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Cerebrosides of baifuzi, a novel potential blocker of calcium-activated chloride channels in rat pulmonary artery smooth muscle cells

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Abstract

Calcium-activated chloride channels (CaCCs) are crucial regulators of vascular tone by promoting a depolarizing influence on the resting membrane potential of vascular smooth muscle cells. However, the lack of a special blocker of CaCCs has limited the investigation of its functions for long time. Here, we report that CB is a novel potential blocker of $I_{Cl(Ca)}$ in rat pulmonary artery smooth muscle cells (PASMC). Cerebrosides (CB) were isolated from *Baifuzi* which is dried root tuber of the herb *Typhonium giganteum* Engl used for treatment of stroke in traditional medicine. Using the voltage-clamp technique, sustained Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) was evoked by a K^+-free pipette solution containing 500 nM Ca^{2+} which exhibited typical outwardly rectifying and voltage-/time-dependence characterization. Data showed that CB played a distinct inhibitory role in modulating the CaCCs. Moreover, we investigated the kinetic effect of CB on $I_{Cl(Ca)}$ and found that it could slow the activation dynamics of the outward current, accelerate the decay of the inward tail current and change the time-dependence characterization. We conclude that CB is a novel potent blocker of CaCCs. The interaction between CB and CaCCs is discussed.

Keywords: Calcium-activated chloride channels; Calcium channels; Pulmonary artery smooth muscle; Cerebrosides; Patch clamp

1. Introduction

Calcium-activated chloride channels (CaCCs) play important roles in cellular physiology, including epithelial secretion of electrolytes and water, sensory transduction, regulation of neuronal and cardiac excitability, and regulation of vascular tone. In vascular smooth muscle cells, a variety of cationic channels (Ca^{2+}, K^+, Na^+) in the plasma membrane have been characterized, and their functional roles in controlling vascular tone have been extensively studied. For example, K^+ channel dysfunction plays an important role in the development of pulmonary hypertension (Yuan et al., 1998). Activity of K^+ channels regulates the membrane potential (E_m) of pulmonary artery smooth muscle cells (PASMC) and in turn elevates $[Ca^{2+}]_i$ by opening voltage-dependent Ca^{2+} channels which are implicated in stimulating vascular SMC proliferation and inducing vasomotor tone (Somlyo and Somlyo, 1994; Platoshyn et al., 2000).

Analogous to K^+ as the predominant intracellular cation, Cl^- is the most abundant intracellular and extracellular anion under physiological conditions. CaCCs have been extensively studied in smooth muscle cells derived from a variety of tissues and appear to be involved in both regulation of myogenic tone and contraction stimulated by agonists (Large and Wang, 1996; Davis and Hill, 1999). Activation of CaCCs in smooth muscle

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can occur by Ca^{2+} entry through voltage-gated calcium channels (VGCCs) or by Ca^{2+} released from intracellular stores by inositol 1,4,5-trisphosphate (IP_3) generated through the phospholipase C (PLC) pathway (Large and Wang, 1996; Davis and Hill, 1999). Because E_{Cl} is positive to the resting potential in smooth muscle, opening CaCCs will produce a depolarization (Chipperfield and Harper, 2000).

Norepinephrine, which contracts smooth muscle by activation of Gq-coupled α-adrenergic receptors, increases membrane Cl^- efflux (Wahlstrom, 1973), which leads to membrane depolarization (Bolton, 1979) by activation of $I_{Cl(Ca)}$ (Byrne and Large, 1985; Byrne and Large, 1988). The depolarization is almost abolished by removing external Cl^- in pregnant guinea pig myometrium, guinea pig mesenteric vein, and the anococcygeus muscle (Van Helden, 1988; Large, 1984). This depolarization could increase the open probability of VGCCs, thereby enhancing Ca^{2+} entry and further increasing muscle contraction. Thus smooth muscle contraction is under the control of the release Ca^{2+} from intracellular stores in response to muscle activators and Ca^{2+} entry through VGCCs activated by the depolarization induced by CaCC activation.

Further support for the role of CaCCs in smooth muscle contraction was gathered using the Cl^- channel blocker NFA (Criddle et al., 1996; Criddle et al., 2002; Greenwood and Large, 1995; Lamb and Barna, 1998; Yuan, 1997). NFA blocks both rabbit portal vein $I_{Cl(Ca)}$ and rat aorta contraction induced by norepinephrine by about 50%. Smooth muscle cells also express calcium-dependent potassium current (I_{KCa}). Thus an increase in $[Ca^{2+}]_i$ can open both CaCC and I_{KCa}, which will induce depolarization and hyperpolarization. Spontaneous depolarizations, which may result from the activation of $I_{Cl(Ca)}$ by Ca^{2+} sparks, have been observed in smooth muscle in the absence of agonists. Although these depolarizations could alter the smooth muscle tone, the precise physiological significance of these depolarizations is unknown (Large and Wang, 1996).

