Effects of volume-activated chloride channels on the invasion and migration of human endometrial cancer cells

M. Li, D.B. Wu, J. Wang

Department of Obstetrics and Gynecology, Anhui Provincial Hospital, AnHui Medical University, Hefei (China)

Summary

Objective: To investigate the role of volume-activated chloride channels (VACC) in invasion and migration of human endometrial cancer cell. *Materials and Methods:* Expression of voltage-gated chloride channel-3 (CLC-3) was detected by employing reverse transcriptase-polymerase chain reaction (RT-PCR) in human endometrial cancer Ishikawa cell line. Cell invasion and cell migration were determined by using the Transwell invasion and migration assay, respectively. NPPB, a Cl^- channel blocker, was treated to observe the effects of volume-activated Cl^- channel on invasion and migration of endometrial cancer cell. *Results:* CLC-3 RNA expression was observed in Ishikawa cell line. The authors showed that blockade of Cl^- channels specifically inhibited invasion and migration of endometrial cancer Ishikawa cell line in a dose-dependent manner. VACC activation and subsequent regulatory volume decrease (RVD) were markedly suppressed by NPPB. Anion replacement studies indicate that permeation of Cl^- ions through endometrial cancer Cl^- channel is obligatory for regulatory volume decrease (RVD) induced by VACC. Moreover, $[Ca^{2+}]_i$ measurements indicated that VACC-mediated increase in $[Ca^{2+}]_i$ was one of the mechanisms of cancer cell invasion and migration. *Conclusions:* These data intensely suggest that VACC in endometrial cancer may facilitate tumor invasion and migration, presumably through inducing RVD and mediating $[Ca^{2+}]_i$ increase.

Key words: Chloride channels; Invasion; Migration; Endometrial cancer.

Introduction

Chloride channels are ubiquitous transmember proteins involved in diverse cellular processes. They are essential for salt and fluid movements across epithelia and volume regulation. According to gating mechanisms, there are from a functional point of view, five classes of chloride channels, including volume-activated chloride channel (VACC) (also named as swelling-activated chloride channel and volume-sensitive or -regulated chloride/anion channel). An increase in cell volume evokes a series of signaling events resulting in activation of volume-sensitive chloride channel. The effluxes of Cl^- ions through the Cl^- channel followed by water lead to a cell volume decrease referred to as regulatory volume decrease (RVD). There is a continuing effort to identify the molecular structure of the VACC. Although CLC-3, an attractive voltage-gated chloride channel (CLC) gene family member, might be involved in the activation or modulation of volume-activated chloride current, the molecular nature of VACC is still controversial. Nevertheless, CLC-3 has been considered the most likely molecular candidate of VACC [1, 2]. Despite difficulties concerning the molecular identification of VACC, there is a good agreement on the properties which include blockade by 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) [3, 4]. The activation of VACC by volume changes and its influence on cell volume make it a prime candidate for participation in the shape-volume changes.

In a previous study [5, 6], the authors found that the chloride channel is correlated with the malignant biology manner of ovarian cancer cell. However, there are no data available as to whether the VACC affects invasion and migration of endometrial cancer. The authors hypothesize that the activation of VACC, with its accompanying water efflux, results in cell shrinkage (RVD) and increase of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) that are a requirement for successful invasion of endometrial cancer cells. Hence, VACC may aid the invasive biology of endometrial cancer cells, a feature that greatly compromises surgical treatment of this disease.

Materials and Methods

Cell culture

Human endometrial cancer Ishikawa cell line was obtained from Basic Medicine Research Institute, Qilu Hospital, Shandong University (China). Cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and maintained at 37°C in a humid atmosphere of 5% CO_2 in air.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of CLC-3 expression

Total RNA was isolated from the cultured Ishikawa cell line using the Trizol reagent according to the manufacture's procedure; mRNA was transcribed into first strand cDNA using oligo-dT primers and M-MLV reverse transcriptase. The sequences used were: CLC-3 primer, sense, 5'GGCAGCATTAACAGTTCTACAC3'; antisense, 5'TTCCAGAGCCACAGGCATATGG3'. β-actin primer, sense, 5'AACTCCATCATGAAGTGTGA3'; antisense, 5'ACTCCTGCTTGCTGATCCAC3'. PCR cycling conditions were as follows: an initial denaturation step of 94°C for 5 min, 94°C for 1 min, annealing at 58°C for 1 min, and elon-

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gation at 72°C for 1 min. A final elongation step of 10 min at 72°C occurred on the last cycle. PCR reactions were cycled 40 times. Control reactions without reverse transcriptase were performed for each PCR amplification experiment. The PCR products were analyzed by electrophoresis in a 2% agarose gel and stained with ethidium bromide (0.5 μg/ml).

