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Vibrio vulnificus Cytolysin Forms Anion-selective Pores on the CPAE Cells, a Pulmonary Endothelial Cell Line

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Cytolysin produced by *Vibrio vulnificus* has been incriminated as one of the important virulence determinants in *V. vulnificus* infection. Ion selectivity of cytolysin-induced pores was examined in a CPAE cell, a cell line of pulmonary endothelial cell, using inside-out patch clamp techniques. In symmetrical NaCl concentration (140 mM), intracellular or extracellular application of cytolysin formed ion-permeable pores with a single channel conductance of 37.5±4.0 pS. The pore currents were consistently maintained after washout of cytolysin. Replacement of Na^+ in bath solution with monovalent ions (K^+, Cs^+ or TEA^+) or with divalent ions (Mg^{2+}, Ca^{2+}) did not affect the pore currents. When the NaCl concentration in bath solution was lowered from 140 to 60 and 20 mM, the reversal potential shifted from 0 to -11.8 and -28.2 mV, respectively. The relative permeability of the cytolysin pores to anions measured at -40 mV was $Cl^- = NO_2^- \geq Br^- = I^- > SCN^- >$ acetate$^-$ > isethionate$^-$ > ascorbic acid$^-$ > $EDTA^{2-}$, in descending order. The cytolysin-induced pore current was blocked by Cl^- channel blockers or nucleotides. These results indicate that *V. vulnificus* cytolysin forms anion-selective pores in CPAE cells.

Key Words: *V. vulnificus*, Cytolysin, Pore, Ion selectivity

INTRODUCTION

Vibrio vulnificus is known to be a life-threatening pathogen that causes septicemia and serious wound infection in human. *V. vulnificus* infection is characterized by the high fatality rate of 70% and the primary attack against people who are immunocompromised or have underlying chronic liver disease such as liver cirrhosis (Blakes et al, 1979). *V. vulnificus* is frequently isolated from oysters and other shellfishes. Since it is naturally found in warm marine waters, people with open wounds can be exposed to *V. vulnificus* through direct contact with seawater. Although *V. vulnificus* infection can be treated with antibiotics such as doxycycline or a third-generation cephalosporin (e.g., ceftazidime), the effectiveness of those kinds of drugs are very low. Since *V. vulnificus* is a virulent pathogen leading to significant morbidity and mortality, it has been thought to relate in part to the production of exotoxin (Wickboldt & Sanders, 1983). The known extracellular cytolytic toxins produced by *V. vulnificus* are cytolysin (Kreger & Lockwood, 1981; Gray & Kreger, 1985), protease (Miyoshi & Shinoda, 1988), collagenase (Smith & Merkel, 1982), antiphagocytic surface antigen (Kreger et al, 1981), lipase (Desmond et al, 1984), siderophore (Simpson & Oliver, 1984), and phospholipase (Testa et al, 1984). Cytolysin showed cytolytic, vascular permeability activities against erythrocytes from animal species and against Chinese hamster ovary cells in tissue culture. Furthermore, it was lethal for mice and had vascular permeability factor activity in guinea pig skin (Gray & Kreger, 1985). Thus, cytolysin was considered as a critical factor in inducing local edema, tissue necrosis, necrotizing vasculitis, hemolytic anemia and death.

Generally, pore-forming toxins are known to cause lysis of nucleated cells such as monocytes, lymphocytes, and endothelial cells as well as erythrocytes by disrupting the membrane of target cells via pore formation. This transmembrane pore allows the leakage of ions and small molecules, and can lead to lysis of the target cell (Huestis, 1977; Bhakdi & Tranum-Jensen, 1988; Song et al, 1996). Kim et al. (1993) reported that cytolysin caused lysis of erythrocytes due to the formation of small pores on erythrocyte membrane by cholesterol-mediated oligomerization of cytolysin. Calcium exerted an inhibitory effect on *V. vulnificus* cytolysin-induced hemolysis as an osmotic protectant in mouse erythrocytes (Park et al, 1994). However, up to now, there is little information about electrophysiological property, especially ion selectivity of the transmembrane pore formed by cytolysin.

In the present study, we observed the transmembrane pore formation by cytolysin in CPAE cells, and identified its ion selectivity.

