Tetrandrine inhibits Ca^{2+}-activated chloride channel in cultured human umbilical vein endothelial cells

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ABSTRACT

AIM: To characterize the electrophysiological and kinetic properties of Ca^{2+}-activated chloride channel (CaCC) in cultured human umbilical vein endothelial cell line (HUVEC), and test the inhibitory effects of tetrandrine (Tet) on CaCC. **METHODS:** Ca^{2+}-activated Cl^- currents ($I_{Cl,Ca}$) were recorded by patch-clamp whole cell configurations. $[Ca^{2+}]_i$ was measured via intracellular Fura-2 fluorescence intensities. **RESULTS:** $I_{Cl,Ca}$ was activated by increasing $[Ca^{2+}]_i$ via direct elevation of intracellular calcium. $I_{Cl,Ca}$ showed an apparent outward rectification properties, and it was activated in a voltage- and calcium-dependent mode. Tet dose-dependently inhibited $I_{Cl,Ca}$, the IC_{50} was (5.2±0.4) μmol/L (n=8 cells). Tet suppressed both voltage-dependent and calcium-dependent activation of $I_{Cl,Ca}$. The activation time constant was (326±12) ms [in the presence of 10 μmol/L Tet, compared to control (175±17) ms, at +100 mV], and Ca^{2+} concentration for half maximal activation was (387±61) nmol/L for Tet (compared to control (287±36) nmol/L. **CONCLUSIONS:** Tet effectively blocked $I_{Cl,Ca}$, and such effects might be due to its inhibitory effects on the activation process of Ca^{2+}-activated chloride channel.

INTRODUCTION

Tetrandrine (Tet, 6,6',7,12-tetramethoxy-2,2'- dimethyl-berbaman) is a purified *bis*-benzylisoquinoline alkaloid derived from the root of a Chinese herb *(Stephania tetrandra* S Moore)[1,2]. It was first shown as an anti-hypertensive agent in both normal and hypertensive subjects in 1950s[3,4]. The primary anti-hypertensive action of Tet is presumably due to its vasodilatory property, which was confirmed both *in vivo* (15 mg/kg in conscious rats) and *in vitro* (1-100 μmol/L, effectivly decreased noradrenaline and high K^+-evoked contraction of isolated aortic rings)[5-8]. It was widely accepted that such vasodilatory effects principally relied on its direct Ca^{2+} antagonism (blocking voltage-dependent Ca^{2+} channels, mainly L-type Ca^{2+} channel, at 1-300 μmol/L with IC_{50} of 14.8 μmol/L) and partially on its interaction with α-adrenoceptor in vascular smooth muscle cells[6,9,10]. Recent studies showed that Tet also inhibited calcium-depletion-induced calcium entry in human and bovine endothelial cells, which might be through its blockade of a Ca^{2+} release-activated Ca^{2+} channels in such cells[11,12]. In addition to the effects on Ca^{2+} release-activated Ca^{2+} channels, Wu *et al* reported that Tet (0.5-50 μmol/L) effectively suppressed large conductance Ca^{2+}-activated K^+ current in human endothelial cell line, and another group found that Tet de-

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pressed inwardly rectifying K^+ current in cultured aortic endothelial cells[13,14]. But Kwan *et al* showed that tetrandrine (30 μmol/L) did not affect either outward K^+ current or inwardly rectifying K^+ current in cultured bovine pulmonary artery endothelial cells, but Tet abolished thapsigargin-stimulated increase in outward K^+ current[11].

