ± 0.5% (n = 18, $p < 0.005$), and 3.4 ± 0.4% (n = 19, $p < 0.005$) when Ter A was increased to 1.25 μM, 2.5 μM, 5 μM, and 10 μM at the intracellular side of the patch, respectively. The Po described above were plotted against [Ter A] and fitted to the Hill equation: $Po = P_{max} \star [\text{Ter A}]^N / (EC_{50}{}^N + [\text{Ter A}]^N)$, where P_{max} is the maximum Po of the channel at the experimental condition, EC_{50} is the [Ter A] required to give half-maximal activity of the BK_{Ca} channels, and N is the Hill coefficient. The best fit to the data was obtained with values of 4.3% for P_{max}, 2.8 μM for EC_{50}, and 1.0 for N, suggesting that there was no cooperation for the stimulation of the BK_{Ca} channel by Ter A. This effect was reversible (Figure 3C), the Po of the channel was 0.5 ± 0.06% under control conditions, increased to 3.2 ± 0.3% by 10 μM Ter A, then returned back to 0.9 ± 0.2 % after washout (Figure 3D, n = 5).

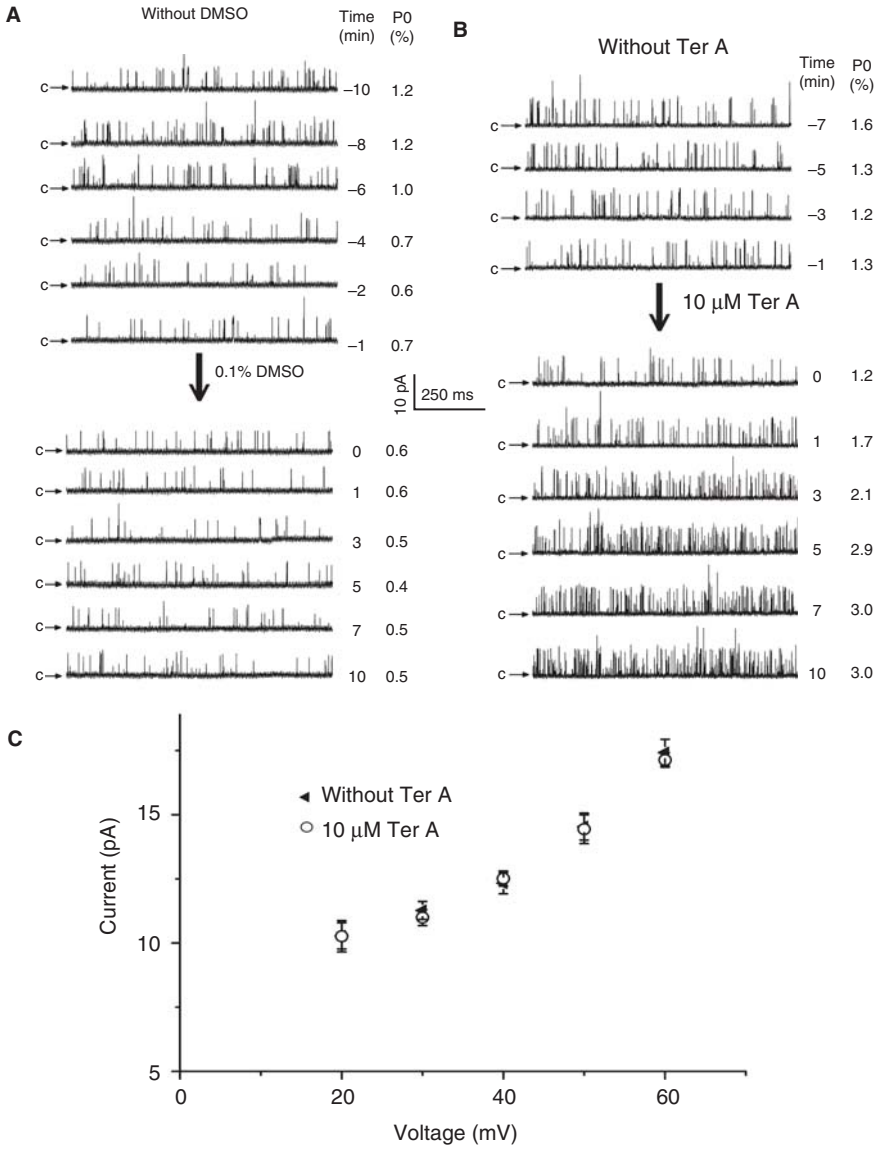

Figure 2. Time course of the BK_{Ca} channel activation by intracellular Ter A. Time course of single channel recordings in control condition (A) or upon intracellularly application of Ter A (B). 0 min denotes the time point of intracellularly application of DMSO or Ter A. (C) Current-voltage relationship for single-channel activities before and after application of Ter A. Each point represents mean ± SEM for at least 4 patches.

