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Cation Flux Studies of the Lesion Induced in Human Erythrocyte Membranes by the Thermostable Direct Hemolysin of *Vibrio parahaemolyticus*

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Vibrio parahaemolyticus*, an important agent of seafood-borne gastroenteritis, expresses several putative virulence factors that could account for the disease symptoms of infected humans, namely, diarrhea, nausea, and abdominal cramps. The pathogenicity of *V. parahaemolyticus* correlates well with the Kanagawa phenomenon (the hemolytic ability of strains grown on Wagatsuma blood agar), implicating the thermostable direct hemolysin (TDH) as the predominant toxin responsible for pathogenicity. TDH-induced hemolysis could be inhibited by the addition of the osmolyte sorbitol to the extracellular solution, supporting the hypothesis that hemolysis occurs through colloid osmosis secondary to an increase in the cation permeability of the membrane. The effect of TDH on cation permeability was investigated by measuring K^+ (congener, $^{86}Rb^+$) influx into human erythrocytes in which the endogenous cation transporters had been blocked (by use of ouabain, bumetanide, and nitrendipine). TDH increased K^+ influx into these cells; this increase was rapid in onset and constant in magnitude, suggesting a direct action by TDH on the membrane. The kinetics of leak generation were examined; the relationship between counts accumulated and hematocrit indicated that the TDH-induced lesion is multihit in nature. TDH-induced K^+ influx was sensitive to Zn^{2+}. Time courses of hemolysis in isosmotic solutions of monovalent cation chlorides were used to obtain the selectivity series for the TDH-induced leak: $Cs^+ > Li^+ > K^+ > Rb^+ > Na^+$. Both the Zn^{2+} sensitivity and this selectivity series were obtained for crude culture supernatants, suggesting that TDH is the predominant leak-inducing agent. Thus, we have identified several features of the TDH-induced leak likely to be important in the diarrhetic action of *V. parahaemolyticus* in the human intestine.

Vibrio parahaemolyticus is an important cause of gastroenteritis, with clinical symptoms of diarrhea, abdominal cramps, nausea, and vomiting (8, 21). The diarrhea is usually watery and self-limiting but can be dysenteric in nature (21). The mechanisms underlying the diarrhetic action of this pathogen are not well understood. However, the pathogenicity of strains correlates well with the Kanagawa phenomenon: the hemolytic ability of strains cultured on Wagatsuma blood agar (25). The relationship between Kanagawa phenomenon hemolysis and pathogenicity suggests that hemolytic factors may underlie disease associated with *V. parahaemolyticus*. The culture supernatants of pathogenic strains have been shown to contain several constituents that promote hemolysis, including a phospholipase A (47), a lysophospholipase (47), thermostable direct hemolysin (TDH) (10, 14, 24, 26, 33, 35, 38, 45, 47), and TDH-related hemolysin (30).

The genes encoding TDH (*tdh-1* and *tdh-2*) and TDH-related hemolysin (*trh-1* and *trh-2*) have been cloned and sequenced (22, 29, 30), and the cytotoxic activities of various preparations have been assayed on several cell types (34, 38). Recent reports indicate that the TDH gene is transferable between members of the *Vibrio* genus, specifically *V. parahaemolyticus* and *V. cholerae* of the non-O1 serogroup (1, 15, 41). Therefore, it has been suggested that TDH (and/or TDH-related hemolysin) may underlie the diarrhea induced by a range of marine vibrios associated with seafoods (41).

Compelling evidence for a pivotal role of TDH in enterotoxigenicity in *V. parahaemolyticus* was obtained recently. Using Ussing chambers, Nishibuchi et al. (28) compared the culture filtrate of a TDH-positive *V. parahaemolyticus* strain with the culture filtrate of a genetically manipulated TDH-negative derivative strain. The culture filtrate of the parental (TDH-positive) strain caused a significant increase in short-circuit current (I_{SC}) across isolated rabbit ileal tissue compared with that induced by the culture filtrate of the TDH-negative mutant derivative. Return of the cloned *tdh-1* gene to the TDH-negative mutant restored the ability of the mutant to increase I_{SC}. Furthermore, the capacity of a TDH-containing filtrate to increase I_{SC} was markedly inhibited by the addition of anti-TDH antiserum. Microscopic evaluation of rabbit ileal mucosa revealed no evidence of histological damage in response to TDH. Thus, at concentrations likely to be relevant pathophysiologically, TDH appears to perturb membrane (and presumably epithelial) transport by a mechanism more subtle than cytolysis.