Despite the fact that CaCCs are so broadly expressed in cells and play such important functions, understanding these channels has been limited by the absence of specific blockers and the fact that the molecular identities of CaCCs remains in question. Here, we report the CB is a novel potential blocker of $I_{Cl(Ca)}$ in rat PASMC. Cerebrosides (CB) were isolated from *Baifuzi*, which is dried root tuber of the herb *Typhonium giganteum* Engl which has been used for treatment of cerebral apoplexy for a long time in China (Chen et al., 2002). Cerebrosides are a kind of glycosphingolipid built a long-chain amino-alcohol known as a sphingoid base or long-chain base (LCB), a fatty acid residue and a saccharine head (Tringal, 2001).

It was reported that cerebrosides may act by signal transduction through cell membranes and exhibit significant activities such as inhibitting the entry of HIV-1 in neural cell lines in 1991 by Harouse et al. (1991) and show ionophoric activity for Ca^{2+} ions in 1990 by Kitagawa (Shibuya et al., 1990). However, the activity of CB on ion channels is completely unknown. We tested the effect of CB on CaCCs because CB was believed as the major pharmacological component for treatment of cerebral apoplexy. And activation of CaCCs induces artery contract is considered a very important process for cerebral apoplexy in arterial smooth muscle. We expected to investigate the relationship between cerebrosides and CaCCs and illustrate possible medicinal mechanism why CB could cure cerebral apoplexy. The present study focuses on the kinetic effect of CB on CaCCs' electrophysiological properties such as the activation, deactivation, voltage-/time-dependence of $I_{Cl(Ca)}$ and the potential role of CB in modulating the $I_{Cl(Ca)}$. We conclude that CB is a novel potent blocker of CaCCs and our finding may open the functional gate of CaCCs.

2. Materials and methods

2.1. Preparation of PASMC

Cells were prepared from isolated rat main pulmonary artery. After dissection and removal of connective tissue the artery was rubbed with incurvate scissors softly to remove endothelial cells in D-Hanks' balanced salt solution containing (in mg/ml) 0.4 KCl, 0.06 KH_2PO_4, 8.0 NaCl, 0.06 Na_2HPO_4, 0.35 $NaHCO_3$, pH7.2. The tissue was then cut into small strips and incubated in 2 mL D-Hanks' balanced salt solution containing 2 mg/mL collagenase for 1 h and then added 1 mL 0.15% trypsinase for 5 min at 37 °C to create a single cell suspension. The digestion was stopped by 2 mL DMEM supplemented with 10% fetal bovine serum and the cells were released by gentle agitation with a wide bore Pasteur pipette. Single pulmonary artery smooth muscle cells were resuspended and plated onto a glass coverslip and incubated in a humidified atmosphere of 5% CO_2-95% air at 37 °C in 20% fetal bovine serum culture medium for 2–3 days before use.

2.2. Preparation of cerebrosides

CB was isolated from *T. giganteum* Engl. The detailed process was described in our previous paper (Chen et al., 2002). Because CB cannot be dissolved in water, we packed it with liposome. CB contained the main cerebroside typhoniside A and some analogue of it. Their difference was the length of the fatty acid, such as 16, 18, 22 carbon atoms.

2.3. Electrophysiology

Conventional whole-cell patch-clamp measurements were performed using an EPC-9 patch-clamp amplifier and PULSE software (HEKA, Lambrecht, Germany). In experiments, $I_{Cl(Ca)}$ were evoked by pipette solutions containing 500 nM Ca^{2+} as this concentration of Ca^{2+} generated large and robust Cl^- currents in pulmonary artery smooth muscle cells (Piper and Greenwood, 2003; Greenwood et al., 2001). The pipette solution contained (mM): TEA-Cl 20; CsCl 106; HEPES 5; BAPTA 10; MgATP 3; $GTPNa_2$ 0.2; $MgCl_2$ 0.42, and the pH was set to 7.2 by adding CsOH. Free $[Ca^{2+}]$ was set at 500 nM by the addition of 7.8 mM $CaCl_2$ determined by the EQCAL buffer program. The external solution contained (mM): NaCl 126; HEPES 10; pH 7.4, glucose 11; $CaCl_2$ 1.8; $MgCl_2$ 1.2; TEA-Cl 10 and 4-aminopyridine 5. All reagents were purchased from Sigma unless otherwise stated.

2.4. Experimental protocols: patch-clamp recording from single cell

Patch pipettes pulled from borosilicate glass capillaries had resistances of 2–6 megohms by excising with internal solution. Currents were obtained by excising the patch from cells in the whole-cell configuration and typically digitized at 20 kHz. Macroscopic records were filter at 2 or 2.9 kHz during digitization. During recording, drugs and control/wash solutions were puffed locally onto the cell via a puffer pipette containing eight solution channels. The tip (300 μm diameter) of the puffer pipette was located about 120 μm from the cell. As determined by the conductance tests, the solution around

a cell under study was fully controlled by the application solution with a flow rate of 100 μl/min or greater. All pharmacological experiments met this criterion. All experiments were performed at room temperature (22–25 °C).