Effect of extracellular Ter A on the BK_{Ca} channels

To determine whether the channel activity could be modulated by extracellular Ter A, the tip of the pipette was filled with the pipette solution and then back-filled with the same solution except containing desired [Ter A]. Probably owing to the slow diffusion of Ter A (150 μM) toward the patched membrane, Po of the channel was increased gradually with the increase in time and reached a plateau after about 10 min of the giga-ohm seal formation (Figure 4A). Statistical data in Figure 4B showed that 10, 75 and 150 μM extracellularly applied Ter A increased Po of the channels from 1.7 ± 0.4% (n = 14) of control to 2.2 ± 0.8% (n = 5), 7.2 ± 0.3% (n = 4) and 10.4 ± 2.7% (n = 5), respectively, indicating that extracellular Ter A could activate the BK_{Ca} channel as well. Interestingly, the Po of the channel in the presence of 10 μM Ter A in the cytoplasmic side (3.4 ± 0.4%, n = 19) was significantly higher than that obtained with the same concentration of Ter A applied extracellularly (2.2 ± 0.8%, n = 5). Besides, it usually took approximately 10 min to get stable upon extracellularly application of Ter A, much longer than that of intracellular application of Ter A. This result indicated that Ter A activated the BK_{Ca} channels more efficiently when it was applied intracellularly, implying that the binding site for Ter A might be located at the cytoplasmic side of the channel.