The precise nature of the membrane lesion induced by TDH remains obscure (7), although Honda et al. (14) recently published hemolysis and electron microscopic evidence favoring the induction of a pore-like lesion. Erythrocytes represent a well-characterized system for the analysis of membrane transport and agents that perturb it; they constitute a particularly appropriate model in which to test the effects of TDH because of the correlation between *V.*

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parahaemolyticus pathogenicity and hemolytic activity (25). By use of hemolytic activity in conjunction with site-directed mutagenesis (2) and chemical modification of aromatic residues (42), two amino acids (Trp-65 and Leu-66) have been identified as important for lytic activity.

We have obtained preliminary evidence that hemolysis caused by a crude TDH preparation occurs secondarily to an increase in the cation permeability of the erythrocyte membrane (16, 17). In this study, we used the erythrocyte model to analyze the nature of the TDH-induced membrane lesion, which is likely to underlie diarrhea induced by *V. parahaemolyticus* infection. Experiments were performed under conditions of a pharmacological blockade of endogenous cation transport pathways by use of maximal inhibitory concentrations of ouabain (5), bumetanide (5), and nitrendipine (12) to block K^+ influx by the Na^+ pump, the Na^+-K^+-$2Cl^-$ cotransporter, and the Gardos channel, respectively. Under these conditions, residual K^+ influx has properties dictated by electrodiffusion, allowing examination of the direct effects of TDH on the basal membrane leak. We delineated features of the TDH-induced lesion and demonstrated that TDH is the component of the *V. parahaemolyticus* culture supernatant responsible for the generation of the cation leak and that it operates through a multihit mechanism.

MATERIALS AND METHODS

Chemicals and reagents. Biochemicals were obtained from either Sigma Chemical Co. (St. Louis, Mo.) or BDH (Poole, Dorset, United Kingdom), except that nitrendipine and ^{86}RbCl (10 mCi/ml) were obtained from Bayer Pharmaceuticals (Newbury, Berkshire, United Kingdom) and NEN Research, Dupont (UK) Ltd. (Stevenage, United Kingdom), respectively. Crude TDH (Kanagawa hemolysin; Sigma) was supplied as a lyophilized powder containing approximately 50% protein balanced with Tris-HCl buffer–EDTA–phenylmethylsulfonyl fluoride–sodium azide (0.05% [wt/vol]). One hemolytic unit is defined by Sigma as the amount that will cause 50% hemolysis of a 1% erythrocyte suspension in phosphate-buffered saline at pH 7.0 after 2 h of incubation at 37°C and then refrigeration for 12 to 24 h at 4°C (36).

Purification of TDH. Kanagawa hemolysin (3.2 mg) was reconstituted in 10 ml of 20 mM Tris-HCl buffer at pH 7.5 and subjected to ion-exchange chromatography with a MonoQ column in the fast protein liquid chromatography system of Pharmacia (Piscataway, N.J.). An NaCl gradient of 0 to 1 M was used to fractionate samples; peak fractions (detected by measurement of the A_{280}) were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, concentrated, and loaded onto a fast protein liquid chromatography Superose 12 gel filtration column. Purified TDH was recognized on the basis of its molecular weight (close to 21,000, previously reported [38]) in SDS-PAGE (Fig. 1). The identity of the protein was confirmed by amino-terminal sequencing (20 residues) by previously described techniques (27). Purified TDH was concentrated to 50 μl, equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 0.05% (wt/vol) NaN_3, and lyophilized by use of a Speedvac. TDH was reconstituted by the addition of 50 μl of double-distilled water and then 3.95 ml of buffer solution [145 mM NaCl, 15 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS); pH adjusted to 7.5 with NaOH]. Aliquots (0.33 ml) were stored at −22°C and thawed immediately prior to use.

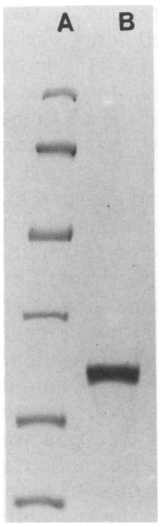

FIG. 1. Purity of TDH, as determined by SDS-PAGE with gradient gels containing 10 to 20% total acrylamide. Lane A contained molecular weight markers (low range; Bio-Rad Laboratories, Richmond, Calif.) in descending order of molecular weight: phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Lane B was loaded with 10 μg of TDH purified as described in Materials and Methods.

