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Potent Membrane-Permeabilizing and Cytocidal Action of *Vibrio cholerae* Cytolysin on Human Intestinal Cells

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Many strains of *Vibrio cholerae* non-O1 and O1 El Tor that cause diarrhea do not harbor genes for a known secretogenic toxin. However, these strains usually elaborate a pore-forming toxin, hitherto characterized as a hemolysin and here designated *V. cholerae* cytolysin, whose action on intestinal cells has not yet been described. We report that *V. cholerae* cytolysin binds as a monomer to Intestine 407 cells and then assembles into detergent-stable oligomers that probably represent tetra- or pentamers. Oligomer formation is accompanied by generation of small transmembrane pores that allow rapid flux of K^+ but not influx of Ca^{2+} or propidium iodide. Pore formation is followed by irreversible ATP depletion and cell death. Binding of fewer than 10^4 toxin molecules per cell in vitro is lethal. The possibility is raised that production of this toxin by bacteria that are in close contact with intestinal cells is rapidly cytocidal in vivo, and death of intestinal cells may be a cause of diarrhea.

Enteropathogenic *Vibrio cholerae* can elaborate two basically different categories of exotoxins. The first is represented by classic cholera toxin (CT), zonula occludens toxin, and accessory cholera toxin (7, 25). The genes encoding the latter are located in the "virulence cassette region" and do not occur independently of the CT gene (6). A membrane-damaging toxin, hitherto referred to as a hemolysin (4, 9, 11, 12, 21, 30, 31) and here designated the *V. cholerae* cytolysin (VCC) to underline its capacity to damage nucleated cells, represents the second type of *Vibrio* exotoxin. Production of VCC is not coupled to the expression of CT.

V. cholerae O1 biotype El Tor, the cause of the present pandemic of cholera, was historically differentiated from classical *V. cholerae* O1 by its ability to produce VCC. Later, Richardson et al. demonstrated that classical *V. cholerae* O1 strains produce another (second) hemolysin. This second hemolysin remains to be more closely characterized, but it appears to be distinct from VCC (21). A pathogenetic role of VCC has not been clearly established, however, and a few conflicting reports exist in the literature. Thus, *V. cholerae* O1 mutants in which both the CT and VCC genes were deleted still caused diarrhea in volunteers (15). On the other hand, deletion of the gene for HlyU, the regulator of VCC gene expression, led to a 100-fold decrease in virulence in an animal model (28). Also, epidemiological studies have shown that gastroenteritis cases due to CT-negative, hemolytic *V. cholerae* O1 El Tor have occurred (10, 17). During a 3-year period from 1989 to 1991, 31 patients infected with *V. cholerae* O1 strains that were hemolytic but lacked the gene encoding CT were identified in Uzbekistan (34). Evidence thus persists that the hemolytic phenotype is, indeed, an indicator of virulence in CT-negative *V. cholerae* O1 strains. In the same context, *V. cholerae* non-O1 strains recognized as important causes of diarrheal diseases and isolated worldwide (3, 16) usually do not produce CT (29) but are hemolytic.

The genes encoding *V. cholerae* O1 El Tor and non-O1 hemolysin have been cloned and sequenced and found to be highly homologous to each other (4). The proteins, which we here collectively designate VCC, have been purified and partially characterized as pore-forming toxins (11, 30, 31). Their membrane-permeabilizing action has been demonstrated in erythrocytes, planar lipid bilayers, and, most recently, liposomes (14, 32, 33). The VCC has been shown to be cytotoxic to various cell lines (11, 32, 33). The VCC from *V. cholerae* non-O1 evokes fluid accumulation in the rabbit ileal loop and suckling mouse models (12), so an investigation into the effects of VCC on intestinal cells appeared warranted. We report on a remarkably potent cytocidal action of VCC on human intestinal cells and provide evidence that cell death is due to generation of pore-forming toxin oligomers on target membranes.