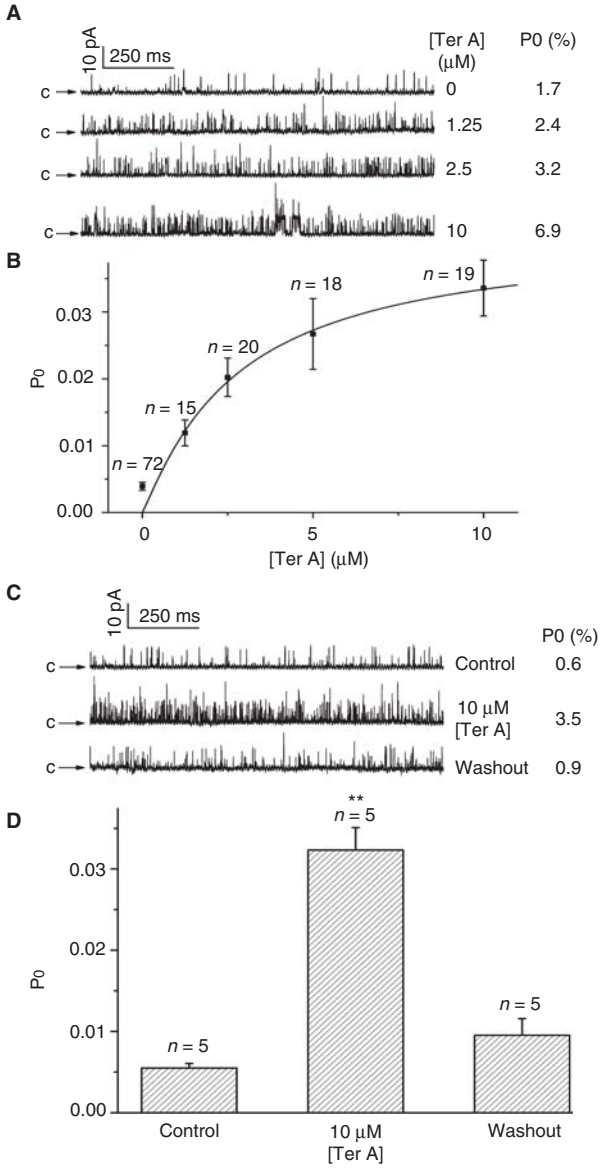

Figure 3. Dose-dependent effects of intracellular Ter A on the BK_{Ca} channels. (A) Representative single BK_{Ca} channel traces at +20 mV in the control and during successive exposure to different concentration of Ter A (1.25–10 μM) in the same patch. (B) Ter A increase the Po of BK_{Ca} channels in a dose-dependent manner. The curve is the best fit to Po against the [Ter A] according to the Hill equation. (C) Current traces before, during application and wash-out of 10 μM Ter A. (D) Statistical summary for the washout experiment. $^{**}p < 0.005$ compared to the control and washout condition.

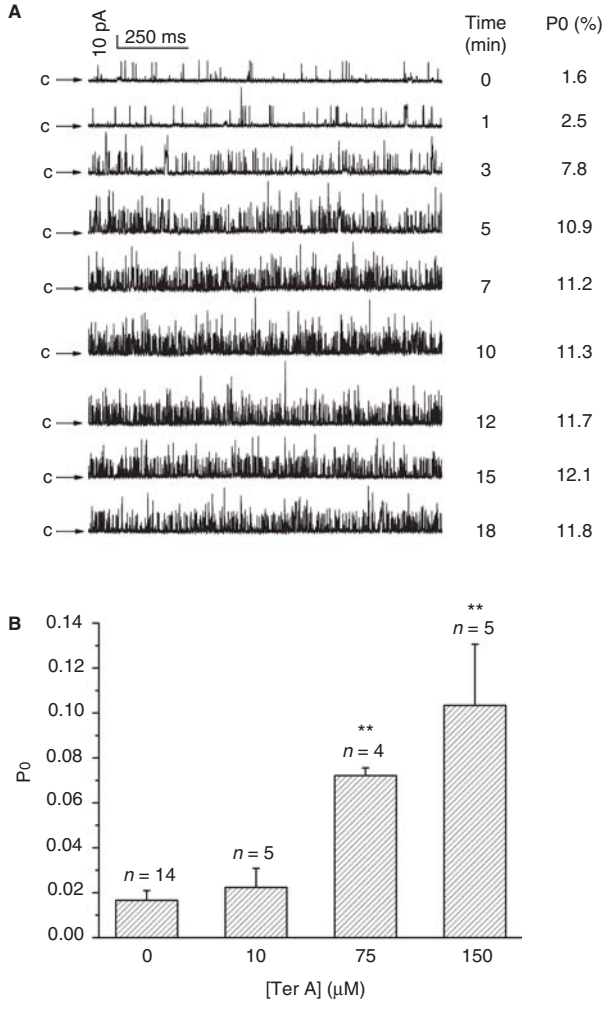

Figure 4. Effect of extracellularly applied Ter A on the BK_{Ca} channels. (A) Effect of 150 μM extracellular Ter A on Po over time at +20 mV. The recordings start (0 min) at the time when the inside-out patch was formed. Arrows indicate the closed state of the channels. (B) Statistical results of extracellular Ter A (10–150 μM) on Po of the channel at 10 min after formation of a giga-ohm seal at +20 mV. Values are mean ± SEM. $^{**}p < 0.005$ compared to the control.

