Korean J Physiol Pharmacol
Vol 3: 199−205, April, 1999

Increase of Intracellular Ca^{2+} Concentration by *Vibrio Vulnificus* Cytolysin in Rat Platelets; Triggering Mechanism of Platelet Cytolysis

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Vibrio vulnificus cytolysin caused platelet cytolysis and increased intracellular calcium concentration ($[Ca^{2+}]_i$) of rat platelets in a concentration-dependent manner. In the presence of *V. vulnificus* cytolysin (3 HU/ml), lactate dehydrogenase (LDH) activity was increased from 1.3±0.4% of control to 64.3±3.4% in platelet suspension buffer. In Ca^{2+}-free platelet suspension buffer, however, *V. vulnificus* cytolysin did not induce $[Ca^{2+}]_i$ increase and LDH release. Addition of EGTA (2 mM) to suspension buffer after the initial Ca^{2+} influx reversed $[Ca^{2+}]_i$ to the control level. However, a Ca^{2+} channel blocker verapamil (20 μM) or mefenamic acid (20 μM) did not inhibit *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and LDH release. Divalent cations such as Co^{2+}, Cd^{2+} or Mn^{2+} (2 mM each) also did not alter *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and LDH release. *V. vulnificus* cytolysin (3 HU/ml)-induced calcium influx was completely blocked by lanthanum (2 mM). Lanthanum (2 mM) also completely blocked *V. vulnificus* cytolysin (3 HU/ml)-induced LDH release. Osmotic protectants such as, raffinose, sucrose or PEG600 (50 mM each) did not inhibit the lytic activity of *V. vulnificus* cytolysin. In conclusion, lanthanum sensitive Ca^{2+} influx plays a significant role in *Vibrio vulnificus* cytolysin-induced platelet cytolysis and thrombocytopenia in *V. vulnificus* infection.

Key Words: *Vibrio vulnificus* cytolysin, Platelet cytolysis, Ca^{2+} channel blocker, LDH, $[Ca^{2+}]_i$

INTRODUCTION

Vibrio vulnificus caused septicemia and serious wound infection in immunocompromised state and underlying diseases such as liver cirrhosis or hemochromatosis in the human (Blake et al, 1979; Park et al, 1991; Gholami et al, 1998; Kumamoto & Vukichi, 1998; Shapiro et al, 1998; Tsusuki et al, 1998). *V. vulnificus* cytolysin which was detected in culture medium of *V. vulnificus* showed hemolytic activity and cytotoxicity in cultured mammalian (Kreger & Lockwood, 1981; Gray & Kreger, 1985). Although the pathogenetic roles of *V. vulnificus* cytolysin are so far controversial (Wright et al, 1990; Oliver et al, 1995), *V. vulnificus* cytolysin is one of the most possible candidates in the pathogenesis of *V. vulnificus* infection (Miyoshi et al, 1988). Even submicrogram of *V. vulnificus* cytolysin is fatal to mice when injected intravenously (Gray & Kreger, 1985). However, the target cells or pathogenesis of *V. vulnificus* cytolysin has not been known enough.

Platelets have been used as a useful model in the study of transmembrane signaling since they are highly responsive to various agents and they have active biochemical and morphological events (Honemann et al, 1998; Shiraga et al, 1998). Platelets play a role in thrombus formation, hemostasis and regeneration of vessels responding to a wide variety of stimuli. These small disc-shaped cells recognize even minor damages occurred on the surface of the endothelial cells lining blood vessels. They promptly respond to physical or chemical stimuli by undergoing adhesion, shape change, secretion and aggregation. Such a series of cell responses ends in the formation of a hemostatic plug or thrombus (Nozawa et al,

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1991). Staphylococcus α-toxin which has a similar action to *Vibrio* cytolysin is known to cause blood coagulation and form small pores on erythrocyte membrane (Bhakdi et al, 1988; Bhakdi et al, 1996). *V. vulnificus* has been known to cause hemolysis and thrombocytopenia (Kim et al, 1993; Park et al, 1994; Hirono et al, 1996). In the present study, the effects of *V. vulnificus* cytolysin on platelet were examined as a possible mechanism for thrombocytopenia in *V. vulnificus* infection.