Blood. Blood was either taken fresh from healthy adult donors (collected into either heparin [10 IU/ml] or acid-citrate-glucose [85 mM Na citrate, 71 mM citric acid, 111 mM glucose]) or obtained as outdated bank blood (Blood Transfusion Center, John Radcliffe Hospital, Oxford, United Kingdom). Erythrocytes were washed five times by centrifugation (in a Sorvall RT6000B centrifuge at 10°C and 2,500 × *g* for 5 min), aspiration, and resuspension in MOPS-buffered saline (MBS; 145 mM NaCl, 15 mM MOPS, 5 mM glucose; adjusted to pH 7.4 by the addition of Tris base). After centrifugation, the plasma and buffy coat were removed by aspiration. Hematocrits (HCts) of cell suspensions were determined by measuring hemoglobin released spectrophotometrically (A_{540}) after the addition of 100 μl of cell suspension to 5 ml of Drabkin's solution (9). All experiments were performed at 37 ± 0.05°C with a thermostatically controlled water bath.

Hemolysis studies. Time courses of hemolysis in MBS were measured to assess the relative potencies of pure TDH and crude TDH as hemolytic agents with the addition of a given volume of reagent. For initiation of an experiment, 100 μl of packed cells was added to 4.9 ml of MBS containing ouabain (0.1 mM), bumetanide (0.1 mM), and nitrendipine (10 μM) (i.e., the maximal inhibitory doses of these inhibitors) and 100 μl of pure or crude TDH (obtained from thawed stock aliquots [see above]). Time courses of hemolysis in these solutions were assayed by measuring the hemoglobin released into supernatants by lysed cells. At each time point, a 0.6-ml sample was taken and the cells were centrifuged (in an Eppendorf 5412/5414 Microfuge at 15,000 × *g* for 10 s). The A_{540} of the supernatant (after dilution) was measured and expressed as percent hemolysis by comparison with the A_{540} yielded for the solution by 100% hemolysis (0.5 ml of cell suspension added to 3 ml of water). The H^{50} index was defined previously (13, 46) as the time taken for 50% hemolysis to occur. This index was calculated for both crude

and pure TDH preparations at the standard doses used. There was a pronounced donor variation in the absolute H^{50} indices; accordingly, the H^{50} index for pure TDH was expressed relative to that for crude TDH.

Isosmotic solutions of the alkali metal chlorides (150 mM) were made in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH adjusted to 7.4 by the addition of Tris base) and adjusted to 291 ± 3 mosmol by the addition of HEPES (as measured by use of a Wescor 5100 C vapor pressure osmometer). For initiation of an experiment, 100 μl of packed cells was added to 4.9 ml of the relevant solution, which also contained ouabain (0.1 mM), bumetanide (0.1 mM), and nitrendipine (10 μM) (as described above) and 30 μl of TDH (obtained from thawed stock aliquots [see above]). Time courses of hemolysis were assayed as described above. At each time point, a 0.6-ml sample was taken and the cells were centrifuged (as described above). In these isosmotic experiments, $(H^{50})^{-1}$ was a semiquantitative indicator of permeability to ion *x* (46). Because of variations in the susceptibility of cells according to donor, permeability to any ion was expressed relative to permeability to Cs, since hemolysis was fastest in $CsCl_2$. The (permeability to ion *x*/permeability to Cs) index was shown as an Eisenman plot (11) (i.e., plotted against the inverse of the crystal ion radius) and used to define the selectivity series for the TDH-induced lesion.