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ABBREVIATIONS: NFA, niflumic acid; FFA, flufenamic acid; DIDS, 4,4'-diisothio-cyanatostilbene-2,2'-disulfonic acid

METHODS

CPAE cell culture

The pulmonary endothelial cell line, CPAE (ATCC CCL 209) was obtained from Korea Cell Bank (College of Medicine, Seoul National University, Korea). CPAE cells were cultured in culture media [RPMI 1640 supplemented with 15% fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and amphotericin B (0.25 mg/ml)] in a humidified atmosphere of 5% CO_2. When CPAE cells (30～32 passages) had grown to confluence, adherent cells were removed under sterile conditions from the culture flask by gentle trypsinization (0.05% trypsin, 0.02% EDTA). The cell suspensions were immediately used for various experiments.

Purification of V. vulnificus cytolysin

A virulent strain of *V. vulnificus* E4125 was kindly supplied by Dr. M. H. Kothary (Department of Microbiology, Virulence Assessment Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Washington D.C.). The strain was cultured in the heart infusion diffusate broth as described by Kreger et al. (1988). Cytolysin was purified to homogeneity from the culture supernatant by a modification of the method developed by Kim et al. (1992).

Recording and analysis of membrane currents

Gigaseals were formed with Sylgard-coated pipettes (borosilicate, Kimax-51) with 4～5 megaohm resistances and single channel currents were recorded as previously described (Hamill et al, 1981). Channel currents were recorded with an Axopatch 1D patch clamp amplifier (Axon Instruments, Union City, California, USA) and stored on video tapes through digital recorder (PCM-501 ES, SONY, Tokyo, Japan) for subsequent computer analysis. Cut-off frequency was 2 kHz for an analysis of a single channel current and 300 Hz for recording on video tape. pClamp 6.03 software (Axon Instruments) was used for data acquisition and analysis. All experiments were carried out at 22±2°C. The pipette and bath solutions contained (in mM) 140 NaCl, 2 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.2). The 140 mM NaCl of bath solutions were replaced depending on the conditions of experiment. The deficit of osmolarity was compensated by adding glucose.

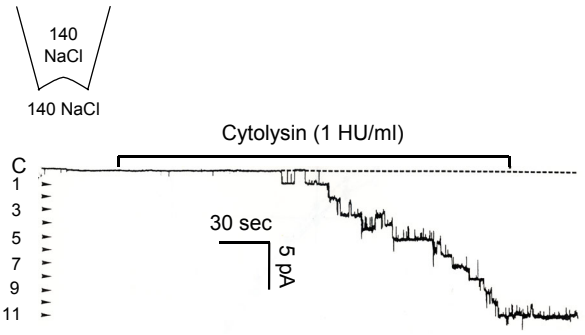

Fig. 1. Effect of intracellular-applied cytolysin on the pore formation measured in a CPAE cell using the inside-out patch clamp technique. The bath and the pipette solutions contained 140 mM NaCl symmetrically and membrane potential was held at -40 mV. The numbers beside the current trace indicate the number of pores. C represents a closed state.

Statistical analysis

Data are presented as meanSE. To calculate statistical significance, we used Student's *t*-test and a P-value of less than 0.05 was considered significant.

RESULTS

Pore formation by cytolysin

Fig. 1 shows the effects of intracellular-applied cytolysin on a pore formation measured from inside-out patch of CPAE cell. The bath and the pipette solutions are identical. In 2 min after treatment of cytolysin (1 HU/ml), the pores started to form and number of pore increased with time. The single pore conductance was 37.5±4.0 pS (n=11). This pore formation was maintained after washout of cytolysin. Similar results were obtained by cell-attached patch of CPAE cell after cytolysin was applied to the pipette solution (data not shown). Because the extracellular or intracellular application of cytolysin had a similar effect on the pore formation, we measured pore currents after 6～9 pores had been formed with cytolysin (1 HU/ml) employing inside-out patch for a convenience of cytolysin washout.

Permeability of the pore to monovalent cations

The cytolysin-induced inward currents (Fig. 1) might result from Na^+ influx. Thus, permeability of the pore to