METHODS

Bacterial strain and culture

A virulent strain of *Vibrio vulnificus* E4125 was kindly supplied by Dr. MH 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 heart infusion diffusate broth (Gibco) at 37°C for 4 hours as described by Kreger et al (1981).

Preparation of cytolysin

The cytolysin was purified from the supernatant of *V. vulnificus* culture by ammonium sulfate fractionation, calcium phosphate gel adsorption, quaternary methylamine anion-exchange chromatography and octyl-Sepharose CL-4B chromatography (Kim et al, 1992).

The hemolytic activity of the cytolysin against mouse erythrocyte was determined using the method developed Bernheimer & Schwartz (1963). Briefly, the cytolysin was diluted with phosphate-buffered saline (67 mM Na_2HPO_4, 77 mM NaCl, pH 7.4) containing 1 mg/ml of bovine serum albumin (PBS-BSA). The cytolysin (1 ml) was mixed with the equal volume of 0.7% mouse erythrocyte suspension in PBS-BSA (final concentration 0.35%). The mixture was incubated at 37°C for 30 min and centrifuged (500 g) at 4°C for 3 min. From the supernatant the absorbance of hemoglobin was measured at the wavelength of 545 nm. One hemolytic unit (HU) was defined as the amount which liberates half of the hemoglobin in the erythrocyte suspension under these experimental conditions.

Preparation of washed platelets

Fresh blood was obtained from adult Sprague-Dawley rat (250~300 g). Blood was collected into plastic tubes containing 1.8% sodium citrate solution (1/9 volume of blood) at pH 4.5 and subsequently centrifuged at 100 g for 10 min to obtain platelet-rich plasma (PRP). PRP was then centrifuged at 1000 g for 20 min at room temperature (20~25°C). The pellet was washed twice with Tyrode/HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) solution (pH 6.35) and centrifuged at 1000 g for 20 min. The resultant pellet was resuspended in the second Tyrode/HEPES solution (pH 7.35) with a final density of approximately 5×10^8 cells/ml. The Tyrode/HEPES solution was composed of (mM) NaCl 138.3, KCl 2.68, $MgCl_2 \cdot 6H_2O$ 1.0, $NaHCO_3$ 4.0, HEPES 10, glucose 5.55 and albumin 1.35% (w/v).

Measurement of lactate dehydrogenase (LDH) release

LDH is known to be released into the supernatant from the cytosol of damaged cells. Therefore, cytolysis was quantified by measurement of LDH activity using colormetric assay. LDH was determined with the use of a commercially available test kit (Cytotoxicity Detection Kit, Boehringer Mannheim, Germany). The cytolysin was diluted with PBS-BSA buffer and the mixture was incubated at 37°C for 5 min and centrifuged. The absorbance of LDH in the supernatant was measured at 495 nm.

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

In resuspended platelet solution, 4 μM fura-2 AM was added and incubated for 30 min at 37°C. Platelets were subsequently washed with the Tyrode/HEPES solution (pH 7.35) to remove extracellular fura-2 AM. Then, the number of platelets was readjusted to 1×10^8/ml. Fura-2 was excited alternately by UV light at 340 nm and 380 nm using a rotating filter wheel. The intensity of fluorescence was measured through a 510 nm filter with a photomultiplier (Photon Technology International, NJ, USA). The signal at 340 nm was divided by that at 380 nm and from this ratio, $[Ca^{2+}]_i$ was calculated. The platelet suspension (1.5 ml) in a quartz cuvette was preincubated at 37°C for 5 minutes except these experiments done at 4°C. 2 minutes after

the addition of $CaCl_2$ at a final concentration of 1 mM, cytolysin was added and the responses were monitored for maximum of 5 min. Various blockers were preincubated for 5 min before the addition of cytolysin.

Statistics

Statistical analysis were performed with the Student's t-test where appropriate. Changes were considered significant at $P<0.01$. Data were expressed as mean±S.E.M..