Effect of Ter A on the Ca^{2+}-dependent activation of the BK_{Ca} channels

To understand the effect of Ter A on the Ca^{2+}-dependent activation of the BK_{Ca} channel, we examined the BK_{Ca} channel activity over a range of $[Ca^{2+}]_i$ (1 nM–50 μM) before and after intracellular application of Ter A. Comparing Figure 5A and 5B, it was clear that application of 10 μM Ter A resulted in significant increase in Po of the channel. In Figure 5C, the mean Po of channels was plotted as a function of $[Ca^{2+}]_i$ before and after application of Ter A. In the absence of Ter A, the relationship was well fitted with the Hill equation: $Po = P_{max} * [Ca^{2+}]_i^N/(K^N + [Ca^{2+}]_i^N)$, where P_{max} is the maximum Po, K is the $[Ca^{2+}]_i$ required for the half activation, and N is the Hill coefficient. Values of 8.4% for P_{max}, 11.2 μM for K, and 2.3 for N were obtained from the best fit in the absence of Ter A. However, in the presence of Ter A, an additional parameter P_{TerA} needs to be added to the Hill equation as:

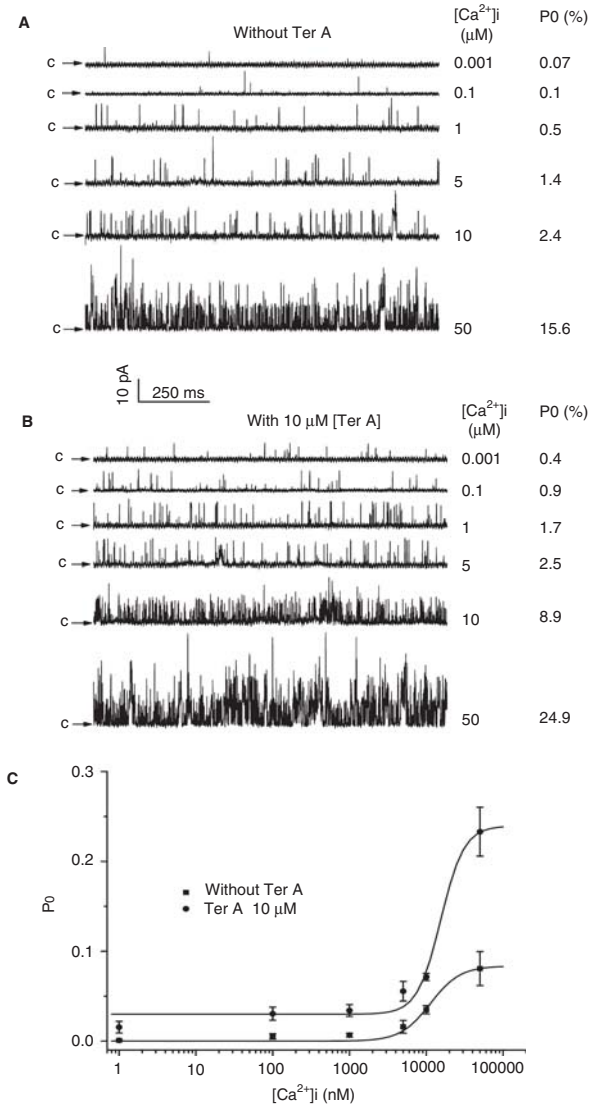

Figure 5. Effect of Ter A on the Ca^{2+}-dependence of BK_{Ca} channel activation. Representative single channel current traces of the BK_{Ca} channels at $[Ca^{2+}]_i$ ranging from 1 nM–50 μM before (A) and after (B) intracellular application of 10 μM Ter A at +20 mV. (C) The relationship between Po and $[Ca^{2+}]_i$ in the absence and presence of 10 μM Ter A are fitted to a modified Hill equation (see text for detail). Each point is the mean ± SEM from at least five experiments.

$Po = P_{max} * [Ca^{2+}]_i^N/(K^N + [Ca^{2+}]_i^N) + P_{TerA}$ to get the best fit. This parameter represents the effect of Ter A on Po of the channel in the condition with no intracellular Ca^{2+}. Values of 20.9% for P_{max}, 15.4 μM for K, 2.8 for N, and 3% for P_{TerA} were obtained by the best fitting to the modified Hill equation in the presence of 10 μM Ter A. This result indicated that Ter A shifted the Ca^{2+}-dependent activation curve of the channel upward, suggesting that Ter A could activate the channels even without intracellular Ca^{2+}.