2.5. Statistical analysis

Data analysis was carried out using IGOR PRO software (Wavemetrics, Lake Oswego, OR, USA). Averaged results were expressed as mean ± SEM. The data fitted by mono-exponential function started after 5 ms of the pulse to avoid the capacitive current. Comparisons between means were performed using Student's *t* test. The difference between groups were considered significant when $P < 0.05$.

3. Results

3.1. Inhibition of $I_{Cl(Ca)}$ by CB

Dialysis of pulmonary artery smooth muscle cells with a K^+ free pipette solution containing 500 nM Ca^{2+} evoked a stable inward current at the holding potential of −50 mV (I_{hold}) that had a mean steady amplitude of -97 ± 13 pA ($n = 16$, Fig. 1A). The current was considered to be carried by Cl^- that is activated at physiological membrane potentials by intracellular calcium concentrations greater than 100 nM (Piper and Greenwood, 2003; Greenwood et al., 2001). To confirm the recorded current was $I_{Cl(Ca)}$, 100 μM niflumic acid (NFA) was used to inhibit the current ($n = 4$, Fig. 1B). Although NFA is not a perfect special blocker of CaCCs, it is commonly used to identify and isolate the CaCCs. $I_{Cl(Ca)}$ exhibited time-/voltage-dependent properties when depolarized from −50 mV to +70 mV for 500 ms. Stepping to +70 mV produced an instantaneous current that was followed by the time-dependent development of an outward current during the voltage step (I_{tdo}). Upon repolarization to −80 mV, a tail current (I_{tail}) was recorded that declined over the course of the step. After an initial period of stabilization of the amplitude of the current at −50 mV, the voltage-dependent outward currents were reproducible for the duration of the experiment under control condition.

In comparison, I_{hold} at −50 mV evoked by 500 nM $[Ca^{2+}]_i$ was reduced 30.9% from -97 ± 13 pA to -67 ± 6 pA by 100 μM CB ($n = 6$, $P < 0.05$, Fig. 1D). Fig. 1C shows the great inhibition of 100 μM CB on the current developed at +70 mV. The mean time was dependent on the outward current (I_{tdo}) decreasing 36.8% from 182 ± 22 pA to 115 ± 5 pA ($n = 6$, $P < 0.01$, Fig. 1D). The mean amplitude of the inward tail current upon repolarization to −80 mV (I_{tail}) was reduced 14.5% from -338 ± 28 pA to -289 ± 22 pA ($P > 0.05$, Fig. 1D). As control, the liposome without CB was tested and had no effect on $I_{Cl(Ca)}$ (data not shown). $I_{Cl(Ca)}$ was time dependently activated at a positive voltage (+70 mV), and deactivated quickly at negative potentials (−80 mV). In order to investigate the effect of CB on the kinetics of $I_{Cl(Ca)}$ evoked by +70 mV