Invasion and migration assays

The invasiveness of endometrial Ishikawa cancer cell was assayed using Transwell chambers. Briefly, polycarbonate filter (pore size, 8 μm) was coated with 100 μl of Matrigel. Serum-free RPMI-1640 containing 1.0×10^5 cells in 100 μl was introduced into the upper compartment and conditioned medium used as chemoattractant was added into the lower chamber. NPPB were added to both upper (with cells) and lower chambers at the desired concentrations. The cells were allowed to invade the Matrigel for 24h at 37°C in a 5% CO_2 atmosphere. Cells that had penetrated the Matrigel were quantified by MTT assay. Cells on the upper surface of the filter that had not invaded through the Matrigel were removed with a cotton swab. Cells that had invaded, remained on the filter and 25 μl MTT solution (5 mg/ml) was added to each well. After four hours of incubation at 37°C, the cells on the filter formed dark-blue crystals. The filter was then placed into another well containing 150 μl of DMSO to dissolve the crystals. After 30 min, the solution was poured into 96-well plates and absorbance was measured. To assess cellular migration potential, the protocol described above was used, except that Matrigel was omitted.

Cell volume measurements and ion replacement assay

Ishikawa cells were perfused with saline solution consisting of (in mM): NaCl, 122.6; KCl, 5.0; $MgCl_2$, 1.2; $CaCl_2$, 2.0; $Na2HPO_4$, 1.6; NaH_2PO_4, 0.4; glucose, 10.5; HEPES 5.0; $NaHCO_3$, 25.0; and Na_2SO_4, 1.2, pH 7.4. For osmotic challenges, this solution was modified by removing 50 mM NaCl in the case of hypo-osmotic solution (200 mOsm/kg) and adding back mannitol for the isotonic (308 mOsm/kg) solution. In this manner, solutions were maintained isoionic. During the ion replacement experiment, cells were superfused with a solution containing (in mM): Na gluconate 130, K gluconate 5.4, $MgSO_4$ 0.8, Ca gluconate 1.2, NaH_2PO_4 1, glucose 5.5, and Tris 5, pH-adjusted to 7.4. To obtain a hypotonic solution, the sodium gluconate was reduced to 80 mM. Then the coverslips were mounted on an inverted microscope with a CCD camera. Cell images were captured every 30 sec. throughout the entire experiments and were then analyzed with an image analysis software. Cell volume was calculated using the equations of $V = 4/3 \times S \times (S/\pi)^{1/2}$, where S is the area ($\mu m^2$). RVD was calculated with the equation of $RVD\ (\%) = (V_{max}\text{-}V_{min}) \div (V_{max}\text{-}V_0) \times 100\%$, where V0 is the cell volume in isotonic solution before hypotonic stimulation, V_{max} is the peak volume in hypotonic solution, Vmin is the volume before return to isotonic solution. Drugs were added to the isotonic and hypotonic solutions to final desired concentrations of NPPB. All experiments were performed at room temperature (22°C - 26°C).

