Volume- and calcium-activated chloride channels in human umbilical vein endothelial cells

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ABSTRACT

AIM：To characterize the properties of chloride currents and its modulation in human umbilical vein endothelial cells (HUVEC). METHODS：Using whole-cell patch-clamp recording techniques. RESULTS：Exposure of HUVEC to 13.5 % and 27 % hypotonic solution (HTS) induced a current $I_{Cl,vol}$. This current was correlated with the changes in cell volume and showed a modest outward rectification. It was slowly inactivated at positive potential (> 50 mV)，and it was time- and voltage-independent in kinetics. The current densities (pA/pF) were 20 ± 3 (13.5 % HTS) and 58 ± 4 (27 % HTS，n = 7)，respectively at + 100 mV test potential. Applying GTPγs (300 μmol·L^{-1}) elicited a current similar to $I_{Cl,vol}$，while cAMP (0.5 mmol·L^{-1}) had no effect on the current. Increase in $[Ca^{2+}]_i$，either by directly loading cells with high concentration of Ca^{2+} ($CaCl_2$)，or by perfusing vasoactive agonist ATP (10 μmol·L^{-1})，activated $I_{Cl,Ca}$. The current density was only (23 ± 5) pA/pF (n = 8 cells). Such current was slowly activated at positive potential，inactivated quickly at negative potential，and showed strong outward rectification. Both currents were inhibited by DIDS and verapamil. Challenging a cell with elevated $[Ca^{2+}]_i$ and HTS activated $I_{Cl,vol}$ on the top of $I_{Cl,Ca}$ in the same cell，suggested co-existence of these two currents and that they were modulated by different ways. cAMP-regulated chloride channel and ClC (chloride channel family) channel were absent. CONCLUSION：HUVEC express two kinds of chloride channels，$I_{Cl,vol}$ activated by change in cell volume and $I_{Cl,Ca}$ by elevation of $[Ca^{2+}]_i$，respectively.

INTRODUCTION

Vascular endothelial cells are important in various functions of blood vessels. Such multiple functions are mediated by the production and release of a variety of vasoactive agents that affect the cells in the vessel wall or endothelial cells. The membrane potential is an important regulator of intra- and intercellular signal transduction in vascular functions. Change in membrane potential is mainly controlled by K^+，Cl^-，and possibly nonselective cation channels. Besides potassium currents，chloride currents are the most dominant currents in vascular endothelial cells. Several types of chloride channels，such as volume-activated，calcium-activated，and high conductance chloride channels (activated by isoproterenol and cAMP) were reported in endothelial cells[1]. These currents are important for regulation of cellular secretion and controlling of volume and intracellular pH. They also control the membrane potential and the driving force for Ca^{2+} influx，and cell proliferation[2].

Volume-activated chloride currents，$I_{Cl,vol}$，have already been described in details in several kinds of endothelial cells including human umbilical vein endothelial cells (HUVEC)[3]. Such currents were activated by cell swelling and changes in cell shape in response to mechanical or humoral stimulation[3,4]. Though calcium-activated chloride current，$I_{Cl,Ca}$，was well studied in vascular smooth muscle cells，only a few papers reported its existence in calf pulmonary artery endothelial cells[5,6]. Presently there is no report of $I_{Cl,Ca}$ in HUVEC. The purpose of the present experiments was to characterize the properties of multiple types of Cl^- currents in HUVEC and to focus on the mode of activation and modulation of such a channel.

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MATERIALS AND METHODS

Cells Cells from a cultured human umbilical vein endothelial cell line (cell line HUVEC, VEC304) were used. The cells were grown in Medium 199 containing 10 % calf serum, 0.03 % *l*-glutamine, benzylpenicillin 2 g·L^{-1}, and streptomycin 2 g·L^{-1}. Cells were detached by exposure to 0.1 % trypsin in Ca^{2+}- and Mg^{2+}-free solution and plated on gelatin-coated coverslips. Cultures were maintained at 37 ℃ in a fully humidified atmosphere of 5 % CO_2 in air. For electrophysiological measurements, only non-confluent, single endothelial cells were used.