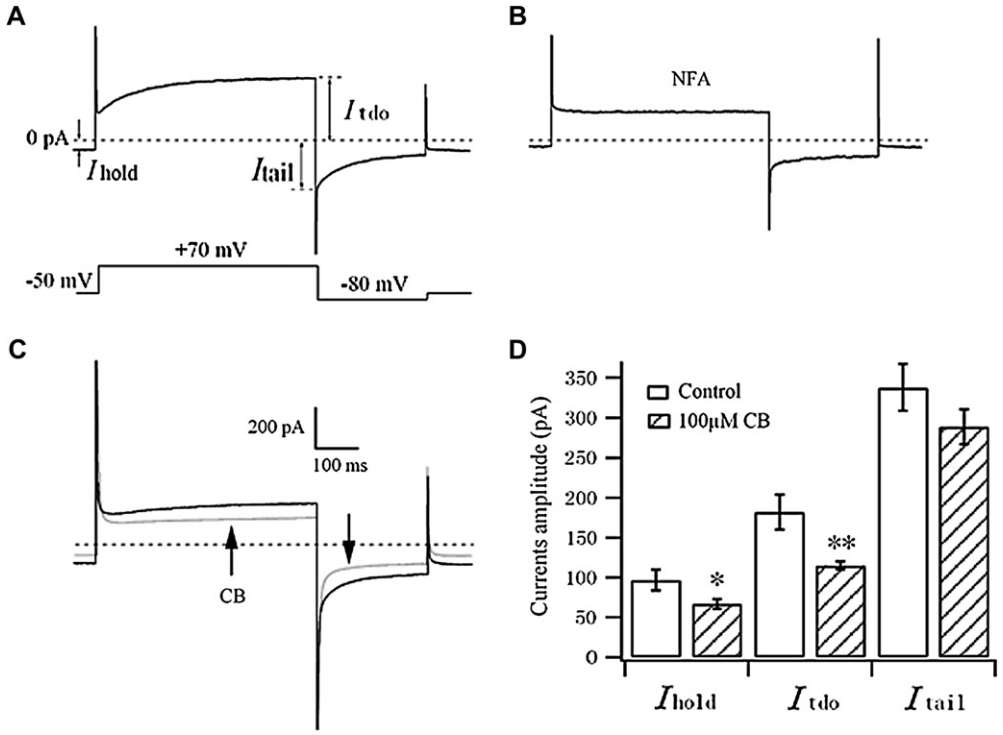

Fig. 1. Inhibition of CB on $I_{Cl(Ca)}$ evoked by 500 nM $[Ca^{2+}]_i$. (A) Representative whole-cell current recorded from a rat pulmonary artery smooth muscle cell. The stimulated voltage protocol was shown below. Cells were held at a holding potential of −50 mV by depolarizing initially to +70 mV for 500 ms. Following the depolarizing pulse, the cell was stepped to −80 mV for 250 ms to study the tail current. The arrows label the current measured methods which were discussed in the text: inward current at the holding potential of −50 mV (I_{hold}), time-dependent outward current (I_{tdo}) and tail current at −80 mV (I_{tail}). Dotted line represents zero current. (B) Application of NFA (100 μM), $I_{Cl(Ca)}$ was significantly inhibited. (C) Mean current curve were shown in the absence (black line, $n = 6$) and presence of 100 μM CB (grey line, $n = 6$). The plot of amplitude of I_{hold}, I_{tdo} and I_{tail} were shown in plane D.

depolarization, we compared the activation and inactivation time constants before and after applying CB by fitting the currents with mono-exponential function. The activation time constants (τ_{act}) of $I_{Cl(Ca)}$ were 156 ± 18 ms and 536 ± 37 ms at +70 mV and the time constants of deactivation (τ_{ina}) were 69 ± 8 ms and 40 ± 4 ms at −80 mV in the absence and presence of 100 μM CB, respectively ($P < 0.01$, Fig. 1C). Data suggested that CB was an effective blocker of $I_{Cl(Ca)}$. In comparison to NFA, which effectively abolished voltage-dependent transitions concomitant with a drastic slowing of the current decay, CB slowed down activation remarkably at positive voltage and accelerated inactivation at negative voltage.

3.2. Effect of CB on activation kinetics of CaCCs

To investigate CB on the activation kinetics of CaCCs, we measured the effect of CB on currents amplitude and activation time constants of $I_{Cl(Ca)}$ at different voltages. Step currents were evoked by depolarizing voltage steps from −80 mV to +80 mV in 10-mV increments from −50 mV (Fig. 2A). The current−voltage relationship of $I_{Cl(Ca)}$ is shown (control, $n = 11$, Fig. 2B), which exhibited a typical outwardly rectifying characterization. After application of 100 μM CB, the outwardly rectifying currents were inhibited remarkably (100 μM CB, $n = 6$). As described in Fig. 1, CB significantly changed the activation time constants at different potentials ($P < 0.01$, Fig. 2C). Data suggests that CB not only reduces $I_{Cl(Ca)}$ currents amplitude but also changes its activation kinetics.

3.3. Effect of CB on the tail currents of $I_{Cl(Ca)}$

$I_{Cl(Ca)}$ has been widely discussed as a tail current in different tissues. I_{tail} is evoked by depolarizing initially from −50 mV to +70 mV for 500 ms and then stepped to testing potentials from +40 mV to −100 mV in 10-mV increments for 500 ms (Fig. 3A). The current−voltage relationship was shown in Fig. 3B in the absence (■, $n = 16$) and presence of 100 μM CB (○, $n = 6$). E_{rev} of I_{tail} was 3.7 ± 3 mV ($n = 6$, Fig. 3A,B), which is close to the theoretical Cl^- equilibrium potential 0.5 mV calculated by Nernst equation. These data confirmed that I_{tail} carried by Cl^-. In order to know the effect of CB on the I_{tail}, we applied 100 μM CB on rat PASMC and found CB inhibited the tail currents and did not distinctly change the reversal potential of $I_{Cl(Ca)}$ (Fig. 3B). The deactivation time constants of I_{tail} increased gradually from −100 mV to −20 mV and decreased remarkably after applying 100 μM CB ($n = 6$, $P < 0.01$, Fig. 3C). The data suggests that CB not only decreases the amplitude of I_{tail} but also accelerates the deactivation of $I_{Cl(Ca)}$.