MATERIALS AND METHODS

Reagents. RPMI 1640, Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), glutamine, penicillin-streptomycin, phosphate-buffered saline (PBS), and Hanks' balanced salt solution (HBSS) were obtained from Biochrom (Berlin, Federal Republic of Germany [FRG]). Chemical reagents were purchased from Sigma (Deisenhofen, FRG). Carrier-free, $Na^{125}I$ was obtained from Amersham Corp. (Braunschweig, FRG). A polyclonal rabbit antiserum against purified VCC was produced as previously described (32). Staphylococcal alpha-toxin, *Escherichia coli* hemolysin, and streptolysin O (SLO) were prepared in our laboratory as previously described (2).

Bacterial strain and purification of VCC. *V. cholerae* O1 El Tor 8731 (9) was kindly supplied by Robert Hall of the U.S. Food and Drug Administration, Washington, D.C. VCC was purified from culture supernatants by ethanol precipitation (final concentration, 40%), preparative isoelectric focusing in a sucrose density gradient, and hydroxyapatite chromatography (35). Specific activity of VCC toward erythrocytes paralleled toxicity for intestinal cells during VCC purification.

Radioiodination of VCC. VCC (20 μg) and 500 μCi of ^{125}I (as NaI) were incubated in siliconized glass tubes coated with 2.5 μg of 1,3,4,6-tetrachloro-3α,6α,-diphenylglucouril (Iodo-Gen; Sigma) for 5 min at room temperature. The reaction was stopped by transferring the mixture to a fresh vial containing dithiothreitol final concentration, 10 mM). Bovine serum albumin was added to 0.1%, and the labelled protein was purified by gel filtration (PD-10 column; Pharmacia-Biotech, Uppsala, Sweden). The specific activity was approximately 700 Ci/mmol of iodinated VCC. Approximately 50% of the radiolabelled toxin exhibited the capacity to bind and lyse rabbit erythrocytes. The labelled VCC was stored in aliquots at −70°C.

Cell culture. The Intestine 407 cell line, which originated from the jejunum and ileum of a 2-month-old human embryo, was generously donated by B. A. M. van der Zeijst of the Department of Bacteriology, Institute of Infectious Diseases and Immunology, University of Utrecht, Utrecht, The Netherlands. The cell line was cultured in DMEM with 2 mM glutamine supplemented with 10% FCS, 100

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U of penicillin per ml, and 100 μg of streptomycin per ml (culture medium) in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were seeded at $3 \times 10^4/cm^2$ in 96- or 6-well flat-bottom tissue culture plates or in 75-cm^2 tissue culture flasks (Nunc, Wiesbaden, FRG) and cultured until confluency was reached (~2 days). The final cell density was approximately $15 \times 10^4/cm^2$. Since 1 well in a 96-well culture plate has an area of 0.33 cm^2, each well contained approximately 5×10^4 cells.

Bioluminescence assay for determination of cellular ATP. Cell monolayers grown in 96-well culture plates were incubated with 100 μl of VCC at different concentrations in culture medium for 2 h. Intracellular ATP was determined by chemiluminescence measurements with luciferase (Boehringer-Mannheim GmbH, Mannheim, FRG) as described previously (1). ATP contents of toxin-treated cells were expressed as percent luminescence relative to that of untreated cells (1, 26).

Measurement of K^+ efflux. Cell monolayers in six-well plates containing 0.5 ml of culture medium were incubated with or without 10 ng of VCC per ml. At the times indicated, cells were washed twice with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–150 mM NaCl, pH 7.2, and lysed with 0.5 ml of 1% Triton X-100. K^+ concentrations were determined by flame photometry (Shimadzu flame emission spectrophotometer AA-660) as previously described (26).