Electrophysiology Whole cell membrane currents were measured. Currents were monitored with a patch 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 ℃). Two voltage protocols were applied. Ramp current recording was designed to determine current-voltage (I-V) relationship. Holding potential was at 0 mV, a step to – 80 mV for 0.6 s then 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. Other voltage steps were applied from a holding potential of – 50 mV to a test potential from – 100 to + 100 mV in 20-mV increments. The duration of the steps is 2 s. In order to compare data obtained from different cells, we have normalized the current amplitudes per unit of membrane capacitance.

To observe the depolarization-induced inactivation of the currents, 1 s lasting steps to potential between + 30 mV and + 140 mV were applied with an increment of 10 mV (holding potential at – 80 mV), followed by a short step to + 120 mV. To analyze voltage-dependent deactivation of current, a step of 1 s to + 100 mV was applied from holding potential of 0 mV and followed by a step of 3 s to voltages ranging from + 75 to – 100 mV, spaced 25 mV.

Solutions and drugs Cells were superfused with normal Krebs' solution, containing: NaCl 150, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, Glucose 10, HEPES 10 (mmol·L^{-1}), adjusted to pH 7.4 with NaOH 1 mmol·L^{-1}. To avoid the influence of inward rectifying K^+ current, Cs^+ substituted K^+ in the Krebs' solution when recording currents.

The pipette solution contained (mmol·L^{-1}): CsCl 40, Cs-aspartate 100, $MgCl_2$ 0.1, egtazic acid 5, $CaCl_2$ 1.93, Na_2ATP 4, HEPES 10, pH adjusted to 7.2 by CsOH 1 mmol·L^{-1}.

To elicit the volume-activated currents, a hypotonic solution (HTS) was used. First, HUVEC cells were superfused with a modified, isotonic Krebs' solution (mmol·L^{-1}): NaCl 105, CsCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, Glucose 10, HEPES 10, mannitol 90, pH adjusted to 7.4 by NaOH 1 mmol·L^{-1}. Omitting mannitol from the above solution resulted in a hypotonic solution with a 27 % reduced osmolarity. Changing the concentration of mannitol to 45 mmol·L^{-1} resulted in 13.5 % reduction in osmolarity.

To activate the Cl^- current directly by Ca^{2+}, pipette solutions were used in which the free Ca^{2+} concentration was adjusted to 500 nmol·L^{-1} by modifying concentration of $CaCl_2$ and egtazic acid in pipette solution (egtazic acid 5, $CaCl_2$ 3.79, and $MgCl_2$ 0.1 mmol·L^{-1}, pH = 7.2).

DIDS (4, 4′-diisothicyano-stilbene-2, 2′-disulphonic acid), verapamil, GTPγs, ATP, and cAMP were purchased from Sigma. DIDS, verapamil, and ATP were administered via extracellular perfusion solution. GTPγs and cAMP were applied via pipette solution by adding these agents directly to pipette solution to a desired concentration.

Statistics Data were expressed as $\bar{x} \pm s$ and compared with *t*-test.

RESULTS

Activation of $I_{Cl, vol}$ by increase in cell volume Cell swelling was induced by superfusing the cells with a hypotonic solution in which the osmolarity was decreased by 13.5 % and 27 %. In an isotonic extracellular solution, only small and time-independent currents were recorded. After perfusing the cells with 13.5 % hypotonic solution for 3 – 5 min, a large current could be elicited, and the current was slowly but incompletely inactivated at positive potentials (> 40 mV). Amplitude of the current was increased with a decrease in osmolarity. The current was stable and no obvious run-down was observed if maintained in 27 % hypotonic solution. On reperfusing the cells with the normal Krebs' solution, the current could return to the control level (Fig 1,2). The current-voltage (I-V) relation curve revealed that the reverse potential (– 28.1 mV) for $I_{Cl, vol}$ was close to

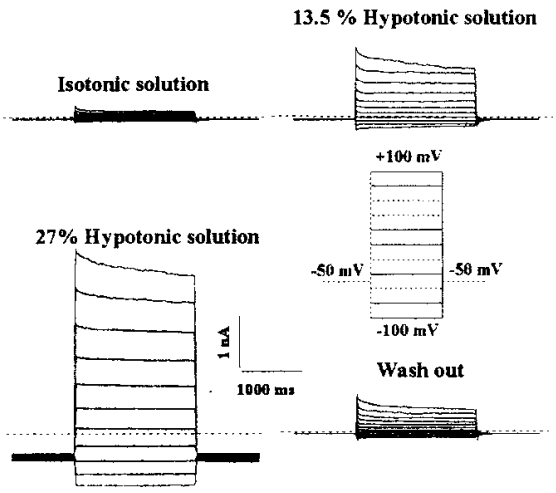

Fig 1. Volume-activated chloride current in cultured human umbilical vein endothelial cells.