$[Ca^{2+}]_i$ measurements

Ishikawa cells were grown overnight in 90% RPMI-1640 supplemented with 10% FBS containing 100 μU/ml penicillin and 100 μg/ml streptomycin on circular discs at 37°C and 5% CO_2 in air. Cells were loaded with Fura-2/AM for 1 hr in the dark at room temperature by incubation with 10 μM membrane-permeant Fura-2/AM in a physiological solution that contained (in mM): NaCl, 122.6; KCl, 5.0; $MgCl_2$, 1.2; $CaCl_2$, 2.0; Na_2HPO_4, 1.6; NaH_2PO_4, 0.4; glucose, 10.5; HEPES 5.0; $NaHCO_3$, 25.0; and Na_2SO_4, 1.2, pH 7.4. For osmotic challenges, this solution was modified by removing 50 mM NaCl in the case of hypo-osmotic solution (200 mOsm/kg) and adding back mannitol for the isotonic (308 mOsm/kg) solution. In this manner, solutions were maintained isoionic. Drugs were added to the hypotonic solutions to final concentrations of NPPB. Ca^{2+} influx was measured by the changes in fluorescent signals as recorded by a LS-50B luminescent spectrometer at excitation wavelength of 340/380 nm and emission wavelength of 510 nm. The ratio of the two images 340/380 was calculated and converted to absolute $[Ca^{2+}]_i$ concentrations.

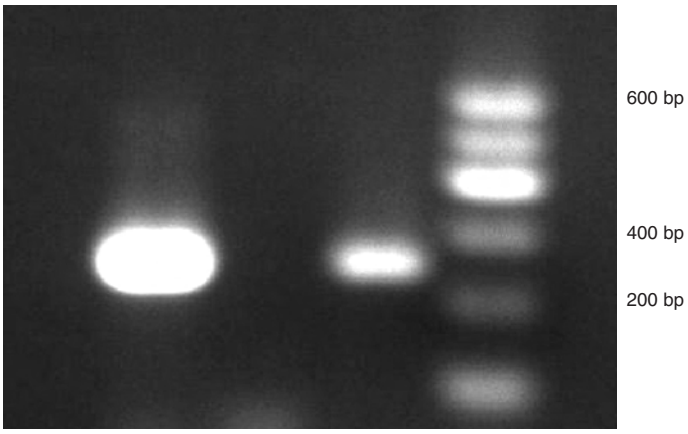

Figure 1. — RT-PCR analysis of the expression of CLC-3 transcript in human endometrial cancer Ishikawa line.

Statistics

Data were presented as the mean ± standard error. Student's t-test was used for statistical analyses and differences were considered significant at $p < 0.05$. All experiments were performed at least three times with representative data presented.

Results

RT-PCR assay

Unfortunately, the molecular structure of VACC is not known. After many candidates, only CLC-3, an attractive member of CLC family, is still considered as being potentially involved in volume-regulated chloride currents. Thus, in trying to find a possible correlation of VACC with biological behavior of endometrial cancer, from the molecular basis, the authors' strategy was firstly to determine whether CLC-3 is expressed in the Ishikawa cell line. Total RNA was extracted and RT-PCR analysis was performed using primers specific for CLC-3 and β-actin. Figure 1 shows that PCR notably amplified a 235-bp CLC-3 and 247-bp β-actin from total RNA isolated from Ishikawa cells. No product was detected in the absence of reverse transcriptase.