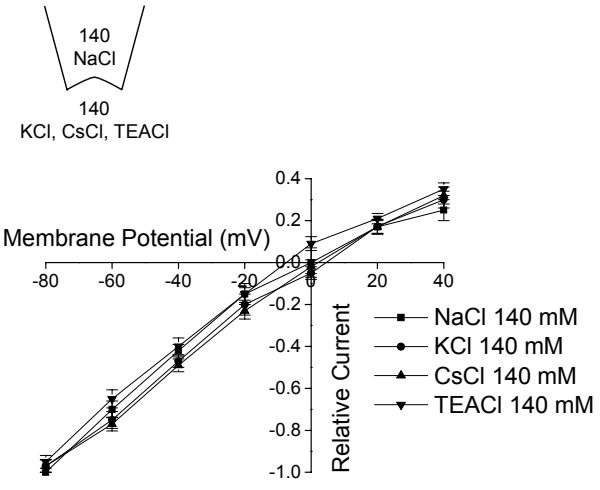

Fig. 2. Permeability of the transmembrane pore formed by cytolysin to monovalent cations. Pipette solution contained 140 mM NaCl. For bath solution, 140 mM NaCl was replaced with equimolar KCl, CsCl and TEACl, respectively. The membrane potential was held at -80 mV and the depolarizing pulses up to +40 mV in 20-mV steps were applied. Current-voltage relationships were normalized to the control current measured at -80 mV holding potential in bath solution containing 140 mM NaCl. Data are presented as meanSE (n=5, respectively).

various monovalent cations was investigated (Fig. 2). For bath solution, 140 mM NaCl was replaced with equimolar KCl, CsCl and TEACl, respectively. When current-voltage (I-V) relations were constructed, there was no statistical difference between permeability of the pore to each monovalent cation.

Permeability of the pore to divalent cations

Fig. 3 shows the ion selectivity of the cytolysin pores to divalent cations. NaCl (140 mM) in the bath solution was replaced with 10 mM NaCl plus 65 mM $MgCl_2$ or with 10 mM NaCl plus 65 mM $CaCl_2$, respectively. The resultant I-V relations showed that there was no statistical difference between permeability of the pore to each divalent cation.

Dependence of reversal potential (V_{rev}) on the intracellular Cl^- concentration

We measured the value of V_{rev} when 140 mM NaCl of bath solutions was replaced with different concentrations of NaCl. V_{rev} was calculated by Nernst equation (Nernst, 1888). As shown in Fig. 4A, the values of V_{rev} were shifted negatively, which is consistant with the values of V_{rev} based on the concentration of Cl^-: 0 mV for 140 mM NaCl, -11.8 mV for 60 mM NaCl and -28.2 mV for 20 mM NaCl. Although the observed values of V_{rev} deviated slightly from the estimated values calculated by Nernst equation (Fig. 4B), the negative shift of V_{rev} indicates that the cytolysin pores are anion-selective.

Permeability of the pore to anions

To investigate the permeability of cytolysin pores to anions, Cl^- in the bath solution was replaced with various anions as shown in Fig. 5. The relative permeability of the cytolysin pores to different anions at -40 mV was $Cl^- = NO_2^- \geq Br = I^- > SCN^- >$ acetate$^-$ > isethionate$^-$ > ascorbic acid$^-$ > $EDTA^{2-}$, in descending order. These results suggest that the cytolysin pore current are highly permeable to anions and furthermore more selective to anions.

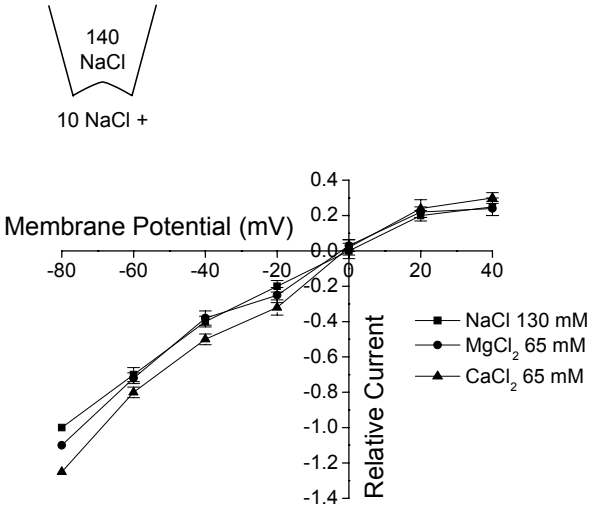

Fig. 3. Permeability of the transmembrane pore formed by cytolysin to divalent cations. Pipette solution contained 140 mM NaCl. And, 140 mM NaCl in the bath solution was replaced with 10 mM NaCl plus 65 mM $MgCl_2$ or with 10 mM NaCl plus 65 mM $CaCl_2$, respectively. The membrane potential was held at -80 mV and the depolarizing pulses up to +40 mV in 20-mV steps were applied. Current-voltage relationships were normalized to the control currents measured at -80 mV holding potential in bath solution containing 140 mM NaCl. Data are presented as meanSE (n=4, respectively).