Endothelium plays an important role in modulating vascular smooth muscle tone by releasing many vasoactive factors, nitric oxide and PGI_2, which are controlled by intracellular Ca^{2+} signals[15]. Since voltage-dependent Ca^{2+} channel is absent in endothelial cells, several types of Ca^{2+} entry channels are major influx route of Ca^{2+} (Ca^{2+} release-activated Ca^{2+} channels, nonselective cation channels etc). However after activation of these entry channels, Ca^{2+} influx depends on the driving force- electrochemical gradient for Ca^{2+}. Endothelial K^+ channels (Ca^{2+}-activated K^+ channels and inwardly rectifying K^+ channel) and Cl^- channels (volume-regulated chloride channels and Ca^{2+}-activated chloride channel) are two major channel types in regulating the driving force for Ca^{2+} entry[16]. In contrast to the fast growing knowledge of Ca^{2+}-activated K^+ channels, the endothelial Ca^{2+}-activated Cl^- channel (CaCC) is not very well studied and only a few papers reported its electrophysiological and pharmacological properties. Besides its role as a modulator in Ca^{2+} signaling, CaCC might also be important for regulating secretion, intracellular pH, and cell proliferation[16-19].

Since both K^+ and Cl^- channels are involved in maintaining normal function of endothelial cells, it is worthwhile to learn the effects of Tet on endothelial Cl^- channels besides its known action on various endothelial K^+ channels. The results might be an important supplement to a clear understanding toward the mechanism of Tet. In our previous report, two types of chloride channels (volume-regulated and Ca^{2+}-activated chloride channels) have been identified in cultured human umbilical vein endothelial cells[20]. In this study, we focused on CaCC. We conducted whole-cell current recording, and in some experiments combined with fluorescence measurement of $[Ca^{2+}]_i$ in order to further characterize the kinetics and electrophysiological properties of CaCC. And the inhibitory effect of Tet on CaCC was tested.

MATERIALS AND METHODS

Cells Cells were recovered and cultured from a human umbilical vein endothelial cells line (cell line HUVEC, VEC304, obtained from the Cell Bank of Chinese Academy of Sciences, Shanghai). The cells were grown in Medium 199 containing 10 % calf serum, 0.03 % *L*-glutamine, benzylpenicillin 2 g/L and streptomycin 2 g/L. Cultures were maintained at 37 ºC in a fully humidified atmosphere of 5 % CO_2 in room air. The culture medium was exchanged every 48 h. Cells were detached by exposure to 0.1 % trypsin in Ca^{2+}- and Mg^{2+}-free solution and plated on gelatin-coated coverslips, and kept in culture for 2-4 d before use. For electrophysiological measurements, only nonconfluent, single endothelial cells were used.

Electrophysiology Whole cell membrane currents were monitored with a patch clamp amplifier (CEZ2300, Nihon Kohden) and recorded in an IBM/PC via a D/A converter (Axon LM-1). Voltage clamping, signal acquisition, and analysis of membrane currents were achieved by computer program pCLAMP 5.55. All experiments were carried out at room temperature (22-25 ºC).

Two voltage protocols were applied to study the Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$). Ramp current recording was designed to determine current-voltage (*I-V*) relationship. Holding potential was 0 mV, a step to -80 mV for 0.6 s was followed by a step to -150 mV for 0.2 s and a 2.6 s linear voltage ramp to +100 mV, thereafter a step to 0 mV. Another voltage steps were applied from a holding potential of –50 mV to a test potential of –100 to +100 mV in 20-mV increments. The duration of the step is 2 s. Junction potential between electrode and bath solution was corrected before experiment. In order to compare data obtained from different cells, the current amplitudes were normalized as per unit of membrane capacitance. Cell capacitance for the whole experiment is (29±9) pF (n=23 cells).

$[Ca^{2+}]_i$ measurement The cells were incubated in the medium containing Fura-2 AM (2 μmol/L) for 30 min at 37 ºC. Then the cells were washed by modified Krebs' solution for another 30 min, allowing the optimal hydrolysis of the AM ester. The dye was excited at wavelength of 350 and 380 nm via a rotating filter wheel. The fluorescence was measured at 510 nm and corrected for autofluorescence. The fluorescent signals were detected by an R928 photo-multiplier tube (PMT; Hamamatsu, Shizuoka, Japan). Calibration and calculation of the free Ca^{2+} concentration from the fluorescence ratio were achieved according to detailed methods described elsewhere[21]. Briefly, $[Ca^{2+}]_i$ was determined from the ratio (R) of the fluorescence signals at

both wavelengths, according to: $[Ca^{2+}]_i=K_{dApp}$ $[(R-R_{min})/(R_{max}-R)]$, where K_{dApp} is the apparent dissociation constant, R_{min} is the minimum ratio in zero Ca^{2+}, and R_{max} is the maximum ratio at saturated Ca^{2+}. These calibration constants were obtained from in-cuvette calibrations. Calibrations used values of R_{max}/R_{min}=22.9 and K_d=523 nmol/L