Effect of Ter A on the voltage-dependent activation of the BK_{Ca} channels

Next, we studied the effect of Ter A on the voltage dependent activation of the BK_{Ca} channel. Figure 6A showed current traces at different membrane potentials without Ter A, indicating that the Po of channels increased with the depolarization of the membrane potential. Application of Ter A significantly increased the Po of the channel at every corresponding membrane potential (Figure 6B). The Po of the BK_{Ca} channels as a function of the membrane potential with or without Ter A (1.25–10 μM) was fitted by the Boltzmann equation: $Po = 1/\{1 + e^{[(V1/2 - V)/k]}\}$, where $V_{1/2}$ is the voltage required for half-maximal activity of the channel and k is the slope factor of the curve. The best fit to the data was obtained with values of 117.4 mV for $V_{1/2}$ and 17.0 mV for k in the absence of Ter A and with values of 88.6 mV for $V_{1/2}$ and 18.4 mV for k in the presence of 10 μM Ter A (Figure 6C). Therefore, Ter A shifted the voltage activation curve toward less positive membrane potentials without affecting the slope factor of the curve, which suggested that Ter A did not alter the voltage-sensitivity of the channel.

STREX domain is required for activation of the BK_{Ca} channel by Ter A

We have previously reported that the STREX domain located in the cytoplasmic side of BK_{Ca} channel is required for activation of the BK_{Ca} channel by a mixture of cerebrosides obtained from *Baifuzi* (Chi et al. 2010). To test whether the STREX domain is also required for the activation of the BK_{Ca} channels by a pure cerebroside compound, we compared the effect of Ter A on the wild-type and STREX-deleted BK_{Ca} channels (Figure 7A, 7B). Ter A (10 μM) significantly increased the Po of the wild-type BK_{Ca} channel from 3.5 ± 0.4% ($n = 7$) of control to 7.1 ± 0.4% ($n = 7$). In contrast, it had no significant effect on the STREX-deleted BK_{Ca} channel (Figure 7C). These data indicated that the STREX domain was necessary for Ter A to activate the BK_{Ca} channels. This is in accordance with the inference that the binding site for Ter A might be located at the cytoplasmic side of the channel.

Discussion

In the present study, we demonstrated that single pure cerebroside compounds, Ter A and Ter B purified from edible mushroom *T. albuminosus*, could activate the BK_{Ca} channel by increasing its Po. Ter A could