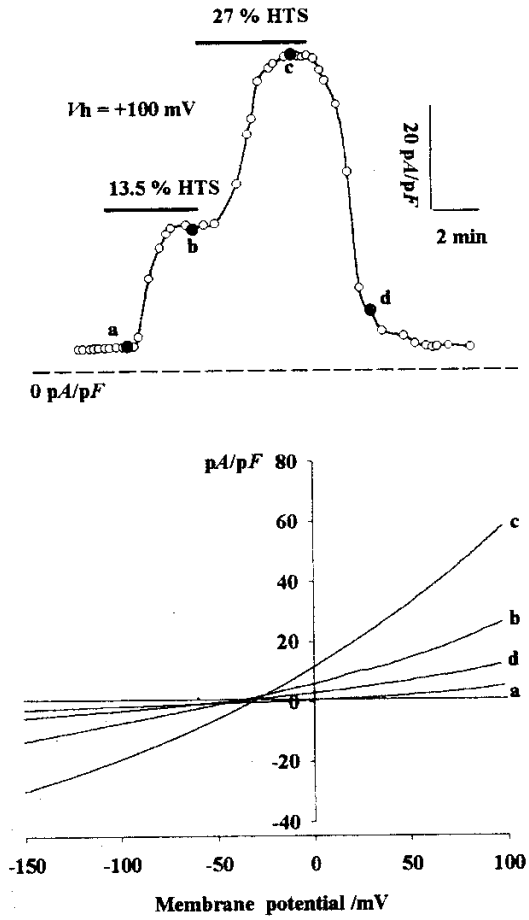

Fig 2. Time course of current at + 100 mV activated by hypotonic solution and current-voltage relationships of volume-activated chloride currents. a) Isotonic solution b) 13.5 % hypotonic solution; c) 27 % hypotonic solution d) wash out.

the theoretical Cl^- equilibrium potential (− 32 mV), and the current showed property of slightly outward rectification. At + 100 mV test potential (Et), current densities for 13.5 % and 27 % hypotonic solution were (20 ± 3) pA/pF and (58 ± 4) pA/pF, respectively. (n = 7, Fig 2).

Calcium-activated Cl^- current, $I_{Cl,Ca}$ To analyze the kinetic properties of this Ca^{2+}-activated current, endothelial cells were loaded with a pipette solution buffered at an elevated Ca^{2+} concentration. After the membrane in the patch was broken and forming the Whole-cell State, the cell was allowed to equilibrate with the pipette solution containing Ca^{2+} (500 nmol · L^{-1}). Just after breaking the membrane, the current recorded before the increase in $[Ca^{2+}]_i$ was rather small. After the membrane was broken, the rise in $[Ca^{2+}]_i$ activated a strong outwardly rectifying current. The current showed a slow activation at positive and a fast decay at negative potential (Fig 3). Similar results were obtained on increasing the $[Ca^{2+}]_i$ by application of ATP 10 μmol · L^{-1}. At + 100 mV test potential the amplitude of the $I_{Cl,Ca}$ was 23 ± 5 pA/pF (n = 8, elevated $[Ca^{2+}]_i$) and