Solutions and drugs At the beginning of each experiments, cells were perfused with modified Krebs' solution, containing (mmol/L): NaCl 150, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH 1 mmol/L. Then through the whole experiment, the cells were superfused with a Cs-Krebs' solution in which Cs^+ substituted K^+ in the Krebs' solution to avoid the influence of any K^+ current.

The pipette solution contained (mmol/L): CsCl 40, Cs-aspartate 100, $MgCl_2$ 1, EGTA 5, $CaCl_2$ 1.93, Na_2ATP 4, HEPES 10, adjusted pH to7.2 by CsOH 1 mmol/L.

To activate the Cl^- current directly by Ca^{2+}, the free Ca^{2+} concentration was adjusted to 500 nmol/L in pipette solutions by modifying concentration of $CaCl_2$ and EGTA in pipette solution (EGTA 5, $CaCl_2$ 3.79, and $MgCl_2$ 1 mmol/L, pH=7.2). To test the Ca^{2+}-dependent activation, free Ca^{2+} was adjusted to 100, 200, 500, and 1000 nmol/L by adding the appropriate $CaCl_2$ (1.94, 2.80, 3.79, 4.32 mmol/L), respectively and calculated by the program CHELATOR, provided by University of California at Berkeley, USA.

Tet (purity >98 %) obtained from Jin-Hua Pharmaceutical Co, Zhejiang, China. 4,4'-Diisocyanato-stilbene 2,2'-disulphonic acid (DIDS), verapamil were purchased from Sigma Co (USA).

Data analysis Inhibitory effects of Tet were defined as percentage of inhibition.

$$\text{Inhibition}=[(I_{max}-I_{blocker})/(I_{max}-I_{rest})]\times 100\ \%$$

where I_{max} and $I_{blocker}$ were the currents at +100 mV testing potential before and during application of Tet respectively. I_{rest} was the membrane current without any stimulation just after the cell membrane was broken. Data were expressed as mean±SD and compared with the *t*-test.

All data and curve fit were achieved via software SigmaPlot2000 (SPSS Inc, USA) or Origin 7.02 (OriginLab Co, USA). Concentration-response curve of the inhibitory action of Tet is fitted by the modified Hill-equation: $Y=(a-d)/[1+(X/c)^b]+d$ using Marquardt-Levenberg methods of nonlinear regression analysis, where *a* and *d* are the effect of concentration at maximum and minimum. *c* refers IC_{50}. *b* is the Hill coefficient[22].

The Ca^{2+}-dependent activation kinetics of $I_{Cl,Ca}$, were fitted by a single exponential function according to the following equation: $I(t)=A_0+A_1exp(-t/t)$. *A* is the amplitude; τ is the time constant of activation (ms). Data of Ca^{2+}-dependent activation of $I_{Cl,Ca}$ were fitted with Hill equations: $I=I_{max}/[1+(K_{Ca}/[Ca^{2+}])^n]$, where *I* is the value of currents, I_{max} is the maximum current, K_{Ca} is the Ca^{2+} concentration for half-maximal activation, *n* is the Hill coefficient[19].