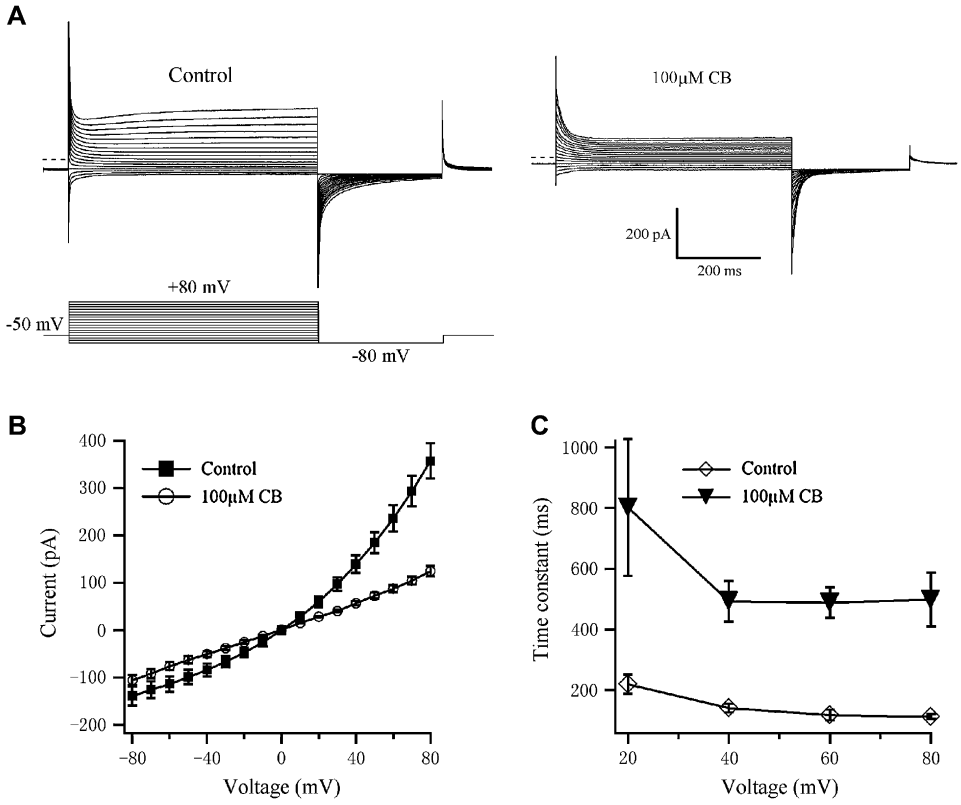

Fig. 2. Effect of CB on I–V relationship and activation time constants of $I_{Cl(Ca)}$. (A) A family of $I_{Cl(Ca)}$ currents evoked by 500 nM $[Ca^{2+}]_i$ at different voltages. Cells were held at −50 mV and stepped to potentials between −80 mV and +80 mV in 10-mV increments for 500 ms shown below. (B) The current−voltage relationship were shown in the absence (■, $n = 11$) and presence of 100 μM CB(○, $n = 6$). (C) The obvious increasing of 100 μM CB of the activation time constants of $I_{Cl(Ca)}$ at +20 mV to +80 mV, respectively (control ▼, $n = 11$; CB ◇, $n = 6$).