Fig. 2

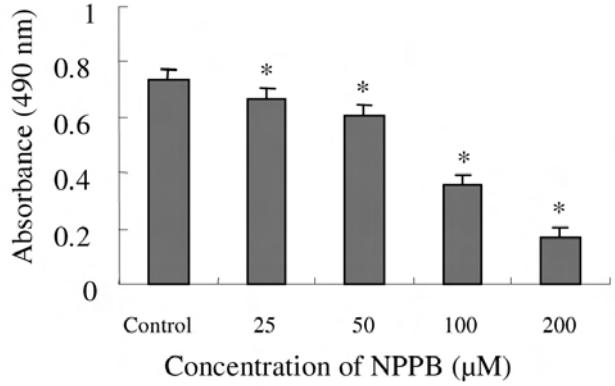

Fig. 3

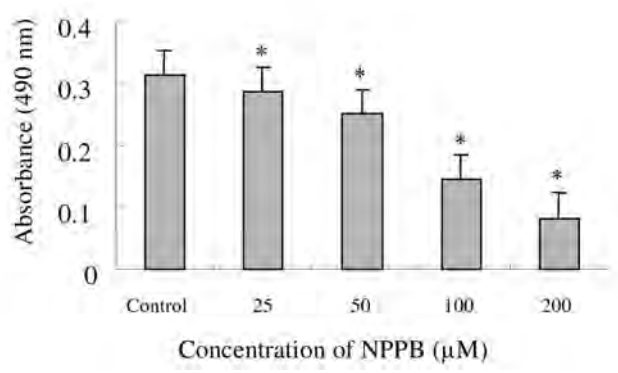

Fig. 4

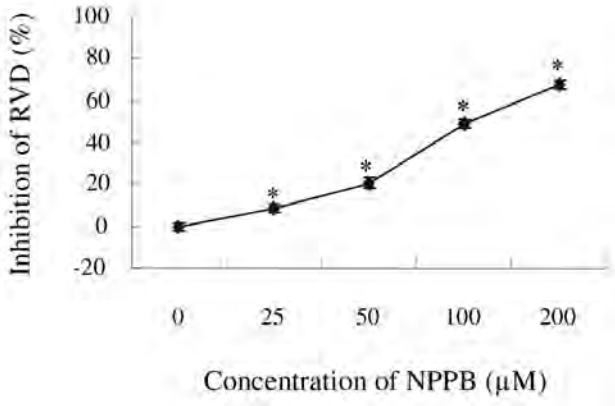

Fig. 5

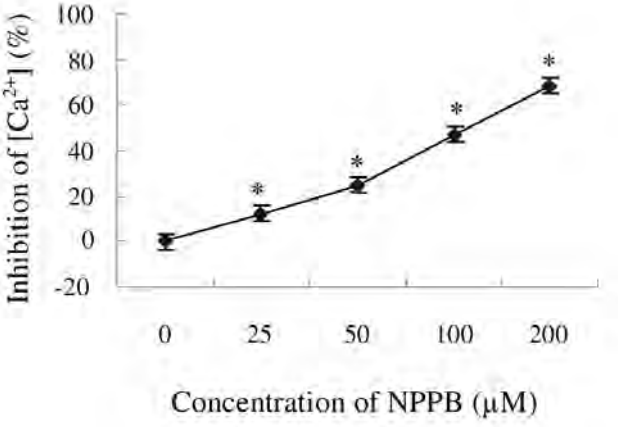

Figure 2. — Inhibition of Ishikawa cell invasion by NPPB. Cells were cultured in medium without (control) or with desired concentrations of chloride channel blocker NPPB. Ability of cell invasion was determined using Transwell chamber. Absorbance value, which indicated relative cell number, was detected employing the MTT method. * $p < 0.05$ compared with control.

Figure 3. — Inhibition of Ishikawa cell migration by NPPB. Cells were cultured in medium without (control) or with desired concentrations of chloride channel blocker NPPB. Ability of cell migration was determined using Transwell chamber. Absorbance value, which indicated relative cell number, was detected employing the MTT method. * $p < 0.05$ compared with control.

Figure 4. — Inhibition Ishikawa cell RVD by NPPB. Cells were perfused in hypotonic solution without (control) or with desired concentrations of chloride channel blocker NPPB. It was shown using cell volume measurement that NPPB inhibited Ishikawa cell RVD induced by hypotonic stimulation in a dose-dependent manner. *$p < 0.05$ compared with control.

Figure 5. — Inhibition Ishikawa cell $[Ca^{2+}]_i$ increase by NPPB. Cells were perfused in hypotonic solution without (control) or with desired concentrations of chloride channel blocker NPPB. It was shown by $[Ca^{2+}]_i$ measurement that NPPB inhibited Ishikawa cell $[Ca^{2+}]_i$ induced by VACC in a dose-dependent manner. *$p < 0.05$ compared with control.