RESULTS

Electrophysiological kinetic properties of the Ca^{2+}-activated Cl^- current $I_{Cl,Ca}$ was directly activated by loading endothelial cells with high concentration of Ca^{2+} (500 nmol/L) via patch electrode. After the membrane was ruptured and formed the whole-cell state, $[Ca^{2+}]_i$ was equilibrated slowly with the pipette solution [from resting level (58±11) nmol/L to the stable level (336±24) nmol/L, *n*=5 cells, Fig 1A], and in the meantime the membrane current slowly increased. The $[Ca^{2+}]_i$ and current amplitude reached to a stable level around 10 min thereafter. Ramp protocol testing of $I_{Cl,Ca}$ showed a significant outward rectifying property (Fig 1B-b), and the reversal potential did not change after $I_{Cl,Ca}$ being activated [(-25±3) mV, compared with (24±5) mV at rest]. The current recorded right after breaking into the cells (when there was no notable increase in $[Ca^{2+}]_i$) was small and used as resting level of the current [I_{rest}, (-0.5±0.4) pA/pF and (3.6±1.0) pA/pF at –80 mV and +100 mV testing potential, respectively, *n*=8 cells, Fig 1C-a]. And the current at the testing potential +100 mV was used to evaluate the effects of channel blockers. The amplitude of $I_{Cl,Ca}$ was (–3.2±1.4) pA/pF and (27±5) pA/pF at –80 mV and +100 mV, respectively (*n*=8 cells, Fig 1C-b). $I_{Cl,Ca}$ was activated slowly at positive holding potential, and inactivated quickly at negative potentials. The activation of $I_{Cl,Ca}$ at positive potential was voltage-dependent and mono-exponential (Fig 2, opened circle). The activation time constant (τ_{act}) was (175±17) ms at +100 mV testing potential (*n*=6 cells).

DIDS (100 μmol/L), a specific chloride channel blocker, reduced the current amplitude by 50.1 %±3.6 % [(12±4) pA/pF at +100 mV, *n* = 4 cells]. Verapamil, acted as non-selective chloride channel blocker, inhibited $I_{Cl,Ca}$ in a concentration-dependent manner. The IC_{50} for its blockade was (120±10) μmol/L (*n*=5 cells).

Quantitative analysis of Ca^{2+} dependence of $I_{Cl,Ca}$ Loading of the endothelial cells with different con-