RESULTS

At a concentration range of 1~10 HU/ml, *V. vulnificus* cytolysin increased the $[Ca^{2+}]_i$ and cytolysis of platelet in a concentration-dependent manner (Fig. 1). At 1 HU/ml, *V. vulnificus* cytolysin increased $[Ca^{2+}]_i$ slightly, which reached the steady state with no further cytolysis within 400 seconds upon addition to the platelet. At concentrations of 2~10 HU/ml, *V. vulnificus* cytolysin increased $[Ca^{2+}]_i$ progressively without reaching the plateau during the period of experiments (Fig. 1A). LDH activity measured as a marker of platelet cytolysis was also increased in a concentration-dependent manner by *V. vulnificus* cytolysin. At 1 HU/ml, less than 5% of the total LDH activity was detected in the platelet suspension buffer (control: 0.89±0.99%, n=5), while the activity increased substantially as the concentration was raised only slightly, such as 40.34±4.68%, 63.54±5.34% and 67.45±4.21% (n=5) by the treatment of 2, 3 and 10 HU/ml *V. vulnificus* cytolysin, respectively (Fig. 1B).

To elucidate a role of extracellular calcium ($[Ca^{2+}]_o$) in *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase, the effects of *V. vulnificus* cytolysin was examined in Ca^{2+}-free buffer added with 2 mM EGTA (Fig. 2). *V. vulnificus* cytolysin (3 HU/ml) caused a slight increase in $[Ca^{2+}]_i$ which was delayed in its onset as compared with when $[Ca^{2+}]_o$ was present (Fig. 2A). *V. vulnificus* cytolysin (3 HU/ml)-induced LDH release was prevented from 64.3±3.4% to 1.3±0.4% when platelets were preincubated with 2 mM EGTA (Fig. 2B). Addition of 2 mM EGTA at 300 seconds after the addition of the *V. vulnificus* cytolysin returned $[Ca^{2+}]_i$ to the control level and prevent LDH activity increase (Fig. 2).

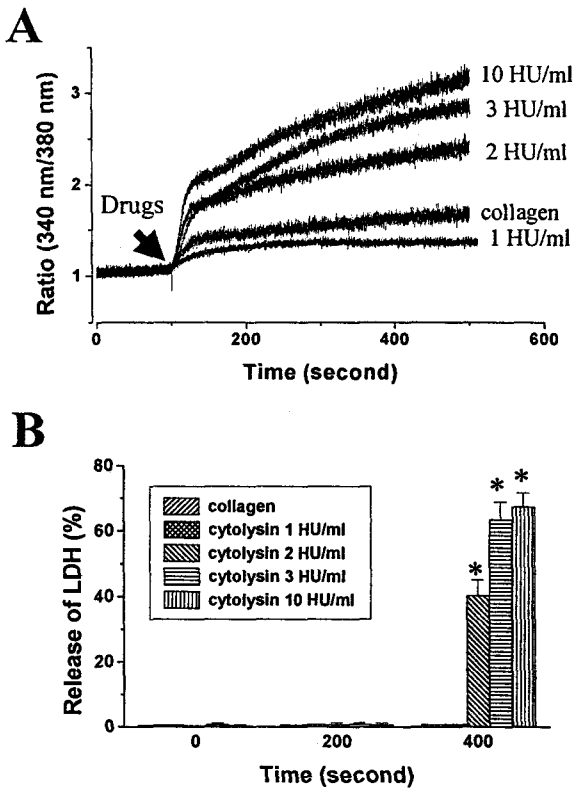

Fig. 1. Effects of *Vibrio vulnificus* cytolysin on intracellular calcium concentration and LDH release from platelets. Traces of $[Ca^{2+}]_i$ change recorded in platelets loaded with fura-2 and stimulated with various concentrations of *V. vulnificus* cytolysin (1~10 HU/ml) (A). Platelet suspension was preincubated at 37°C for 5 minutes before the addition of *V. vulnificus* cytolysin. LDH activities were measured before (0 sec) and after (200 sec and 400 sec) the addition of *V. vulnificus* cytolysin. Values were expressed as mean±S.E.M. from five independent experiments. * indicate $P<0.01$, (B). Collagen (1 μg/μl) was used as a positive control.