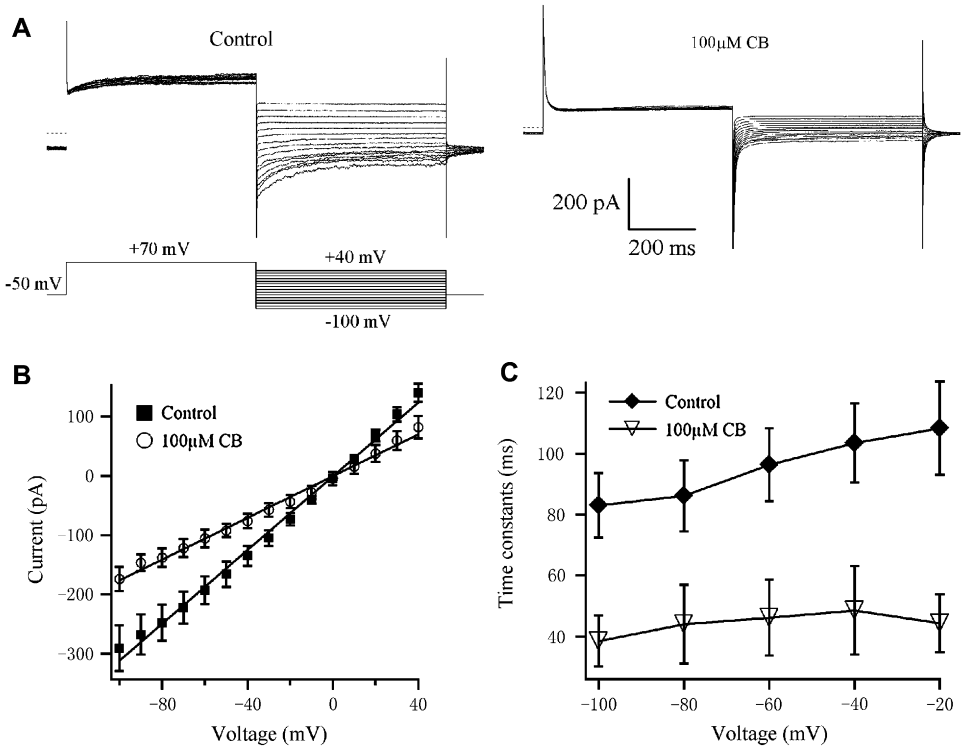

Fig. 3. Effect of CB on $I_{Cl(Ca)}$ deactivation characterizations. (A) Representative currents were shown in the absence and presence of 100 μM CB. The stimulated voltage protocol was shown below. Cells were holding at −50 mV and stepped to +70 mV for 500 ms followed by a 500 ms test step to different potentials between −100 mV and +40 mV in 10-mV increments. (B) The current–voltage relationship of the currents recorded after stepping to the test potentials in the absence (■, $n = 16$) and presence of 100 μM CB(○, $n = 6$). (C) 100 μM CB reduced the deactivation time constants of $I_{Cl(Ca)}$ in different negative testing potentials. I_{tail} was fitted by mono-exponential function (control ◆, $n = 16$; CB ▽, $n = 6$).

3.4. Effect of CB on the time dependence of $I_{Cl(Ca)}$

Time-dependence activation is a classic property of CaCCs at positive voltage by appropriate Ca^{2+} concentration. In our work, extending the depolarization duration of +70 mV from 200 ms to 1400 ms in increments of 100 ms increased I_{tdo} and I_{tail} ($n = 11$, Fig. 4A). With longer periods of prior activation, the time constants for I_{tail} inactivation were also slightly increased ($n = 11$, Fig. 4B). However, increasing the step duration had no effect on the activation time constant of I_{tdo}. Its activation kinetics followed the same mono-exponential time course as shown in Fig. 4A. The I_{tail} currents amplitude was slightly increased but not significantly when $[Ca^{2+}]_i$ was settled at 500 nM $[Ca^{2+}]_i$, which could activated the CaCCs thoroughly and stably (Piper and Greenwood, 2003; Greenwood et al., 2001). After application of 100 μM CB, the amplitude of I_{tdo} ($n = 6$, $P < 0.05$, Fig. 4C) and I_{tail} ($n = 6$, $P > 0.05$, Fig. 4C) were both inhibited to different degrees.

4. Discussion

Many cell types express a type of Cl^- channel that is activated by cytosolic Ca^{2+} concentration in the range of 0.2–5 μM. Ca^{2+}-activated Cl^- channels has been studied less than other chloride channels, such as volume-regulated chloride channel, or cystic fibrosis trans-membrane conductance regulator (CFTR) chloride channels. Nevertheless, CaCCs, in diverse cell types, are clearly an important channel type involved in various physiological functions (cell secretion, anion transport, cell adhesion, etc.) (Fuller et al., 2001; Nilius and Droogmans, 2003). In Xenopus oocytes, where these channels were first described in the early 1980s (Miledi, 1982), increased in $[Ca^{2+}]_i$ that occur upon fertilization cause CaCCs to open. This produces a depolarization of the membrane that somehow prevents the fusion of additional sperm.

Similar channels have subsequently been found in many different cell types including neurons; various epithelial cells; olfactory and photo-receptors; cardiac, smooth, and skeletal muscle; mast cells, etc. CaCCs exist in cells isolated from a variety of types of vessels smooth muscle cells (Greenwood and Large, 1996; Pacaud et al., 1989; Piper and Greenwood, 2003; Greenwood et al., 2001; Toland et al., 2000). In smooth muscle cells, the speculated roles of CaCCs are regulation of membrane potential and modulation of agonist-induced store-depletion dependent intracellular calcium signaling, including regulation of calcium influx. The calcium influx sequentially regulates the contraction of vascular smooth muscle cells and plays an essential role in controlling vascular tone and other disease such as pulmonary hypertension (Nilius and Droogmans, 2003).

In PASMC, the resting E_m, ranging from −35 mV to −55 mV (Toland et al., 2000; Yuan, 1995), is more positive than E_k but more negative than E_{Cl}. Accordingly, under these resting conditions, alteration of sarcolemmal Cl^- channel