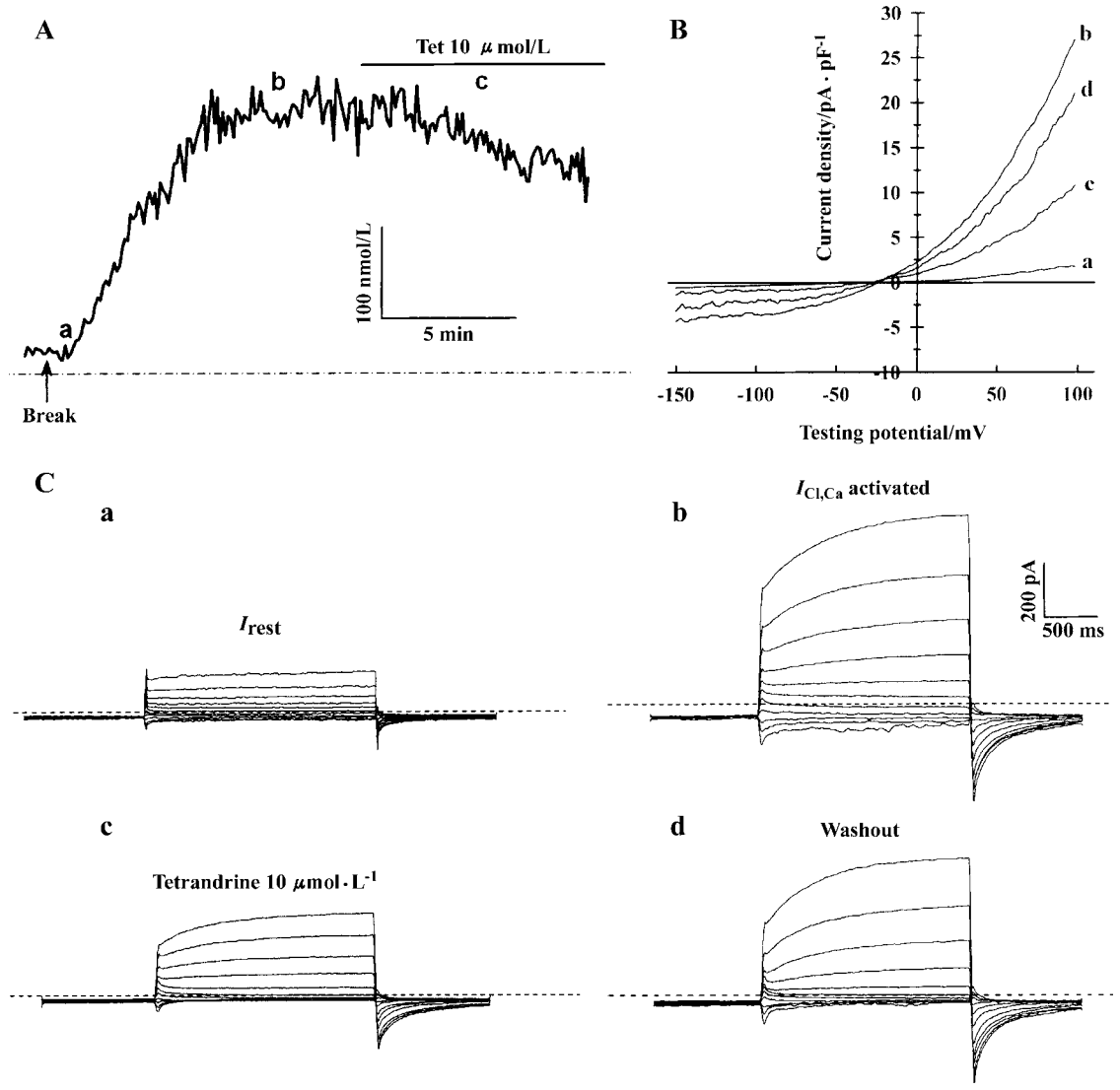

Fig 1. Ca^{2+}-activated Cl^- current ($I_{Cl,Ca}$) in HUVEC and inhibition by Tet 10 μmol/L. A) $[Ca^{2+}]_i$ measurement, "break" and arrow indicate the time point that patch electrode rupture the cell membrane. A-b, A-c is the time point sample current recorded. *I-V* relationship tested via a ramp protocol (B) and current trace tested via a step protocol (C) of $I_{Cl,Ca}$. B,C-a: current at resting level; B,C-b: $I_{Cl,Ca}$ activated; B,C-c: current suppressed by Tet 10 μmol/L; B,C-d: current recovered from washout.

centration of Ca^{2+} enabled us to measure the relation between current amplitude and $[Ca^{2+}]_i$. Fig 3 showed the steady state current densities at +100 mV as a function of $[Ca^{2+}]_i$ (Fig 3 opened circle). The steady state current–$[Ca^{2+}]_i$ relationship was fitted with Hill equations. At +100 mV testing potential, the maximal current was (39±8) pA/pF and the concentration for half maximal activation (K_{Ca}) was (287±36) nmol/L (n=5 cells). The Hill coefficient n_H was 1.89.

Tetrandrine inhibits Ca^{2+}-activated Cl^- current After the amplitude of $I_{Cl,Ca}$ reached to the stable level, Tet (1-100 μmol/L) was bath perfused. The outward current (testing at +100 mV) was decreased by 15 %± 11 %, 35 %±12 %, 71 %±9 %, 88 %±7 %, and 96 %±4 % by tetrandrine 1, 3, 10, 30, and 100 μmol/L, respectively. The inward current was decreased to a similar extent (Fig 1B-c, Fig 1C-c). The inhibition by Tet was reversible after washout by perfusion with Cs^+- Krebs' solution (Fig 1B-d, 1C-d). $[Ca^{2+}]_i$ were monitored in some experiments, slight decrease of $[Ca^{2+}]_i$ was noticed after perfusion with Tet [(293±13) nmol/L, n=3 cells]. Fig 4 showed a summary of the concentration-dependent blockade by Tet. Dose-response curve was fitted by modified Hill equation. The half-maximal blockade by Tet was observed at around 5 μmol/L [(5.2± 0.4) μmol/L, n=8 cells].