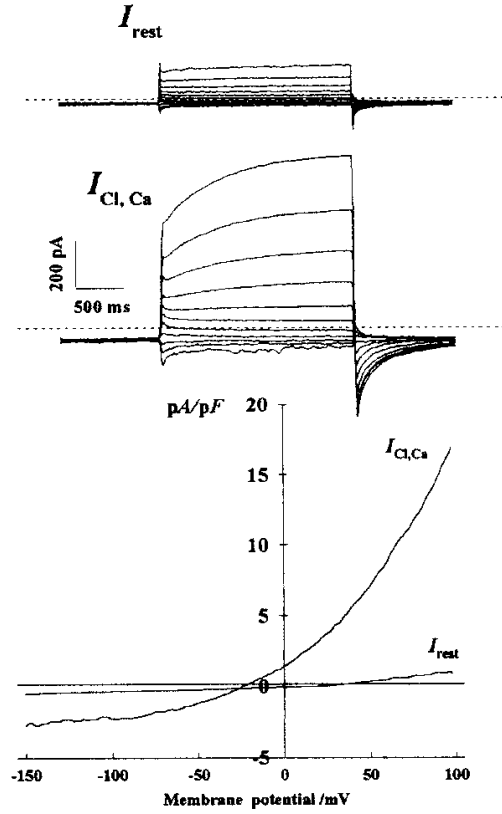

Fig 3. Calcium-activated cholride current in HUVEC.

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(19 ± 4) pA/pF (n = 5, perfused ATP), much lower than that of $I_{Cl,\ vol}$.

Co-activation of $I_{Cl,vol}$ and $I_{Cl,Ca}$ We found that $I_{Cl,vol}$ and $I_{Cl,Ca}$ could be recorded in the same cell. As mentioned above, $I_{Cl,Ca}$ was measured by applying high concentration of Ca^{2+} in pipette solution. After $I_{Cl,Ca}$ reached a stationary level, the cell was superfused by hypotonic solution. Both inward and outward currents were activated. It clearly demonstrated the characteristic of $I_{Cl,vol}$. Resuperfusing the cell with normal isotonic solution deactivated the current. Only the Ca^{2+}-activated current was left (Fig 4).

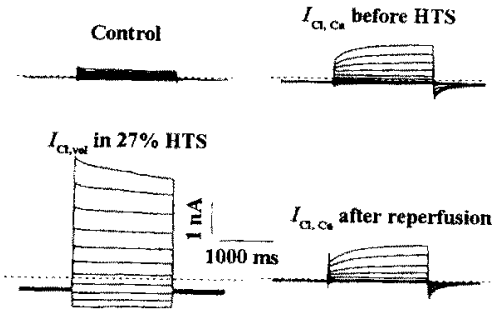

Fig 4. Co-activation of $I_{Cl,\ Ca}$ and $I_{Cl,Vol}$ in the same single HUVEC cell.

Voltage-dependence and kinetic property of $I_{Cl,vol}$ We noted that at more positive potential (> 40 mV), $I_{Cl,vol}$ was slowly inactivated, which was different from $I_{Cl,Ca}$ activated at positive potential. To further investigate the voltage-dependence and electrophysiological properties, inactivation and deactivation of both currents were studied. When observing depolarization-induced inactivation of the current, no significant inactivation of $I_{Cl,vol}$ was recorded. Similar result was got for $I_{Cl,Ca}$. To observe possible tail currents and fast deactivation properties, current was firstly depolarized to + 100 mV and then stepped back to different voltage (steps from + 75 mV to − 100 mV with increment of 25 mV). Neither tail currents nor time and voltage-dependent change could be recorded for $I_{Cl,vol}$. But unlike $I_{Cl,vol}$, tail currents of $I_{Cl,Ca}$ quickly deactivated at negative potential (Fig 5).

A Cl^- current elicited by GTPγs and absence of cAMP-activated current Intracellular application of GTPγs activated a current similar to the HTS-induced $I_{Cl,vol}$. GTPγs (300 μmol·L^{-1}) was added to normal

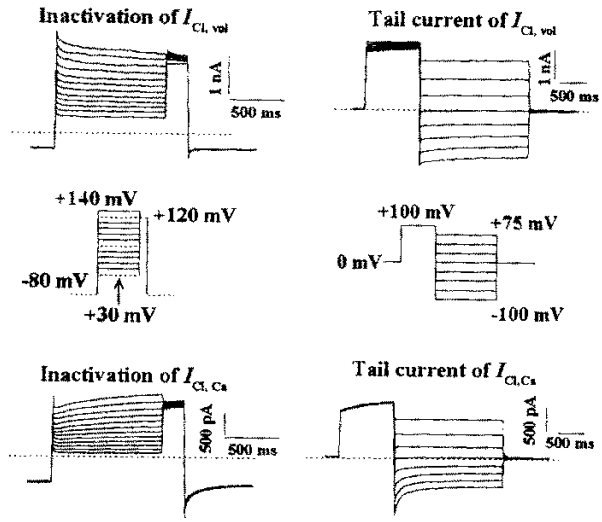

Fig 5. Inactivation and deactivation of $I_{Cl,\ Vol}$ and $I_{Cl,\ Ca}$.