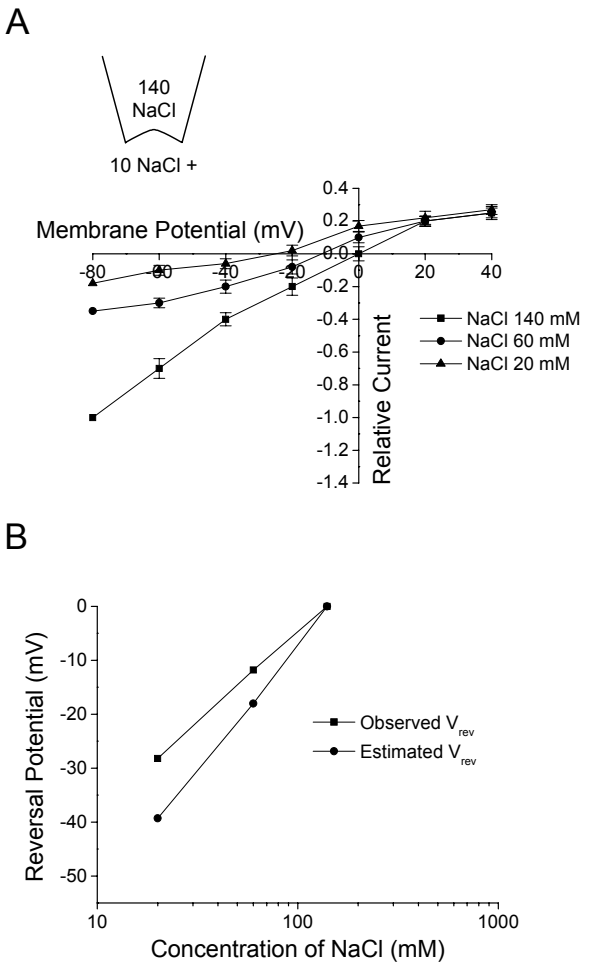

Fig. 4. Dependence of reversal potential (V_{rev}) on intracellular Cl^- concentrations. (A) Current-voltage relationships. The membrane potential was held at -80 mV and the depolarizing pulses up to +40 mV in 20-mV steps were applied. Current-voltage relationships were normalized to the control currents measured at -80 mV holding potential in bath solution containing 140 mM NaCl. And, 140 mM NaCl of bath solution was replaced with different concentrations of NaCl. Data are presented as meanSE (n=4, respectively). (B) Observed or estimated V_{rev} values. The estimated V_{rev} values were calculated by Nernst equation.