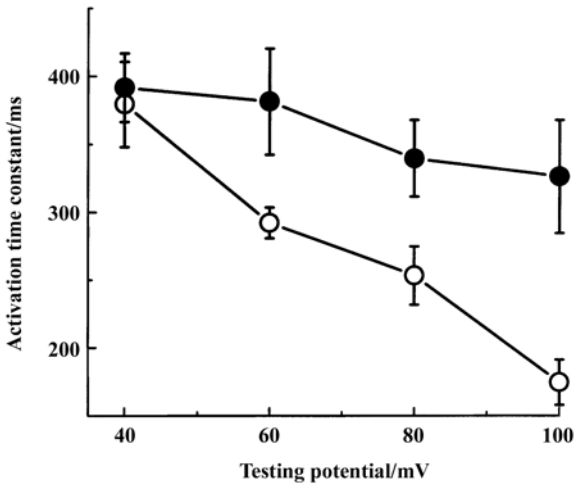

Fig 2. Voltage-dependent activation of $I_{Cl,Ca}$ at positive testing potential. Activation time constants are plotted as function of testing potential. Open circle: control; closed circle: Tet 10 μmol/L.

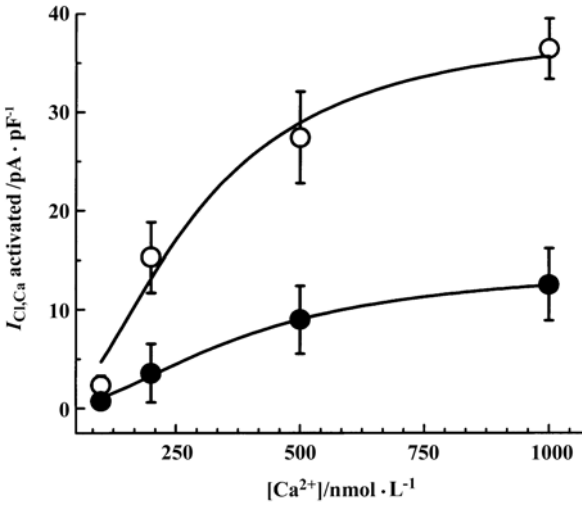

Fig 3. Calcium-dependent activation of $I_{Cl,Ca}$. Open circle: control; closed circle: Tet 10 μmol/L.

Tet did not affect the reversal potential of $I_{Cl,Ca}$ (25±6 mV, Fig 1B-c). But Tet significantly affected voltage-dependent activation of $I_{Cl,Ca}$ at positive testing potential (Fig 2 closed circle). When applying Tet 10 μmol/L, τ_{act} was increased to (392±25), (381±39), (339±28), and (326±12) ms (n=8 cells) at +40, +60, +80, +100 mV, respectively [compared with control: (379±31) ms, (292±11) ms, (253±22) ms, and (175±17) ms]. Apparently, the blocking effects were more significant at more positive testing potential.

The effect of Tet on calcium-dependent activation of $I_{Cl,Ca}$ was also tested (Fig 3, closed circle). Tet

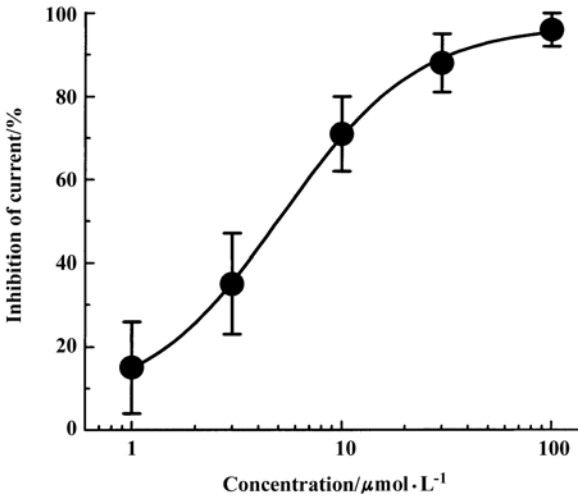

Fig 4. Concentration−response curve of blockade of $I_{Cl,Ca}$ by tetrandrine.