Determination of Ca^{2+} flux. Cell monolayers cultured in six-well plates containing 0.5 ml of RPMI 1640 with glutamine supplemented with 10% FCS and penicillin-streptomycin were incubated with 10 ng of VCC per ml at 37°C for 60 min. For comparison, cells were incubated with *E. coli* hemolysin (0.5 μg/ml) (2) for 10 min. The medium was then recovered and centrifuged ($7,000 \times g$ for 5 min) to remove cellular debris. Determination of Ca^{2+} in the supernatants was performed by flame photometry.

Detection of membrane-bound VCC. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, cell membranes of rabbit erythrocytes treated with ^{125}I-labelled VCC were washed with 5 mM Na_2HPO_4 buffer, pH 8.0. To obtain membranes of VCC-treated intestinal cells, monolayers cultured in 250-ml flasks were incubated in 10 ml of culture medium with or without 10 ng of ^{125}I-labelled VCC per ml for 60 min at 37°C. The cell monolayers were washed thrice with 10 ml of 10 mM PBS, pH 7.3, and then incubated for 30 min at 37°C with 10 ml of buffer containing 138 mM NaCl, 15 mM KH_2PO_4, 0.6 mM EDTA, pH 7.3, to detach the cells from the culture flasks. Cells were centrifuged ($10 \text{ min at } 500 \times g$) at 4°C and washed three times with 10 ml of 10 mM PBS at 4°C. Membranes were solubilized by resuspension in lysis buffer (20 mM Tris-HCl [pH 7.4], 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]). After incubation for 30 min on ice, cellular debris was removed by centrifugation ($14,000 \times g$, 30 min, 4°C) and the supernatant was subjected to SDS-PAGE. The dried gels were exposed to Kodak X-Omat AR X-ray films.

Analysis of propidium iodide influx. Cells grown in six-well plates were washed with DMEM, and VCC (2 to 30 ng/ml) was added in a volume of 2 ml/well in culture medium. The cells were incubated for 30 min at 37°C and washed, and 5 μg of propidium iodide per ml was added. After 10 min of incubation at 37°C, the cells were washed once with DMEM and observed in a Zeiss UV microscope at a magnification of ×400.

Analysis of DNA fragmentation. Cell monolayers cultured in 250-ml flasks were incubated in 10 ml of culture medium with or without 5 ng of VCC per ml for 12 h at 37°C. Thereafter, DNA was isolated and analyzed by gel electrophoresis as previously described (13).

RESULTS

Toxicity of VCC for intestinal cells. By light microscopy, a cytotoxic effect became manifest as rounding up of the intestinal cells after a 4-h incubation with 10 ng of VCC per ml (Fig. 1). The threshold of cytotoxicity was approximately 2 ng/ml. When VCC was applied at 50 ng/ml, gross distortion of the cellular structures (not shown) occurred. Intermediate doses of 5 to 40 ng/ml were cytotoxic without rendering the cells stainable with trypan blue or propidium iodide. This suggested that VCC at these concentrations did not create large defects in the plasma membranes. SLO, a bacterial toxin that forms large pores (2), caused the cells to stain with propidium iodide and trypan blue (Fig. 1). In contrast, Intestine 407 cells were resistant to *Staphylococcus aureus* alpha-toxin; high concentrations of this toxin (up to 20 μg/ml) caused no alterations in cell morphology and no ATP depletion (data not shown).

Cell monolayers were incubated with VCC at 37 and 4°C at the concentrations shown in Fig. 2A, and ATP levels in cell lysates were determined after 2 h. ATP levels decreased in a dose-dependent manner. Approximately 50% ATP reduction was noted at a toxin concentration of approximately 4 ng/ml after 2 h at 37°C and at 200 ng/ml after 2 h at 4°C.

Cell monolayers were incubated with 5 ng of ^{125}I-labelled VCC per ml. After 2 h at 37°C, the medium was removed, the monolayers were washed twice, and the cells were solubilized with 1% Triton X-100 to assess the amount of bound toxin. Under the conditions used, ~6% of the labelled toxin became cell bound.