V. vulnificus cytolysin binds to the cell membrane and oligomerizes to form K^+-permeable but Ca^{2+}-impermeable small pores on the erythrocyte membranes (Kim et al, 1993). The *V. vulnificus* cytolysin-induced pore of pulmonary endothelial cell was shown to be blocked by a chloride channel blocker, DIDS (Han, 1997). In the present study, we have examined the effects of the organic and inorganic ion channel blockers on *V. vulnificus* cytolysin-induced Ca^{2+} influx in attempts to identify the ionic mechanism involved. Pretreatment with lanthanum (2 mM) completely abolished *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and LDH release (Fig. 3). However, verapamil (20 μM), a voltage dependent calcium

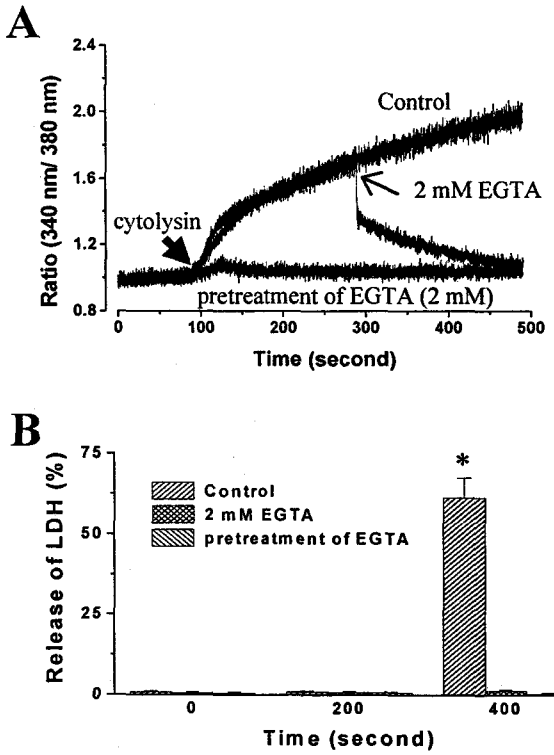

Fig. 2. Effects of extracellular Ca^{2+} on *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase (A) and LDH release (B). Traces represent $[Ca^{2+}]_i$ change in platelets stimulated with *V. vulnificus* cytolysin (3 HU/ml) in the presence of EGTA (2 mM). EGTA was applied before and after cytolysin (A). Effects of EGTA on LDH levels measured as described in Fig. 1 (B).

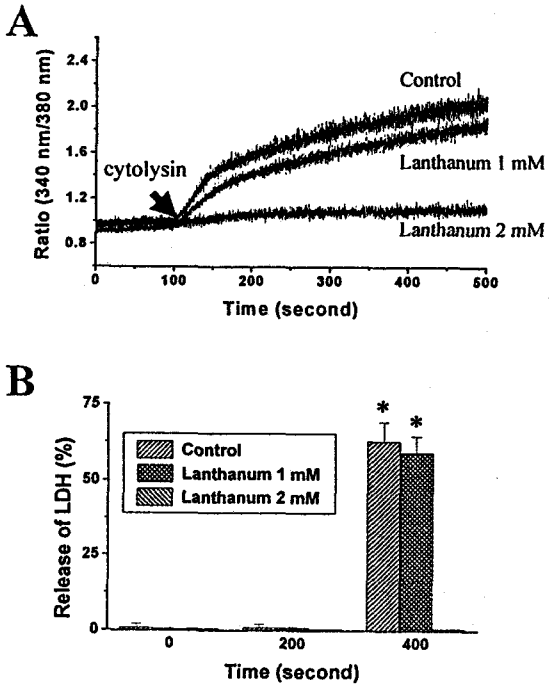

Fig. 3. Effects of lanthanum on *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase (A) and LDH release (B). Traces represent $[Ca^{2+}]_i$ change stimulated with *V. vulnificus* cytolysin (3 HU/ml) in the presence of 1 or 2 mM lanthanum (A). Platelet suspension was preincubated at 37°C for 5 minutes with lanthanum before the addition of *V. vulnificus* cytolysin. LDH activities were given as described in Fig. 1.