Effects of Cl^- channel blocker on Ishikawa cell invasion and migration in vitro

To investigate the roles that VACC plays in the invasive behavior of endometrial cancer, the authors used a Transwell migration assay frequently used to assess cell chemotaxis and invasiveness. Ishikawa cells were plated on the upper side of a filter insert containing eight μm pores and were attracted to migrate through these pores towards the Matrigel. After 24 hrs incubation, the number of invaded cells was quantified using MTT assay. Invasion of Ishikawa cells was greatly reduced by 9.2 ± 1.3% ($p < 0.05$), 17.3 ± 3.1% ($p < 0.05$), 51.8 ± 3.9% ($p < 0.05$), 77.1 ± 4.2% ($p < 0.05$) in the presence of 25 μm, 50 μm, 100 μm, and 200 μm NPPB (Figure 2). After six hrs incubation, migration of Ishikawa cells was significantly inhibited by 8.6 ± 1.5% ($p < 0.05$), 20.3 ± 2.8% ($p < 0.05$), 53.6 ± 3.5% ($p < 0.05$), and 73.7 ± 3.6% ($p < 0.05$) in the presence of 25 μm, 50 μm, 100 μm, and 200 μm NPPB, respectively (Figure 3).

The effect of Cl^- channel inhibitor on RVD

Cell volume adaptive changes are critical for any cell survival. To investigate whether inhibition of Cl^- channel by the drug treated for invasion and migration assays perturb the tumor cells, ability to regulate cell volume, the authors experimentally altered the cell volume of Ishikawa cells by changes in bath osmolality and observed the effects of Cl^- channel inhibitor on volume regulation. They also rendered Ishikawa cells to a brief hypo-osmotic swelling and monitored their gradual volume decrease towards the original cell size. Data demon-

strated that exposure of cultured Ishikawa cells to a hypotonic solution (200 mOsm) induced rapid cell swelling, which was followed by a slow recovery up to 80% of the initial volume. Subsequently, the authors carried out an ion replacement experiment to prove Cl^- role in the RVD. Cells were perfused with a gluconate-based isotonic chloride-free solution for three hrs to deplete the intracellular Cl^-, and then perfused with a gluconate-based hypotonic chloride-free solution. Results indicated that RVD was almost completely abolished. However, cells recovered RVD when gluconate-based hypotonic solution was replaced with chloride-based hypotonic solution. Pretreatment with NPPB, which was added to the hypotonic solution, resulted in inhibition of RVD in a dose-dependent manner (Figure 4). NPPB at 25, 50, 100, and 200 μm inhibited RVD by 8.7 ± 2.1% ($p < 0.05$), 20.8 ± 2.3% ($p < 0.01$), 49.2 ± 3.7% ($p < 0.01$), and 67.4 ± 3.5% ($p < 0.01$), respectively. Taken together, these experiments showed that Cl^- plays a key role in cell swelling-mediated RVD; and NPPB, a conventional chloride channel blocker, can inhibit cell swelling-mediated RVD in a dose-dependent response.

The effect of Cl^- channel inhibitor on $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ is a important parameter in modulating actin filament turnover, and increase of $[Ca^{2+}]_i$ is part of the regulatory volume decrease. To further explore the mechanisms of VACC involvement in invasion and migration of endometrial cancer, the authors examined the effect of Cl^- channel inhibitor on Ca^{2+} influx. Exposure of cultured endometrial Ishikawa cancer cell to a hypotonic solution induced rapid cell swelling. This was followed by a slow recovery within the next 15 min, also referred to as RVD. Interestingly, the application of hypotonic media elicited a sharp rise of $[Ca^{2+}]_i$ to a peak concentration with 600 nM above its levels in isotonic conditions. Pretreatment with NPPB which was also added in the hypotonic solution, significantly reduced the peak Ca^{2+} response in a dose-dependent manner (Figure 5). NPPB at 25, 50, 100, and 200 μm inhibited the peak Ca^{2+} response by 12.1 ± 2.8% ($p < 0.01$), 24.6 ± 3.4% ($p < 0.01$), 47.0 ± 3.1% ($p < 0.01$), and 68.1 ± 4.5% ($p < 0.01$), respectively.