pipette solution. Within 2 − 3 min after breaking the cell membrane, a current was induced that never occurred in normal whole-cell experiments in absence of GTPγs. The currents bore the characteristics of $I_{Cl,vol}$, modest outward rectification and slight inactivation at positive potential. It was (49 ± 9) pA/pF (n = 5). These results hinted to a possible contribution of G-proteins to the activation of this current.

Using the same method, cAMP (0.5 mmol·L^{-1}) was loaded into cell through pipette solution. No obvious current was recorded. And such intervention did not affect the HTS-induced chloride current. Current density was (56 ± 8) pA/pF with cAMP (Et = + 100 mV, n = 5, 27 % HTS).

Blockade of chloride current A chloride channel blocker and a stilbene derivative, DIDS, inhibited the HTS- and Ca^{2+}-induced current. Applying DIDS 100 μmol·L^{-1} decreased the current at + 100 mV by (48 ± 3) % for $I_{Cl,vol}$ and (50 ± 3) % for $I_{Cl,Ca}$ (n = 5). Verapamil inhibited both currents in a concentration-dependent way. Concentration of Half-maximal inhibition was up to 100 μmol·L^{-1} (140.15 for $I_{Cl,vol}$ and 120.1 for $I_{Cl,Ca}$). Verapamil 100 μmol·L^{-1} reduced the current by (48 ± 8) % for $I_{Cl,vol}$ and (52 ± 10) % for $I_{Cl,Ca}$ (n = 5). Tab 1 illustrates the effects of different interventions upon the membrane currents of HUVEC.

DISCUSSION

HUVEC express two types of outwardly rectifying

Tab 1. Effects of different interventions on membrane currents of HUVEC. $n=5-8$. $x\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* I_{rest}.

	$I_{Cl,vol}$		$I_{Cl,Ca}$
I_{rest}	2.1 ± 0.8	I_{rest}	1.2 ± 0.6
$I_{Cl,vol}$ 13.5 % HTS	20 ± 3.5^b	$I_{Cl,Ca}$	23.4 ± 4.7^c
27 % HTS	58.2 ± 4.6^c	ATP	18.9 ± 3.7^c
cAMP	5.27 ± 2.3	DIDS	12.0 ± 3.7^b
cAMP + 27 % HTS	56.2 ± 7.5^c	Verapamil	14.6 ± 3.2^b
GTPγs + 27 % HTS	49.2 ± 9.7^c		
DIDS + 27 % HTS	28.2 ± 8.7^b		
Verapamil + 27 % HTS	31.7 ± 6.4^b		

* DIDS (100 μmol·L^{-1}), verapamil (100 μmol·L^{-1}) and ATP (10 μmol·L^{-1}) were administered via extracellular perfusion solution. GTPγs (300 μmol·L^{-1}) and cAMP (0.5 μmol·L^{-1}) were applied via pipette solution by adding these agents directly to pipette solution to a desired concentration.

Cl^- currents, $I_{Cl,vol}$ was gated by changes in cell volume, and $I_{Cl,Ca}$ by elevation of $[Ca^{2+}]_i$. Both currents could be blocked by chloride channel blocker. Other types of chloride channels reported in endothelial cells, such as high conductance Cl^- channel (cAMP-activation) could not be detected. Different from those voltage-gated ClC-type currents, there was no voltage-dependence in $I_{Cl,vol}$. With different protocol, no voltage-dependent inactivation and tail current was found in $I_{Cl,vol}$. The kinetic properties of $I_{Cl,vol}$ in endothelial cells were not compatible with any member of ClC family.