channel blocker, mefenamic acid (20 μM), a non-selective calcium channel blocker failed to inhibit *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase. Divalent cations such as Cd^{2+}, Co^{2+} and Mn^{2+} are also known to interfere the calcium influx and platelet aggregation (Renterghem & Lazdunski, 1994; Rho et al, 1995). However, Cd^{2+}, Co^{2+} or Mn^{2+} (2 mM each) did not block *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase (data not shown). Thus, the Ca^{2+} channel blockers tested failed to alter *V. vulnificus* cytolysin-induced LDH release. DIDS also failed to block *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and LDH release (Fig. 4).

It is known that at low temperature (4°C) a cytolysin binds to the cell membrane but can not oligomerize in the cell membrane (Kim et al, 1993). The oligomerization of cytolysin bound to the membrane is necessary to the pore formation (Fussle et al, 1981; Buckingham & Duncal, 1983; Bhakdi et al, 1986; Bhakdi & Tranum-Jensen, 1991). It was tested, therefore, whether *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase was originated from binding to the membrane or the formation of pore by the cytolysin (Fig. 5). *V. vulnificus* cytolysin (3 HU/ml) neither increase $[Ca^{2+}]_i$ nor the LDH activity at 4°C. As the temperature was increased subsequently by incubating the platelets in a fresh buffer at 37°C, *V. vulnificus* cytolysin (3 HU/ml) increased the $[Ca^{2+}]_i$. The LDH activity also increased from 1.55±0.55% to 58.55±6.44% in the platelets reincubated at 37°C (Fig. 5B).

Raffinose (MW, 504.5) as an osmotic protectant was known to prevent *V. vulnificus* cytolysin-induced hemolysis (Kim et al, 1993). In the present study, raffinose (50 mM; MW, 504.5), sucrose (50 mM; MW, 342.3) or PEG 600 (50 mM; MW, 600.0) failed to inhibit *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase or LDH release (Fig. 6).

Liou et al (1993) reported that Taiwan cobra venom cardiotoxin induced-platelet cytolysis caused rapid incorporation of actin monomers into cross-

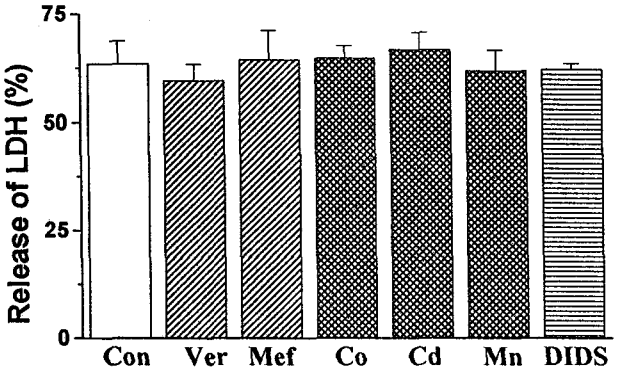

Fig. 4. Effect of various ion channel blockers on *V. vulnificus* cytolysin induced LDH release. One of the following blockers was added to platelet suspension buffer; verapamil (Ver, 20 μM), mefenamic acid (Mef, 20 μM), cadmium (2 mM), cobalt (2 mM) manganese (2 mM), lanthanum (2 mM) and DIDS (100 μM). LDH activities were measured at 300 seconds after the treatment of *V. vulnificus* cytolysin (3 HU/ml). Values are given as mean±S.E.M. of five independent experiments.