Discussion

There is ample evidence that indicates an important role for volume-activated chloride currents in regulating the proliferation and multidrug resistance of tumor cells [7, 8]. Currently, little information is known about its effect on malignant biology behavior of endometrial cancer cells. Although the molecular nature of VACC is not determined, CLC-3 which is a member of the voltage-gated CLC, has been considered the most likely molecular candidate of the VACC. The data in this present study showed that endometrial cancer Ishikawa cell line notably expressed CLC-3 by RT-PCR assay. Moreover some studies demonstrated that VACC contribute to the migration and invasion of cancer cells [9, 10]. In the present study, treatment with NPPB significantly inhibited invasion and migration of endometrial cancer cells in a dose-dependent response. At the same time, the authors found that Cl^- was necessary for RVD by ion replacement experiment and VACC-dependent RVD was positively correlated with invasion and migration of endometrial cancer cells. It is implicated that the VACC may play an important role in the invasion and migration of endometrial cancer cells.

How VACC are involved in control or regulation of cell invasion and migration is at present not clearly understood. VACC activation results in Cl^- efflux that is accompanied by cation and water efflux causing cell RVD. Cytoskeletal reorganization which is related to cell motility and migration is markedly affected by cell volume changes. In many cell types, RVD is associated with an increase, and cell swelling with a decrease in F-actin content [11]. Volume-sensitive Cl^- channels are also proposed to be related to the cytoskeleton or motility of the cells [12]. The authors, therefore speculate that it is possible that the activation of VACC and subsequent RVD may confer the endometrial cancer cells to a selective advantage for invasion and migration by cytoskeletal mechanisms. In fact, this conclusion is supported by the authors' studies in which VACC-dependent RVD is positively correlated with invasion and migration of endometrial cancer cells. Moreover, ion replacement experiment and endometrial cancer cell volume showed that Cl^- channel blocker (NPPB) reduced transmembrane Cl^- fluxes and greatly reduced osmotically-induced cell volume changes.

Ca^{2+} is an intracellular second messenger that plays a central role in signal transduction for many cellular responses. Many reports suggest that exposure of cells to hypoosmotic solutions has been shown to increase intracellular Ca^{2+} levels [13, 14]. RVD in epithelial cells is thought to be dependent on a rise in $[Ca^{2+}]_i$ [15]; moreover the distribution of intracellular free Ca^{2+} is not uniform. The rise of $[Ca^{2+}]_i$ which is more prominent in the cell body than in the lamellipodium [16] can loosen cell-matrix connections at the rear end [17] and isolate the cortical actomyosin gel, thus allowing a contraction at the rear end [18]. Thus, VACC-mediated volume loss and $[Ca^{2+}]_i$ increment mechanisms may act in concert during retraction of the rear end of a migrating cell. In this study, hypotonic stimulation caused a sharp rise of $[Ca^{2+}]_i$ in endometrial cancer cells. Interestingly, NPPB significantly inhibited the increase of $[Ca^{2+}]_i$ evoked by external Ca^{2+} in a dose-dependent manner, indicating that VACC may modulate Ca^{2+} influx into endometrial cancer cells, and subsequently regulate the invasion and migration of these cells by the cytoskeletal mechanisms. Considering the present study, the authors would like to present a network model to interpret how VACC affects invasion and migration of cancer cells. VACC activation results in RVD which affects the cytoskeletal reorganization, probably contributing to the invasion and migration of endometrial cancer. Meanwhile VACC activation gives rise to $[Ca^{2+}]_i$ increase which mediates RVD, loses cell-matrix connections, and is linked to cytoskeletal depolymerization and polymerization. A final result is that cancer cells develop

more invasively. In contrast, VACC blockers could interrupt these pathways, therefore inhibiting the invasion and migration of Ishikawa cancer cell. As to how the VACC affects Ca^{2+} influx, as well as what the link is between RVD and $[Ca^{2+}]_i$ in endometrial cancer, the authors are ready to investigate these in future work.

In summary, the present findings indicate that VACC may be essential for invasion and migration of endometrial cancer cell. It is likely that they modulate invasion and migration through RVD and $[Ca^{2+}]_i$ changes. Therefore, targeting VACC may provide a novel way to inhibit invasion and migration within tissues. It could be worthwhile to further explore the possibility of employing Cl^- channel blockers as a new class of antineoplastic drugs.

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Address reprint requests to:
M. LI, M.D.
Department of Obstetrics and Gynecology
Anhui Medical University Lujiang Road 17
Hefei 230001 Anhui (China)
e-mail: liminzhi2009@yahoo.com.cn