In the experiments whose results are shown in Fig. 2B, cells monolayers were incubated with 20 ng of VCC per ml at 4 or 37°C for the indicated periods. Thereafter, the culture medium containing VCC was removed, the monolayers were washed twice with ice-cold HBSS, and fresh culture medium was added. The cells were then further incubated at 37°C for 2 h, and intracellular ATP levels were measured. A binding phase of 5 min at 37°C sufficed for the first cytotoxic effects to become manifest. Maximal toxicity was observed after a binding phase of 40 min. At 4°C, a binding period of 15 to 20 min was required for cytotoxicity to become apparent, indicating a slower rate of binding at low temperatures. This agreed with binding studies done with erythrocytes in which binding at 4°C was found to be slower but not less efficient than at 37°C (35). The observed reduction in cellular ATP was not accompanied by a reduction of total cellular protein, indicating that ATP depletion was not due to gross cell lysis.

VCC forms pores in the membranes of Intestine 407 cells. To examine the physical state of bound toxin, intestinal cells or rabbit erythrocytes were treated with 10 ng of ^{125}I-labelled VCC per ml. SDS-PAGE of solubilized membranes demonstrated the presence of oligomers identical in molecular weight on the membranes of both cells (see Fig. 4). The oligomers were thermolabile and dissociated into monomers when boiled in 2% SDS. The monomers thus generated exhibited no detectable difference in apparent molecular weight from the native toxin monomer, indicating that no proteolytic processing occurred during oligomer formation (Fig. 3). At 4°C, VCC monomers bound to intestinal cell membranes oligomerized very slowly at low toxin concentrations of 5 to 20 ng/ml and oligomers could not be detected after 3 h of incubation.

To test the ability of intestinal cells to repair cytolysin lesions, cells were treated with 4 to 10 ng of VCC per ml in culture medium. After 1 h of incubation at 37°C, the culture medium containing toxin was replaced with fresh medium without toxin and measurements of cellular ATP levels were performed. No restoration of ATP levels was detected during 24 h, indicating that intestinal cells were unable to repair the toxin-induced lesions (data not shown).

The presence of dextran 4 (30 mM) did not prevent cellular ATP depletion. Cells exposed to VCC in medium in which NaCl was replaced by 150 mM KCl were also not protected from toxin action (data not shown).

Low doses of VCC induce efflux of intracellular K^+. Kinetics of K^+ efflux from intestinal cells were determined at a low VCC concentration of 10 ng/ml. At time intervals, the cell monolayers were washed and concentrations of cellular ATP and K^+ were quantified. As shown in Fig. 4, VCC rapidly induced leakage of cellular potassium. Approximately 70% efflux was noted 10 min after toxin treatment and after 20 min, efflux was complete. K^+ efflux was sequentially followed by depletion of cellular ATP, which first became noticeable 20 min after toxin application and was complete at 60 min.

Under the conditions applied, the cellular membranes had not grossly lost their integrity, since they remained impermeable to trypan blue. Further, K^+-depleted cells had an unaltered total protein content, so loss of K^+ was not due to formation of large membrane defects.