Influx measurements. $^{86}Rb^+$ was used as a congener for K^+. The assumption underlying this technique is that Rb^+ is processed by membrane transport systems in a manner very similar to that found for K^+. Although this assumption has been validated for many membrane transporters (37), it is still questionable for most pore-forming systems. However, in this study, we produce evidence that K^+ and Rb^+ are processed in very similar ways by the TDH-induced lesion (see Fig. 6, the Eisenman plot for isosmotic hemolysis data). MBS containing sucrose (40 mM) (pH 7.4) was used as the basic flux medium to protect cells from lysis. The final amount of radioactivity per 1-ml Eppendorf tube was ca. 1 μCi/ml. Two techniques were used to measure K^+ ($^{86}Rb^+$) influx. The first technique was the conventional $MgCl_2$ wash technique, done as described previously (5); the results are expressed either as influx (millimoles per liter of cells per hour) or as the counts-per-minute index (CPMI). CPMI is defined as [10 × (counts per minute − background counts)]/(standard counts per minute − background counts), where standard counts per minute is the number of counts produced by 10 μl of the KCl (plus trace $^{86}RbCl$) solution (150 mM) used to initiate ($t = 0$) the experiment. The second technique was the dibutyl phthalate (DBP) multiple-wash technique (12), which was used in experiments delineating the dynamics of the TDH-induced leak (see Fig. 2) because it allows rapid separation of cells from the medium; flux is terminated by the addition of a 200-μl aliquot of the suspension to 800 μl of ice-cold saline layered over 250 μl of DBP, through which the cells are then centrifuged (as described above). In both protocols, 150 mM KCl (plus trace $^{86}RbCl$) was added to the cell suspension to initiate the flux time course, such that the final concentration of extracellular K^+ was 15 mM. Study of the dynamics of pore induction (see Fig. 2) by the DBP technique uses the counts-per-minute ratio (CPMR) as the index of accumulated K^+. CPMR is defined as counts per minute/standard counts per minute.

Multihit analysis. Inoue et al. (18) developed an analytical method for the estimation of the number of hits necessary to kill or lyse cells. A hit is envisaged as the physical interaction between a single toxin entity and the membrane. For these experiments, various concentrations of target cells were treated with a constant amount of lytic agent in a constant reaction volume. For a given agent, if the absorbance (an index of total [as opposed to fractional] number of cells lysed) is found to decrease as the HCt is increased beyond a certain level, then it can be concluded that cell death is multihit (as opposed to single hit) in nature. By using K^+ influx rather than hemolysis as our assay of TDH action (counts accumulated being the corrollary of the A_{540} in the method of Inoue et al. [18]), we examined the nature of the lesion itself rather than the number of hits required for cell death.

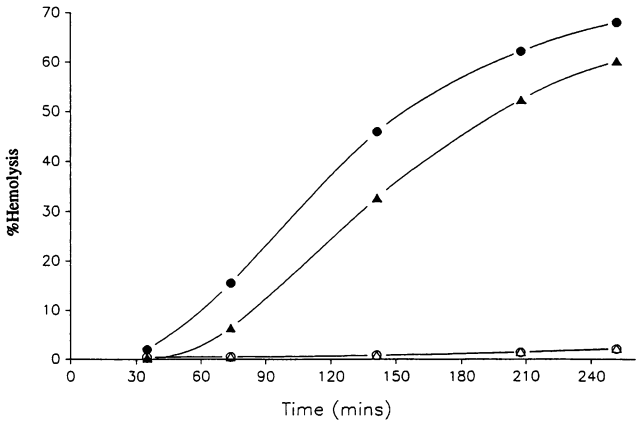

FIG. 2. Osmotic attenuation of TDH-induced hemolysis. Packed human erythrocytes were added to MBS (containing saturating doses of ouabain, bumetanide, and nitrendipine) to initiate the time course. Open symbols indicate control results, obtained in the absence of TDH; filled symbols represent results obtained in the presence of 50 μl of TDH per 5 ml of cell suspension, in the presence (▲) and absence (●) D-sorbitol (23.5 mM).

Data presentation. Data are given either as the mean ± the standard error of the mean for at least three separate experiments (see Table 1 and Fig. 6B) or as typical results (see Fig. 2, 3, 4, 5, and 6A).

RESULTS

The addition of an osmolyte (sorbitol at 23.5 mM) to the extracellular solution significantly inhibited TDH-induced hemolysis, increasing the H^{50} index by 18.7% ± 2.4% (Fig. 2). The protective effect of increased extracellular osmotic pressure suggests that hemoglobin is released to the extracellular solution after membrane rupture because of volume expansion (because of colloid osmosis secondary to an increased net cation influx). Such a lytic process indicates an increase in membrane cation permeability. Thus, hemolysis appears to be the end result of colloid osmosis.

Using the DBP multiple-wash technique for measuring K^+ ($^{86}Rb^+$) influx into ouabain-, bumetanide-, and nitrendipine-inhibited cells, we examined the dynamics of the putative cation leak induced by TDH. The addition of 15 μl of TDH (at a twofold dilution of the stock aliquot) caused a large increase in K^+ influx (Fig. 3). This increase in flux was rapid in onset and constant in magnitude over a 9-min time course. The extent of the increase in flux varied according to donor. Two donors showed large increases of similar magnitudes in CPMR with time after TDH addition; however, a third donor was less responsive.