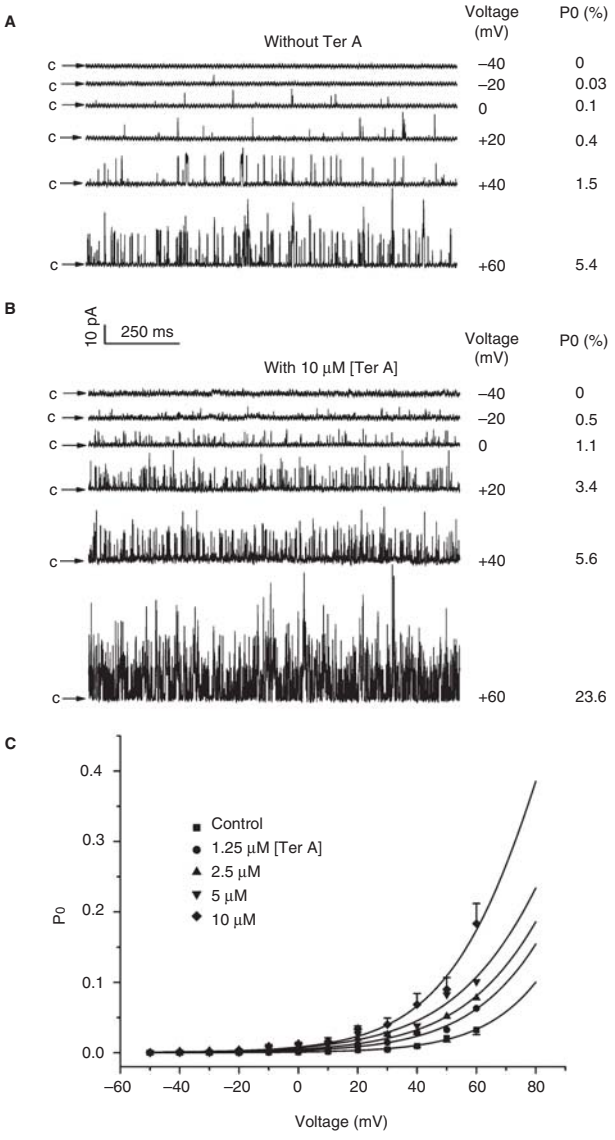

Figure 6. Effect of Ter A on the voltage-dependence of the BK_{Ca} channel activation. Representative single channel current traces of the BK_{Ca} channels at membrane potentials ranging from −50 to +60 mV in the absence (A) and presence (B) of 10 μM Ter A. (C) The relationships between the mean Po of the BK_{Ca} channels and the membrane potentials in the absence and presence of Ter A (1.25–10 μM) are fitted with the Boltzmann equation (see text for detail). Only the error bars for the control and 10 μM Ter A are shown for clarity.

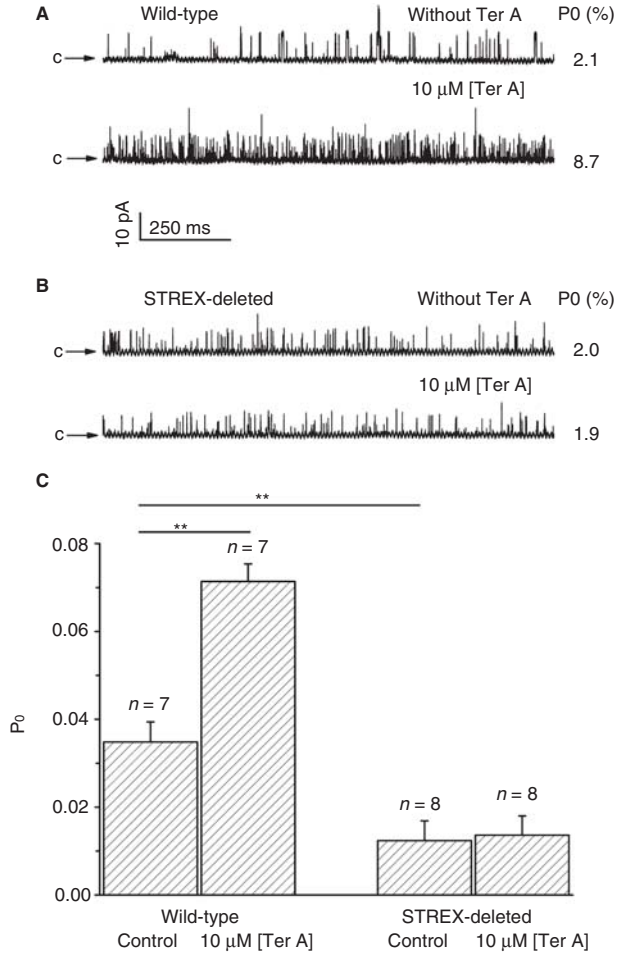

Figure 7. Comparison of the effect of Ter A on the activation of wild-type and STREX-deleted BK_{Ca} channels. Representative single channel current traces of the wild-type (A) and STREX-deleted (B) BK_{Ca} channels in the absence and presence of intracellularly applied Ter A. The membrane potential of the patch is +20 mV and $[Ca^{2+}]_i$ is 10 μM. Arrows indicate the closed state of the channels. (C) Summary of the effect of Ter A on the mean Po of the wild-type and STREX-deleted BK_{Ca} channel. $^{**}p < 0.005$ compared to the control.

activate the channel even without intracellular Ca^{2+} as it shifted the Ca^{2+}-dependent activation curve of the channel upward. Ter A also shifted the voltage-dependent activation curve to the less positive potential. In our previous study, we have shown that a mixture of cerebrosides, isolated from traditional Chinese medicine *Baifuzi*, could potently activate the BK_{Ca} channel (Chi et al. 2010). Taken together, these results might suggest that this type of chemicals might be the BK_{Ca} channel openers. Therefore, individual cerebroside is merit to be explored to find better BK_{Ca} channel openers.