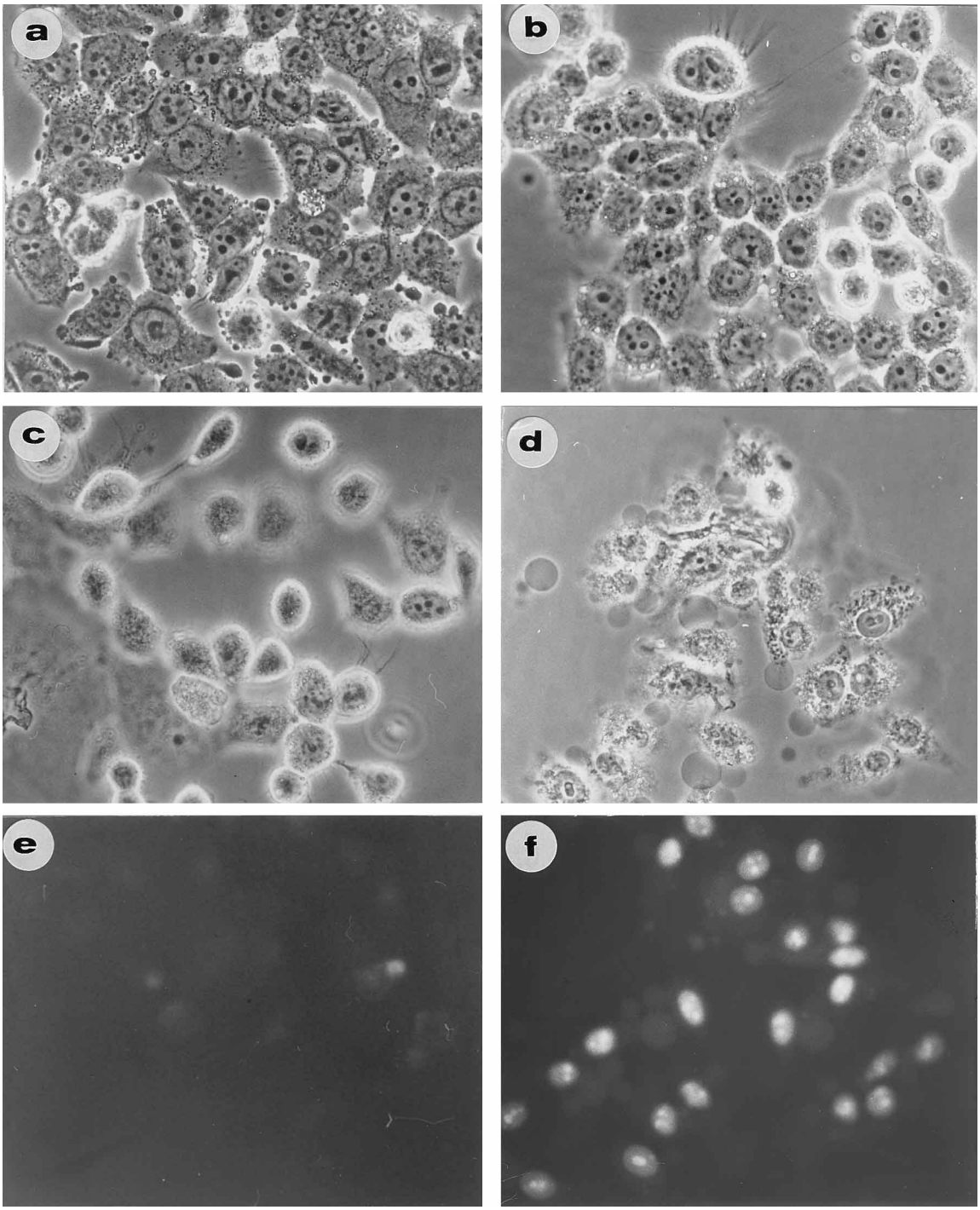

FIG. 1. Phase-contrast microscopy of Intestine 407 cells. Panels: a, control cells (untreated); b, cells exposed to VCC at 10 ng/ml for 4 h; c, cells exposed to VCC at 30 ng/ml for 4 h; d, cells treated with SLO at 1 μg/ml for 30 min; e and f, UV microscopy of Intestine 407 cells permeabilized with VCC at 30 ng/ml for 4 h (e) and 1 μg of SLO per ml for 30 min (f) and incubated with propidium iodide as described in Materials and Methods. Note the uptake of propidium iodide into these cells in contrast to VCC-treated cells. Magnification, ×400.