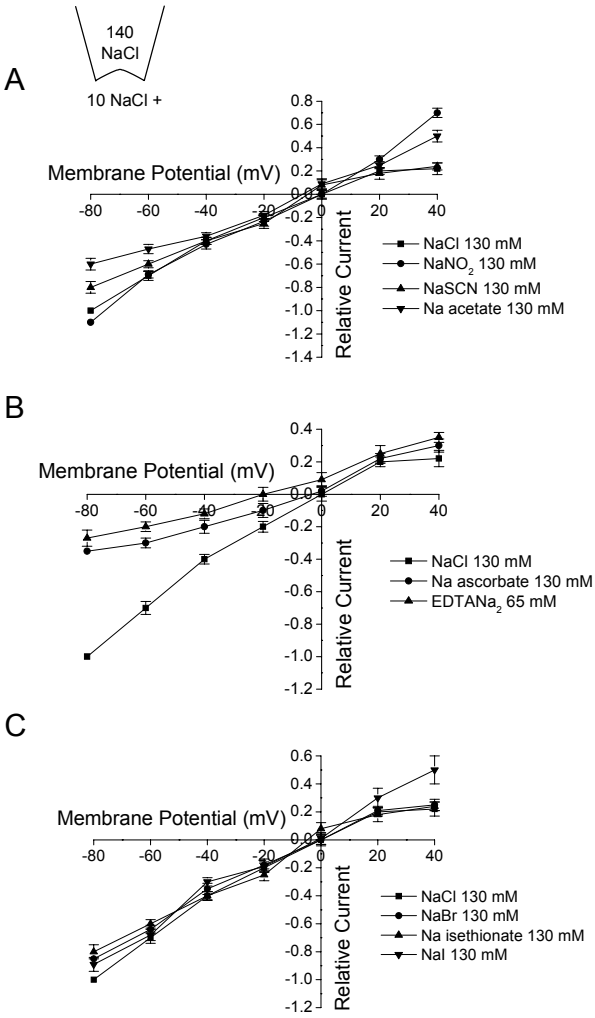

Fig. 5. Permeability of the cytolysin pores to anions. NaCl (140 mM) of bath solution was replaced with 10 mM NaCl plus chemicals that were marked as labels of line graphs, respectively. The membrane potential was held at -80 mV and the depolarizing pulses up to +40 mV in 20-mV steps were applied. Current-voltage relationships were normalized to the control currents measured at -80 mV holding potential with bath solution containing 140 mM NaCl. Data are presented as meanSE (n=4, respectively).

Effect of chloride channel blockers on cytolysin pore current

As shown in Fig. 6, we examined the effect of various Cl^- channel blockers on cytolysin-induced pore current. Although 160μM niflumic acid (NFA) and 60μM flufenamic acid (FFA) had no significant effect on the cytolysin-induced pore current (88.0±8.7 and 85.0±6.9% of the control, respectively, n=4), 4,4'-diisothio-cyanatostilbene-2, 2'-disulfonic acid (DIDS, 100μM) inhibited the pore current to 4.6±3.7% of the control (n=4).

Effects of nucleotides on cytolysin pore current

It has been reported that Cl^- channels are activated or inhibited by nucleotides (Gadsby & Nairn, 1999). Thus, we

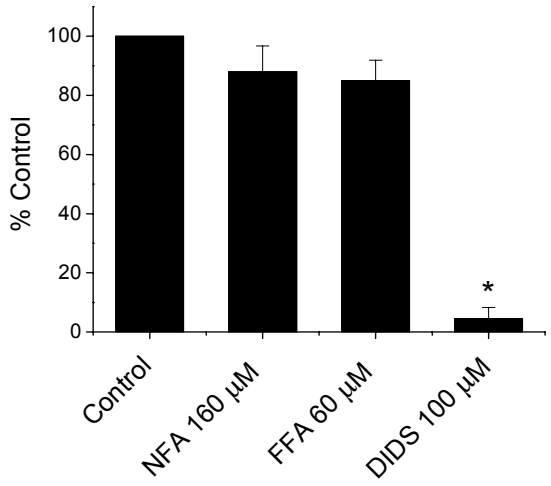

Fig. 6. Effects of various Cl^- channel blockers on cytolysin pore currents. The bath and pipette solutions contained 140 mM NaCl symmetrically and membrane potential was held at -40 mV. The currents were normalized to the control currents obtained in the absence of chemicals. Data are presented as mean±SE (*$P < 0.05$, n=4, respectively).

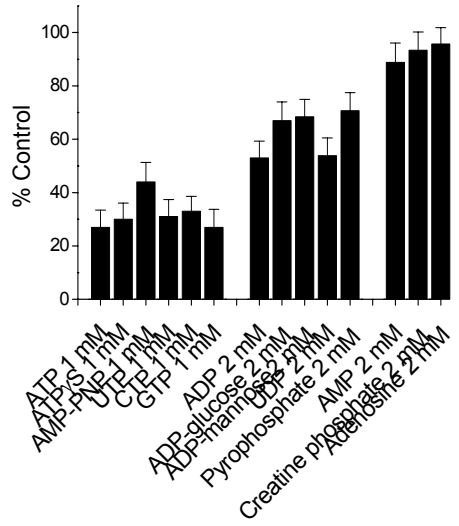

Fig. 7. Effects of nucleotides on cytolysin pore currents. The bath and pipette solutions contained 140 mM NaCl symmetrically and membrane potential was held at -40 mV. The currents were normalized to the control currents obtained in the absence of chemicals. Data are presented as meanSE (n=4, respectively).

examined the effect of nucleotides on cytolysin pore current by using various nucleotides and their derivatives (Fig. 7). Their inhibitory effects on the pore currents were detected, which were dependent on number of phosphate nucleotides.