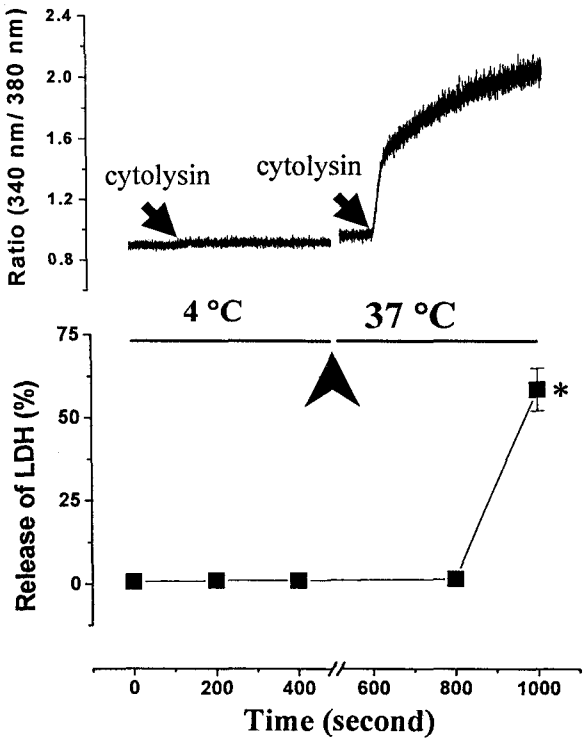

Fig. 5. Effects of temperature on *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase (A) and LDH release (B). Platelet suspension was preincubated at 4°C for 5 minutes before addition of *V. vulnificus* cytolysin (3 HU/ml). Platelets were resuspended in fresh buffer to remove *V. vulnificus* cytolysin and incubated at 37°C for 5 min before 2nd treatment of *V. vulnificus* cytolysin (3 HU/ml). Traces represent $[Ca^{2+}]_i$ change by *V. vulnificus* cytolysin (3 HU/ml) at 4°C and 37°C (A). LDH activities were measured at given times and values were represented as mean±S.E.M. of five independent experiments. * indicate $P<0.01$ (B).

linked actin filaments. Therefore, we examined whether cytoskeleton inhibitors prevented *V. vulnificus* cytolysin-induced platelet cytolysis. Phalloidin (20 μM) an actin depolymerization agent, colchicine (20 μM) an actin polymerization inhibitor or cytochalasin B (20 μM), a microtubule depolymerization agent applied at 100 seconds before addition of *V. vulnificus* cytolysin (3 HU/ml) had no effects on *V. vulnificus* cytolysin-induced platelet cytolysis (Fig. 6).

DISCUSSION

Although thrombocytopenia was observed in *V. vulnificus* infection (Park et al, 1991), the target cells or pathogenesis of thrombocytopenia in *V. vulnificus* infection has not been known. The aim of the present study was to prove that platelet is a target cell for *V. vulnificus* infection resulting in thrombocytopenia. Two major findings confirmed that *V. vulnificus* cytolysin elicited platelet cytolysis triggered by $[Ca^{2+}]_i$ increase in rat platelets. First, *V. vulnificus* cytolysin increased the $[Ca^{2+}]_i$ of platelets and induced LDH release from rat platelets. Second, block of Ca^{2+} influx always diminished *V. vulnificus* cytolysin-induced platelet cytolysis.

It has not been established whether calcium is required for the hemolytic action of bacterial toxin (Jorgensen et al, 1983; Bhakdi et al, 1986; Park et al, 1994). The observation that a small increase in $[Ca^{2+}]_i$ to 1 HU/ml of *V. vulnificus* cytolysin could not induce platelet cytolysis (Fig. 1) suggest that some kind of $[Ca^{2+}]_i$ threshold may exist for cytolysis to occur. Within at least 200 seconds after the addition of *V. vulnificus* cytolysin, inhibition of continuous increase of $[Ca^{2+}]_i$ prevented platelet cytolysis measured as LDH activity (Fig. 2). These results suggest that a continuous increase in $[Ca^{2+}]_i$ for enough time is required for *V. vulnificus* cytolysin to induce cytolysis in platelets. The present study also showed that external calcium was essential for the *V. vulnificus* cytolysin-induced platelet cytolysis. It was characterized by the dependence on the presence of