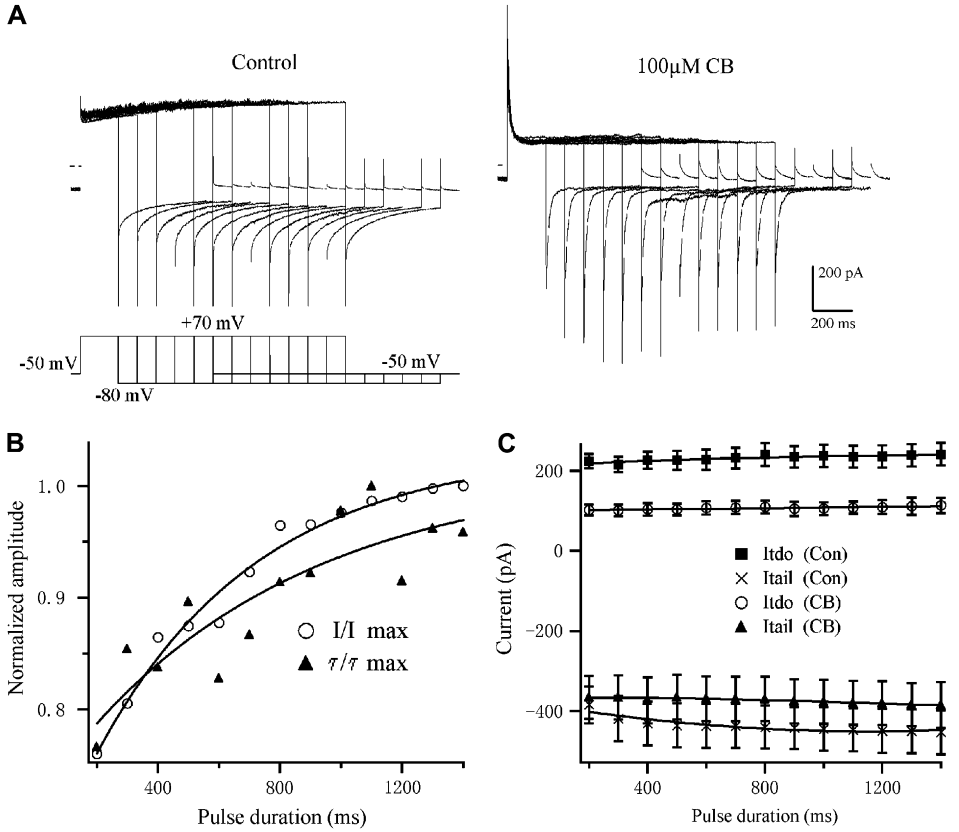

Fig. 4. Effect of CB on time dependence of $I_{Cl(Ca)}$. (A) Currents were recorded from depolarizing the pulmonary artery smooth muscle cells from a holding potential of −50 mV to +70 mV for durations ranging from 200 ms to 1400 ms in increments of 100 ms before and after application of 100 μM CB. (B) Plot of normalized amplitude (I/I_{max}, ○, $n = 11$) and deactivation time constants (τ/τ_{max}, ○, $n = 11$) of I_{tail}. These dots were fitted by mono-exponential function. (C) Effect of 100 μM CB on currents amplitude of I_{tdo} and I_{tail} (control ■ ×, $n = 11$; CB ○ △, $n = 6$).

activity would substantially contribute to the regulation of E_m, which dominates the activity of voltage-gated Ca^{2+} channels (Yuan, 1995). Activation of Cl^- channels, by facilitating Cl^- efflux, would thus result in membrane depolarization and activation of VGCCs, increasing $[Ca^{2+}]_i$ and causing pulmonary vasoconstriction. The Ca^{2+} concentration threshold for activation of $I_{Cl(Ca)}$ in portal vein smooth muscle cells is 180 nM, with full activation at 600 nM. In PASMC, the resting $[Ca^{2+}]_i$ is 50−100 nM, whereas agonist-induced increases in $[Ca^{2+}]_i$ usually range from 200 nM to 1000 nM.

Many vasoactive agents mediate vascular contraction in association with an initial transient increase in $[Ca^{2+}]_i$ followed by a sustained $[Ca^{2+}]_i$ plateau. The Ca^{2+} transient is often due to Ca^{2+} release from intracellular stores and serves to trigger contraction (Hartzell et al., 2005). Although the precise mechanism is not completely known, Ca^{2+}-induced activation of CaCCs like 5-TH and PE, in addition to increasing $[Ca^{2+}]_i$, also causes membrane depolarization and sustained vasoconstriction. To date, at least 10 isoforms of CaCCs have been identified from bovine, human, mouse and porcine (Pauli et al., 2000). Although CaCCs have been studied for more than 20 years, their physiological roles and mechanisms of regulation have remained somewhat cloudy. Despite the fact that CaCCs are so broadly expressed in cells and play such important functions, understanding these channels has been limited by the absence of specific blockers and the fact that the molecular identities of CaCCs remain in question.

CaCCs have not been well studied because of poor pharmacological tools. Even niflumic acid, which is commonly used to identify and isolate CaCCs, cannot be considered a CaCCs-specific probe since it also blocks VRAC in some cell types albeit at higher concentration. This lack of specific tools seriously handicaps CaCCs research not only by impeding the molecular identification and functional characterization of CaCCs but also by reducing the possibilities for *in vivo* interventions. In this study, we found that CB may be a potential blocker of CaCCs. Cerebrosides (CB) were isolated from *Baifuzi* which is dried root tuber of the herb *T. giganteum* Engl which has been used for treatment of cerebral apoplexy for a long time in China (Chen et al., 2002).