(10 μmol/L) significantly reduced the maximal current to (15±3) pA/pF (n=4 cells), and the K_{Ca} was increased to (387±61) nmol/L. But no apparent change of n_H (1.87).

DISCUSSION

Ca^{2+}-activated chloride channel are less well studied than other chloride channels, such as cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels, or volume-regulated chloride channel. Nevertheless, CaCC, in diverse cell types, is clearly an important channel type involved in various physiological functions (cell secretion, anion transport, electrode flux, cell adhesion etc)[23,24]. The first distinct CaCC was identified in the bovine airway, with an ion selectivity of I>Cl, and sensitive to DIDS and DTT[25]. To date, at least ten isoforms of CaCC (found from bovine, human, mouse and porcine) have been identified and published on NCBI GenBank database[26]. The electrophysiological features of CaCC are very similar in the various cell types[27]. The current-voltage relationship described here had the same outward rectification as in other cells[24], and the activation kinetics of CaCC in HUVEC cells was similar to the time course of the Ca^{2+}-activated Cl^- currents demonstrated in cultured calf pulmonary artery endothelial cells and in rat parotid acinar cells[17,28]. Thus, CaCC presently tested belongs to general CaCC family. In endothelial cells, the speculated roles of CaCC are control of membrane potential and modulation of agonist-induced or store-depletion-dependent intracellular calcium signaling, including regulation of calcium influx. The calcium influx sequen-

tially regulates the secretion of vasoactive compounds, which plays an essential role in controling vascular tone[24].

Tet, in the concentration range from 0.1 to 100 μmol/L, not only shows a prominent blocking effect on L- and T-type channels, but also inhibits other ion channels, such as Ca^{2+}-activated K^+ channel, inward rectifying K^+ channel, and Ca^{2+} release-activated Ca^{2+} channel in different cells or tissues[11,29]. It suggests that Tet is not a selective Ca^{2+} channels, but display a rather wide spectrum effects on many ion channels. Our results demonstrated a concentration-dependent blockade of CaCC by Tet. The IC_{50} was 5 μmol/L, which was at the therapeutically relevant concentration range. And compared with verapamil, a calcium channel blocker that exhibits a non-specific inhibitory effect on CaCC (IC_{50} around 100 μmol/L), Tet is rather a potent blocker for endothelial CaCC. We also showed that Tet effectively suppressed both voltage- and calcium-dependent activation of CaCC. With the quantitative methods, we fitted the calcium-dependent activation curve with Hill equation. Hill coefficient >1 (n_H: 1.89) suggested that more than one Ca^{2+} ion bound to the channel to activate it. This suggests the existence of multiple Ca^{2+}-liganded closed states in a voltage-dependent equilibrium with Ca^{2+}-liganded open states. Tet did not affect n_H (1.87). It strongly indicates that Tet does not affect the Ca^{2+} binding sites of the channel. We presume that the mechanism of Tet blocking CaCC might be due to its effects on the activation process. However, the possibility of Tet directly binding to channel can not totally be excluded. The novel finding of the inhibitory effects of Tet on CaCC might be one of the underlying ionic mechanism of action of Tet on endothelial cells. Recent report showed that Tet exerted dual effects on vascular activities. As a vasodilator, it directly relaxes vascular smooth muscle. But since it blocks endothelial Ca^{2+}-activated K^+ channel and Ca^{2+}-release activated Ca^{2+} channel, Tet might impede the NO production in endothelial cells, which leading to a transient vasoconstriction[30]. Kwan pointed out, *in vivo*, such effects of Tet on endothelial cells might be overwhelmed by its sustained direct vasorelaxation on vascular smooth muscle cells[31]. It is worth looking into the physiological role of its inhibitory effects on CaCC. In a separate experiment, we found that in the concentration range similar to that of blocking CaCC, Tet effectively blocked the volume-regulated chloride channel and endothelial cell proliferation (our unpublished data). The blocking CaCC by Tet might also take part in such regulating process since the anti-angiogenic effects of Tet may be related to its ability to inhibit the non-voltage dependent Ca^{2+}-entry pathway.

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