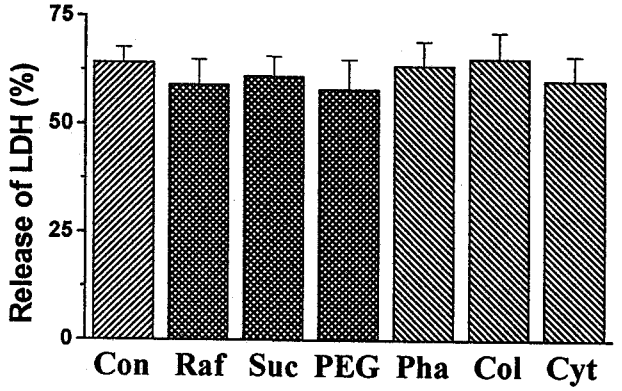

Fig. 6. Effects of osmotic protectants and cytoskeleton inhibitors on *V. vulnificus* cytolysin-induced LDH release. Platelet suspensions were preincubated with one of the cytoskeleton inhibitors, phalloidin (Pha, 20 μM), colchicine (Col, 20 μM) and cytochalasin B (Cyt, 20 μM) or the osmotic protectants, raffinose (Raf, 50 mM), sucrose (Suc, 50 mM) and PEG 600 (PEG, 50 mM) before the treatment of *V. vulnificus* cytolysin (3 HU/ml). LDH activities were measured at 300 seconds after the treatment of *V. vulnificus* cytolysin and values are given as mean±S.E.M. of four independent experiments.

external Ca^{2+} (Fig. 2). It was also supported by the observation that lanthanum sensitive Ca^{2+} influx was solely responsible for *V. vulnificus* cytolysin-induced platelet cytolysis (Fig. 3).

Membrane perturbation of peptides could stimulate phosphoinositide turn over resulting in Ca^{2+} release from the intracellular Ca^{2+} store (Suh et al, 1996). Although lanthanum sensitive-Ca^{2+} influx was essential for *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and platelet cytolysis, Ca^{2+} may be also released from the intracellular store. When the cells were pretreated with EGTA (Fig. 2), *V. vulnificus* cytolysin was able to increase $[Ca^{2+}]_i$ slightly after the time lapse. However, it was clear that *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and the subsequent platelet-cytolysis were significantly dependent on Ca^{2+} influx through the cell membrane.

Hemolysis caused by pore-forming bacterial cytolysins has been classified into colloid-osmotic (Fussle et al, 1981; Bhakdi et al, 1996) and noncolloid-osmotic process (Buckingham & Duncal, 1983). Kim et al (1993) reported that *V. vulnificus* cytolysin caused colloid-osmotic hemolysis. However, our observations that *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and LDH release were not altered by osmotic protectants (Fig. 6) suggest that *V. vulnificus* cytolysin-induced platelet cytolysis may not colloid-osmotic.

In the platelets, cytoskeleton regulates properties of the membrane such as its contours and stability. In the unstimulated platelet, 60~70% of the actin is thought to be prevented from polymerizing. When platelets are activated, there is a rapid increase in actin polymerization (Fox, 1993). Polymerization of actin monomers was also reported in snake venom-induced platelet cytolysis (Liou et al, 1993). However, *V. vulnificus* cytolysin-induced LDH activity increase was not affected by cytoskeleton inhibitors (Fig. 6), suggesting that the cytoskeleton is not involved in *V. vulnificus* cytolysin-induced platelet cytolysis. The idea that *V. vulnificus* cytolysin-induced platelet cytolysis was mediated by pore formation resulted from oligomerization *V. vulnificus* cytolysin was supported by the temperature dependence of *V. vulnificus* cytolysin-induced $[Ca^{2+}]_i$ increase and LDH release (Fig. 5).

In conclusion, *V. vulnificus* cytolysin-induced platelet cytolysis should be triggered by lanthanum-sensitive Ca^{2+} influx and it would be a possible ionic mechanism for thrombocytopenia in *V. vulnificus* infection.

ACKNOWLEDGEMENTS

This study was, in part, supported by a grant CHMP-97-M-2-0025) of the 97 good heath R & D project, Ministry of Health & Welfare, R.O.K..

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