$I_{Cl,vol}$ and $I_{Cl,Ca}$ could be distinguished by their different kinetic and rectifying properties. $I_{Cl,vol}$ and $I_{Cl,Ca}$ differed from each other firstly by the fact that they were activated in distinct ways. One was induced by cell swelling or mechanical stress, the other one by increasing $[Ca^{2+}]_i$ via direct elevation of intercellular calcium or responses to agonist such as ATP. The current densities of $I_{Cl,vol}$ were much larger than those of $I_{Cl,Ca}$. At + 100 mV test potential, $I_{Cl,vol}$ [(58 ± 4) pA/pF, induced by 27 % HTS] was almost twice as much as $I_{Cl,Ca}$ (23 ± 5) pA/pF. It is obvious that $I_{Cl,Ca}$ has the much pronounced outward rectification in comparison with $I_{Cl,vol}$. Contrary to $I_{Cl,vol}$, $I_{Cl,Ca}$ was activated at positive potential and decayed at negative potentials. Such kinetic properties were similar with the findings reported in calf pulmonary artery endothelial cells[5]. $I_{Cl,vol}$ lacked voltage-dependent tail currents; while $I_{Cl,Ca}$ tail current deactivated quickly at negative potential (with holding potential at 0 mV). Nilius *et al* reported that $I_{Cl,Ca}$ had outward tail current when holding potential at − 50 mV[5].

Though $I_{Cl,vol}$ was elicited by cell swelling, the mechanism for $I_{Cl,vol}$ activation was not well interpreted. Protein kinase C, cAMP-activated protein kinase is not involved in the signal pathway for activating $I_{Cl,vol}$[3]. In our results, intercellular cAMP (0.5 mmol·L^{-1}) loading did not affect the amplitude of HTS-induced $I_{Cl,vol}$. It was reported that PLA_2 or protein tyrosine kinase influence the process of $I_{Cl,vol}$ activation[7,8]. GTP_{rs} could elicit a current similar to $I_{Cl,vol}$ in electrophysiological properties. Tilly *et al* reported that cell swelling activated small molecule G protein p^{21Rho}[9]. G protein may increase the sensitivity of channel responding to volume changes rather than directly interact with the channel[10]. But it needs more investigation and evidence.

Another perplexity while studying chloride channel is the molecular identification of channel protein. Though several groups have reported different protein candidates for volume-activated chloride channel, no definite results have been reached[11]. Moreover RT-PCR experiments did not provide any evidence that the endothelial Ca^{2+}-activated Cl^- channel might be identical with a recently cloned Ca^{2+}-sensitive Cl^- channel (CaCC)[12]. For this reason no specific and high affinity blocker was discovered. DIDS and verapamil belong to different categories of chloride channel blocker. DIDS is an anion transporter inhibitor, while verapamil is a multidrug resistance reverser. Although such agents could inhibit the currents, higher concentrations (up to mmol/L) were needed and they might have played a role in modifying but not directly blocking the channel. In summary, HUVEC express $I_{Cl,vol}$ and $I_{Cl,Ca}$, but not cAMP- or voltage-dependent ClC types. And furthermore our data demonstrates co-existence of two such channels in cells, which are activated in different ways. They might be co-activated under various physiological conditions.

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人脐静脉血管内皮细胞容量激活和钙激活氯离子通道

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关键词 血管内皮；膜片箝技术；氯通道；脐静脉；环腺苷一磷酸；钙；腺苷三磷酸；维拉帕米

目的：研究人脐静脉血管内皮细胞的氯离子通道及其不同的调节机制. 方法：全细胞膜片箝技术. 结果：13.5 %和 27 %的低渗液体灌流细胞激活一外向电流($I_{Cl,vol}$). 该电流有弱的外向整流特性，无明显时间和电压依赖性；电流大小为(58 ± 5) pA/pF；分别为(20 ± 3)，(58 ± 4)增加细胞内钙或胞外应用 ATP 能激活 $I_{Cl,Ca}$. 该电流幅值较小(23 ± 5) pA/pF；外向整流特性明显，在正电压下缓慢激活. 两种电流均被 DIDS 及维拉帕米抑制. 在同一个细胞上，在激活 $I_{Cl,Ca}$ 的基础上灌流低渗液体可进一步激活 $I_{Cl,vol}$. 结论：HUVEC 表达有两种氯通道，改变细胞容积可激活 $I_{Cl,vol}$，而增加细胞内钙则诱导出 $I_{Cl,Ca}$.

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