VCC pores are relatively impermeable to Ca^{2+}. Many pore-forming toxins, including *E. coli* hemolysin and *V. parahaemolyticus* thermostable direct hemolysin, produce Ca^{2+}-permeable pores in nucleated cells (2, 23, 24). The influx of Ca^{2+} through the membrane pores causes a fall in extracellular Ca^{2+} concentrations, which can be determined by flame photometry (26). In contrast to *E. coli* hemolysin, VCC induced no significant flux of extracellular Ca^{2+} into cells (Fig. 5). This indicated that VCC produces pores that are relatively impermeable to Ca^{2+}.

Formation of K^+-permissive pores in intestinal cells is not accompanied by internucleosomal DNA degradation. Formation of *S. aureus* alpha-toxin pores in lymphocytes triggers internucleosomal DNA cleavage that is characteristic of pro-

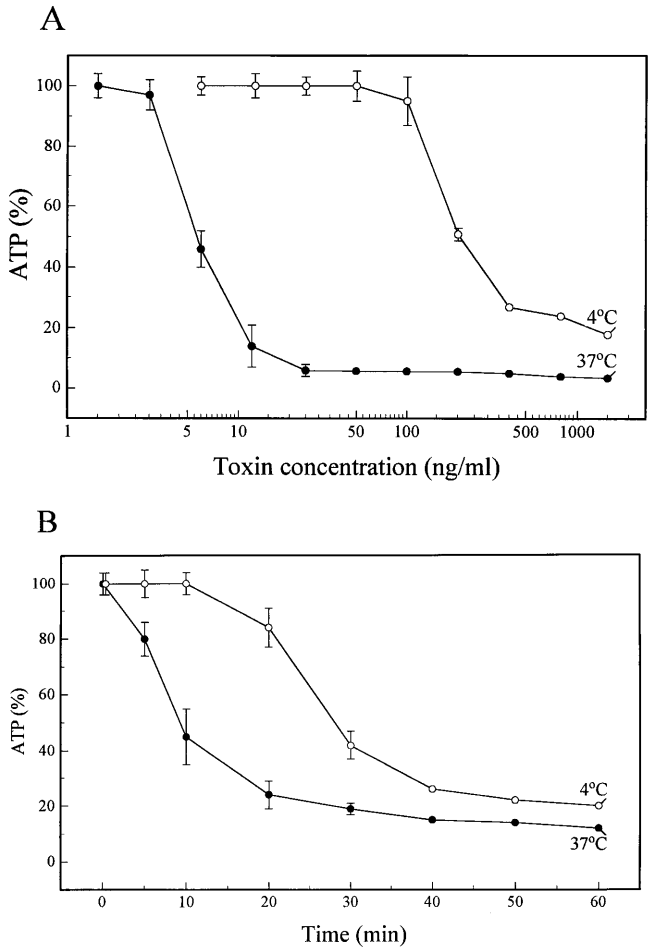

FIG. 2. (A) Depletion of cellular ATP in Intestine 407 cells induced by VCC. Cell monolayers were incubated with VCC in culture medium for 2 h at 37°C (●) or 4°C (○). Relative luminescence was determined as described in Materials and Methods. Data are means of six values, and error bars indicate standard deviations. (B) Retarded binding of VCC at low temperatures. A 50-μl volume of VCC (20 ng/ml) was incubated with cell monolayers at 37°C (●) or 4°C (○) for the indicated periods. Thereafter, the cells were washed with ice-cold HBSS, and 100 μl of DMEM was added. The cells were then incubated at 37°C for 2 h, and ATP in the cell lysates was determined. Each value is the mean of six determinations ± the standard deviation.