As integral membrane proteins nestled in the lipid environment of the plasma membrane, ion channels are modulated by many lipid-soluble molecules in the membrane (Ordway et al. 1989, Barrantes 2002, Tillman and Cascio 2003, Suh and Hille 2005, Maguy et al. 2006, Beech et al. 2009, Reichow and Gonen 2009). BK_{Ca} channels have also been shown to be activated by many kinds of lipid-soluble molecules, including cerebrosides (Chi and Qi 2006, Chi et al. 2010), fatty acid (Denson et al. 2000, Clarke et al. 2002, Sun et al. 2007), steroid (White et al. 1995, Nishimura et al. 2008), PIP_2 (Vaithianathan et al. 2008), and sphingomyelin

derivate (sphingosine-1-phosphate) (Kim et al. 2006). This study demonstrated that single pure cerebroside compounds Ter A and B could activate the BK_{Ca} channel as well. Then, what is the molecular mechanism of the BK_{Ca} channel activation by termitomycesphins as well as other kinds of cerebrosides? Both our previous and the present study showed that BK_{Ca} channels could be activated by cerebrosides even at the inside-out patch configuration. This result strongly suggests that the intracellular signaling milieu is not required for the channel activation. Furthermore, the STREX domain is necessary as its removal abolished the effect of cerebrosides, suggesting that there is interaction between them. How STREX domain that is located in the cytoplasmic side of the membrane could interact with the cerebroside that is highly likely in the lipid membrane? The following two studies indicate that STREX domain could dock on the plasma membrane: (1) STREX-GFP fusion proteins are preferentially located at the plasma membrane (Naruse et al. 2009); and (2) Cytoplasmic side of the BK_{Ca} channel could interact with the plasma membrane via palmitoylation of its STREX domain (Tian et al. 2008). Our recent study has shown that there is a direct interaction between STREX domain and *Baifuzi*-CB (Chi et al. 2010); therefore, it is reasonable to assume that cerebrosides activate the BK_{Ca} channel through its interaction with the STREX domain of the channel. A further study to identify the binding sites of cerebrosides on the channel is necessary to confirm this hypothesis.

The blood-brain barrier (BBB) functions to hinder the efficient delivery of many potentially important therapeutic agents to the brain. Overcoming the BBB to deliver therapeutic agents to specific regions of the brain presents a major challenge to treat most brain disorders. Without specific transport systems, only small molecules with high lipid solubility actually cross the BBB (Oldendorf 1974, Johansson 1990, Pardridge 2003). Due to their intrinsic property of crossing the BBB, lipid-soluble small molecules have potential as therapeutic drugs to treat brain disorders. It has been shown that activation of the BK_{Ca} channel by its openers could minimize neuronal depolarization, reduced neurotransmitter release and significantly attenuated infarct growth during ischemic stroke in animal models (Gribkoff et al. 2001, Hong et al. 2006; Chi et al. 2010). Moreover, BK_{Ca} channels have been suggested to be a specific target for selectively pharmacological modulation of blood-brain tumour barrier to increase delivery of chemotherapeutic drugs to brain metastases (Ningaraj et al. 2002, Hu et al. 2007). Cerebrosides, such as termitomycesphins, are lipid-soluble small molecules which should cross the BBB effectively. Thus, with its effect on activation of BK_{Ca} channels, cerebrosides have potential to become novel therapeutic agents to treat brain diseases that are associated with BK_{Ca} channels.

Acknowledgements

We thank Dr Sokabe and Dr Naruse for their generous gift of the BK_{Ca} channel and its STREX-deleted form.

***Declaration of interest*:** This work was partly supported by the National Basic Research Program of China (2005CB522804) and the National Science Foundation of China (31070741). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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