That is, the nucleotides containing triphosphate showed the strongest inhibitory effect on the pore currents, while the nucleotides containing two phosphates inhibited with less inhibitory potency compared with those of triphosphate-containing nucleotides: 1 mM of ATP, ATPγS, AMP-PNP, UTP, CTP and GTP having three phosphates reduced the pore current to 27.0±6.5, 30.0±6.1, 44.0±7.3, 31.0±6.4, 33.0±5.6 and 27.0±6.7% of the control, respectively (n=4), while 2 mM of ADP, ADP-glucose, ADP-mannose, UDP and pyrophosphate reduced the pore current by 53.0±6.3, 67.0 ±7.0, 68.4±6.6, 53.8±6.7 and 70.7±6.8% of the control, respectively (n=4). Additionally, monophosphate-containing drugs had little effect on the pore currents: 2 mM of AMP, creatine phosphate and adenosine reduce the pore current by 88.8±7.3, 93.4±6.8 and 95.7±6.1% of the control, respectively (n=4).

DISCUSSION

In the present study, we investigated the transmembrane pore formation by *V. vulnificus* cytolysin in CPAE cells, and its ion permeability. Our study clearly showed that the pores were formed by cytolysin and were more permeable to anions. And the pore current was found to be blocked by DIDS and some nucleotides.

Generally, pore-forming toxins are known to cause lysis of nucleated cells by disrupting the membrane of target cells via pore formation. This transmembrane pore can lead to lysis of the target cell by allowing the leakage of ions and small molecules (Huestis, 1977; Bhakdi & Jensen, 1988; Song et al, 1996). The pathogenetic roles of cytolysin in *V. vulnificus* infection are controversial (Oliver et al, 1986; Wright et al, 1991). However, cytolysin is very powerful and is still one of the most likely candidates for the pathogenesis of disease (Miyoshi et al, 1993). Furthermore, Park et al. (1996) reported that cytolysin increased vascular permeability and neutrophil sequestration in the lungs and those were important factors in lethal activity by cytolysin. Our study showed that cytolysin induced a pore current in pulmonary endothelial cells. This result suggests that cytolysin can increase the ionic permeability of the pulmonary vessels and cause lethal activities.

It has been known that hemolysins secreted in *Proteus vulgaris* and *Morganella morganii* formed pores in artificial membranes which had similar channel properties with exclusive cation selectivity (Benz et al, 1994). However, α-toxin of *Staphylococcus aureus* formed pores which were anion-selective (Korchev et al, 1995). In the present study, there are several evidences showing that the pore formed by *V. vulnificus* cytolysin in CPAE cells is more selective to anions than cations. First, the pore current was not affected when Na^+, the major cation in the bath solution was replaced with various monovalent or divalent cations. Second, the reversal potential measured under various concentrations of NaCl in bath solution was dependent on the concentration of Cl^- rather than Na^+. Third, cytolysin-induced current was blocked by Cl^- channel blocker (DIDS), although it remains to be identified which types of anion channels are involved. The relative permeability sequence of the cytolysin pores to anions was $Cl^- = NO_2^- \geq Br^- = I^- > SCN^- >$ acetate$^-$ > isethionate$^-$ > ascorbic acid$^-$ > $EDTA^{2-}$. The permeability of anions to the pore is opposite to their size. However, I^- is more permeable than isethionate$^-$ (almost the same molecular weight as I^-) or SCN^- (less than half molecular weight of I^-). These results indicate that size or three dimensional structures are important factors to determine the permeability of the pore to different anions.

Although we little know the detailed electrophysiological mechanisms of cytolysin-induced cytotoxicity *in vivo*, we could hypothesize that cytolysin formes pores through which Cl^- influx occurs, resulting in cell swelling. This swelling activates a stretch-activated Ca^{2+} channel followed by increase in intracellular Ca^{2+} concentration. Simultaneously, loss of K^+ is followed in order to regulate cell volume (Rothstein & Mack, 1992; Pasantes-Morales & Morales, 2000). Thus, the cell will die by unbalance of electrolytes which is maintained by Na^+-K^+ pump or Ca^{2+} pump. This hypothesis could be applied to develop new drugs for preventing from cytolysin-induced cytotoxicity. Our results showed that a chloride channel blocker (DIDS) and nucleotide triphosphates or their derivatives blocked cytolysin pore current. Thus, the channel-like pore formed by *V. vulnificus* cytolysin can be a new target for treating diseases related with *V. vulnificus* infection if further studies done.

In conclusion, this is the first report showing that *V. vulnificus* cytolysin forms anion-selective pores in a cell line of pulmonary endothelial cells, which were blocked by DIDS and nucleotides. These results could propose a new concept in drug development for the treatment of *V. vulnificus*-related diseases.

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