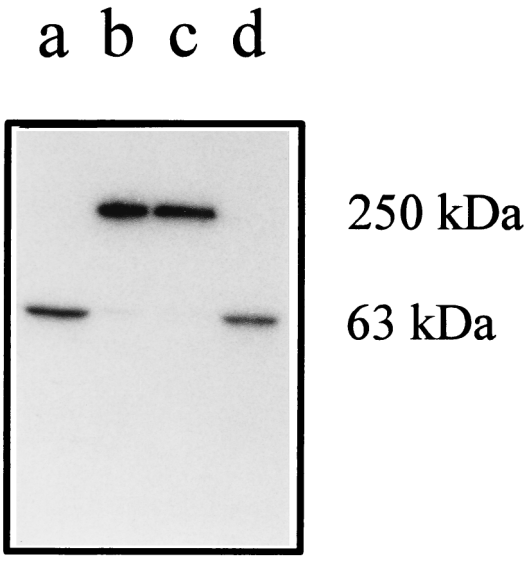

FIG. 3. Autoradiographic analysis of ^{125}I-labelled VCC bound to rabbit erythrocytes or Intestine 407 cells. Lanes: a, radiolabelled VCC; b, VCC-treated erythrocyte membranes; c, VCC-treated Intestine 407 cell membranes solubilized with SDS at room temperature; d, same as lane c but boiled in SDS to dissociate the oligomers.

grammed cell death (13). To test if VCC similarly induces apoptosis, VCC-treated intestinal cells were subjected to chromosomal DNA analysis. However, VCC-treated cells did not show the DNA fragmentation characteristic of programmed cell death (data not shown).

DISCUSSION

Bacterial toxins known to be relevant to the pathogenesis of acute diarrheal disease fall into two main groups, i.e., cytocidal agents with Shiga toxin and Shiga-like toxins as prototypes and toxins that derange cellular ionic homeostasis, with CT and CT-like toxins as classic representatives. All of these toxins enter target cells, where they act as enzymes. The possibility that nonenzymatic, membrane-permeabilizing toxins are, in some instances, also important warrants consideration because a number of organisms that are not known to produce a classic diarrheagenic toxin are enteric pathogens. Examples include *V. parahaemolyticus*, *Aeromonas hydrophila*, and several strains of *V. cholerae*. Indeed, most *V. cholerae* non-O1 strains that are recognized worldwide as frequent causes of diarrhea usually do not produce CT (5, 20, 22). Analogously, *V. cholerae* O1 El Tor strains that lack the CT gene have also been isolated from patients. One of the commonly observed features among all of the above agents is their elaboration of pore-forming cytotoxins. Studies performed with erythrocytes or liposomes as targets have shown that VCC oligomerize in target membranes to form water-filled channels (14, 32). Conceptually, their mode of action is analogous to that of classic pore-forming toxins such as staphylococcal alpha-toxin (2) and aerolysin (8).

Evidence that *Vibrio* cytolysins, indeed, produce diarrhea has been obtained. Ichinose et al. first reported the enterotoxicity of El Tor-like hemolysin from non-O1 *V. cholerae* in 1987 (12), a finding that was later confirmed (32). Analogously, Nichibuchi et al. (18) and Raimondi et al. (19) recently showed that the *V. parahaemolyticus* cytolysin (hemolysin) is entero-

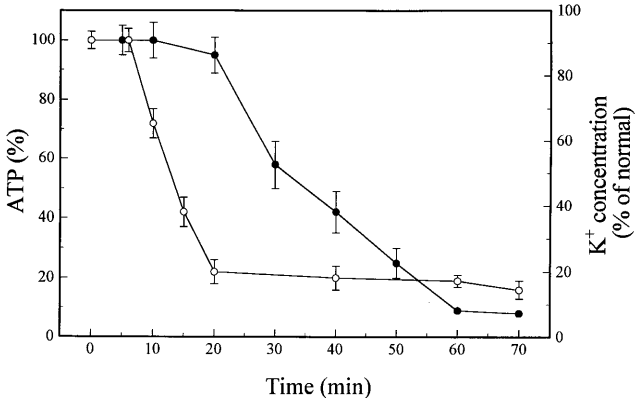

FIG. 4. Kinetics of K^+ and ATP depletion in Intestine 407 cells evoked by VCC. Cell monolayers were treated with 10 ng of VCC per ml. Relative luminescence (●) and K^+ concentrations (○) in toxin-treated cells were determined at various times. Data are means of five values ± the standard deviations.