Cerebrosides are a kind of glycosphingolipids built from a long-chain aminoalcohol known as a sphingoid base or long-chain base (LCB), a fatty acid residue and a saccharine head (Tringal, 2001). They are important membrane lipids found in virtually all vertebrate cells. While some cerebrosides have been characterized, their significant ion channels activities have not been reported. From the experimental data, CB not only blocked the outward time-dependent currents but also inhibited the tail currents, both of which are considered

to be calcium-activated chloride currents. This may be a novel light to study the special pharmacological tools of CaCCs because it is absolutely different in structural characters and possible block mechanisms from the common blocks such as NFA.

CB is a kind of cerebrosides which are the important composes of cell membrane while NFA is aromatic acids. In the similar conditions in rabbit PASMC, Piper AS, etc., showed 100 μM NFA inhibited outward current at positive potentials but increased inward current at negative potentials by binding to an extracellular site (Piper et al., 2002). Because CB is cerebrosides and was packing by liposome, CB may be in both sides of the cell membrane. So we propose that CB affected the CaCC protein from both intracellular sites and extracellular sites. It may be a real specific pharmacological tool of CaCCs to help us study CaCCs comprehensively. But also another possible pathway is that membrane lipids leading to different bilayer thickness can indeed change ion channel behaviors i.e. ion selectivity.

The electrophysiological characterizations of CaCCs are similar in the various cell types, like Ca^{2+} activated, voltage-/time-dependence, outwardly rectifying at constant $[Ca^{2+}]_i$ (Fuller and Benos, 2000; Nilius and Droogmans, 2003; Toland et al., 2000). At subsaturating $[Ca^{2+}]_i$, $I_{Cl(Ca)}$ showed slow activation (>100 ms) at positive potentials and fast deactivation at negative potentials (Piper et al., 2002). In our work, CaCCs had same activation kinetics evoked by 500 nM $[Ca^{2+}]_i$ which showed in Fig. 2. Importantly, we found that CB blocked $I_{Cl(Ca)}$ in a manner of voltage-dependence both in current amplitude and activation/deactivation kinetics. The block was remarkable at positive potential but slight at negative potential (Figs. 2C and 3B). Moreover, the activation time constants increased outstandingly but without changing the decreasing trend with testing potentials after applying CB (Fig. 2C). The results suggested that CB likely acted in the channel pore to block $I_{Cl(Ca)}$ but did not interact CaCC proteins directly.

$I_{Cl(Ca)}$ has an inward "tail" current (I_{tail}) on stepping back to the holding potential which generate the depolarizing afterpotential that is observed following the action potentials occurring in some smooth muscle (Greenwood and Large, 1996; Pacaud et al., 1989). The duration of I_{tail} is usually greater than 100 ms, but an interesting observation is that the decay to I_{tail} appears to vary greatly depending on the different tissues used. I_{tail} decayed exponentially at negative potentials in rat portal vein and rabbit coronary artery, and it was postulated that the decline of I_{tail} might represent channel kinetics (Greenwood and Large, 1996; Pacaud et al., 1989). The tail current amplitudes at different potentials were inhibited slightly (Fig. 3B), whereas the deactivation time constants of CaCCs were reduced markedly by CB (Fig. 3C). CaCCs have little time-dependence activation in our research which evoked by a fixed 500 nM $[Ca^{2+}]_i$. The plot of normalized amplitude and time constants of I_{tail} deactivation as function of pulse duration were shown in Fig. 4B. Compared with Yuan's studies, their results were different that increasing the duration of a +10-mV test pulse significantly augmented the amplitude of I_{tail} (Yuan, 1997). The difference was considered from that the currents were evoked via a gradual accumulation of Ca^{2+} through Ca^{2+} influx through membrane Ca^{2+} channels and/or Ca^{2+}-induced Ca^{2+} release from intracellular Ca^{2+} stores (Yuan, 1997).

CB has an obvious effect on $I_{Cl(Ca)}$ of PASMC evoked by 500 nM $[Ca^{2+}]_i$. If the current results observed in single cells also occur in intact preparations, it is possible that there may be significant physiological responses in cells where $[Ca^{2+}]_i$ is elevated by extracellular activation factors. We provide a hypothesis that attempt to uncover the therapeutic mechanism of *T. giganteum* Engl on cerebral apoplexy. We propose that blocking CaCCs by CB could reduce the depolarization of cell membrane and inhibit the influx of extracellular Ca^{2+}. It tends to slacken the tensional vasomotor state of smooth muscle cells and decrease the vascular hypertension some diseases such as pulmonary hypertension and cerebral apoplexy.

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