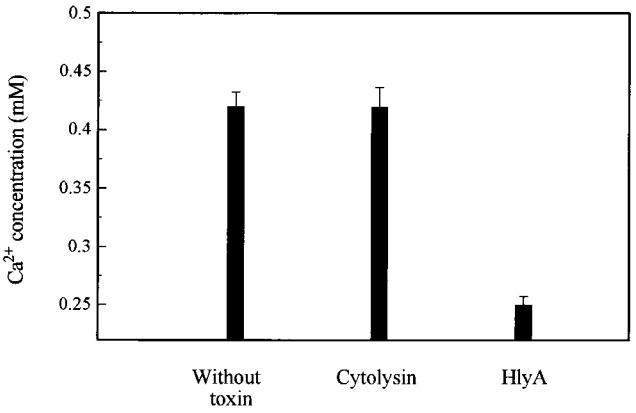

FIG. 5. VCC causes no significant reduction of the extracellular Ca^{2+} concentration. Cell monolayers were treated with 10 ng of VCC per ml or 0.5 μg of *E. coli* alpha-hemolysin (HlyA) per ml. In contrast to HlyA, VCC did not induce significant influx of extracellular Ca^{2+} into the cells. Data are means of seven determinations ± the standard deviations (error bars).

toxigenic, causing intestinal chloride secretion in a rabbit model.

In the light of the above findings, we directly examined the effects of purified VCC on putative physiological target cells. Only one other study on the cytotoxic effects of a membrane-damaging toxin from an enteropathogen on intestinal cells has been published, i.e., that of Tang et al. (24) on the action of *V. parahaemolyticus* thermostable direct hemolysin, which is structurally unrelated to VCC. Our study discloses a remarkably potent cytocidal action of VCC. The results show that cytotoxicity is due to the formation of small pores and indicate that the pores are generated through the assembly of toxin oligomers in the membrane in the absence of any proteolytic processing. Functionally, the VCC pores resemble those created by staphylococcal alpha-toxin (2). They permit rapid flux of K^+ but not of Ca^{2+}, and the attacked cells, accordingly, do not stain with trypan blue or propidium iodide. We have previously shown that production of such small channels by alpha-toxin in membranes of keratinocytes leads to breakdown of mitochondrial function and to ATP depletion (26). Presumably, depletion of cellular ATP provoked by VCC in intestinal cells follows the same course of events.

Quantitative aspects are noteworthy. VCC provoked irreversible ATP depletion in intestinal cells when applied at only 2 to 5 ng/ml, corresponding to $<10^{-10}$ M. Under our experimental conditions, 0.1 ml of a toxin solution containing 5 ng of protein per ml, i.e., approximately 5×10^9 molecules, was applied to $\sim 5 \times 10^4$ adherent cells. Thus, 10^5 molecules were given per cell for 120 min, during which time approximately 6% became cell bound. Hence, binding of only 6,000 toxin molecules to a cell is lethal. We have determined that binding of ~500 toxin molecules is required to lyse one rabbit erythrocyte (unpublished data). The possibility that VCC interacts with high-affinity binding sites on susceptible cells is being investigated, and affirmative preliminary (unpublished) data have been obtained. It is further of interest that cells permeabilized even with very low doses of VCC do not recuperate. This is in contrast to the situation with fibroblasts, in which it has been shown that cells can recuperate from membrane permeabilization events caused by both alpha-toxin (27) and VCC (33).

K^+ leakage appears to trigger apoptotic death in activated lymphocytes (13), but we obtained no evidence of internucleosomal DNA degradation in intestinal cells permeabilized by VCC. The most straightforward explanation for the cytocidal effects of VCC is that it directly kills intestinal cells through its pore-forming action. In an in vivo setting, it is possible that minute quantities of this toxin produced by adherent bacteria suffice to elicit this detrimental effect, and death of intestinal cells may be the single cause of diarrhea.

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