The magnitude of K^+ influx also varied with the hemato-

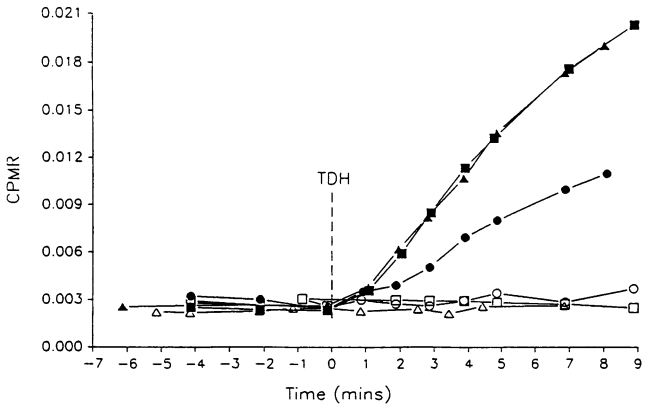

FIG. 3. Dynamics of leak induction. Each symbol shape represents results from a different donor. Flux was initiated by the addition of 0.5 ml of KCl (plus trace ^{86}RbCl) (150 mM) to 4.5 ml of cell suspension (MBS plus ouabain, bumetanide, and nitrendipine), such that $[K^+]_o$ = 15 mM, $[Na^+]_o$ = 135 mM, and HCt = 4%, and 200-μl samples of the flux suspension were taken at 1- to 2-min intervals and centrifuged through DBP. After three such samples were taken, 15 μl of TDH (filled symbols) or MBS (no TDH) (open symbols) was added to the remaining 4.4 ml of the flux suspension. Subscript O indicates extracellular.

crit (HCt), as shown in Fig. 4A. The same patterns were seen in all three experiments, although the results were not directly quantitatively reproducible. Increasing the HCt (i.e., the target cell concentration) beyond a certain level (between 0.015 and 0.025, depending on the donor) caused the induced flux to fall dramatically before reaching a plateau phase (at an HCt of >0.035). Note that the basal level of leak in TDH-treated cells, obtained at an HCt of >0.035, did not change with increasing HCt but was higher than the basal level of leak (by approximately 1 order of magnitude) in untreated, control cells (the control data showed that in untreated cells, K^+ influx did not change with increasing HCt). Furthermore, as shown in Fig. 4B, the index of total counts accumulated (CPMI) by the cells was observed to increase with increasing HCt in the lowest HCt range (up to ca. 0.02). In the middle HCt range (ca. 0.02 to 0.03), CPMI actually decreased rather than levelling off at a higher value. This result suggests that there was a protective effect of a higher target cell concentration, CPMIs being lower than those that would have been predicted if TDH acted only through a single-hit mechanism. In the highest HCt range, CPMI increased again; the slope of this relationship was less marked than that observed in the initial phase but more marked than that for the control cells, not treated with TDH (reflecting the increased basal level of leak caused by TDH treatment).

It was important to compare the potencies of the leaks induced by pure and crude TDH preparations. Evidently, some of the TDH-induced leak activity was lost during the purification protocol; pure and crude preparations were reconstituted into 4 and 10 ml of MBS (pH 7.5), respectively. At the concentrations thus obtained (with the crude preparation having an activity of 100 hemolytic units per ml), pure TDH was found to be a more potent leak-inducing agent than the crude preparation. This result was confirmed in experiments in which both preparations at 20 μl/ml of cell suspension (HCts, 0.020 to 0.025; experiments were performed on three separate donors) caused a dramatic increase in K^+ influx, the magnitude of the leak induced by the crude preparation being 68.7% ± 5.4% of that produced by pure TDH. At the same relative dose (i.e., 100 μl of agent added to 4.9 ml of cell suspension at an HCt of 0.02), the crude preparation had a higher level of hemolytic activity (Fig. 5), the time courses for hemolysis being determined with sucrose-free MBS. The H^{50} indices were calculated as described in Materials and Methods; the H^{50} index for pure TDH was 96% ± 4.5% higher than that for the crude preparation.

Table 1 shows the effect of Zn^{2+} (100 μM) on TDH-induced influx. For all four donors tested, Zn^{2+} (100 μM) was inhibitory to TDH-induced K^+ influx but caused a comparatively small increase in the level of leak in control cells, not treated with TDH. However, there was an extensive variation in the degree of inhibition; i.e., the quantitative reproducibility of the effect was poor. This result was reflected in the high standard error of the mean for the Zn^{2+}

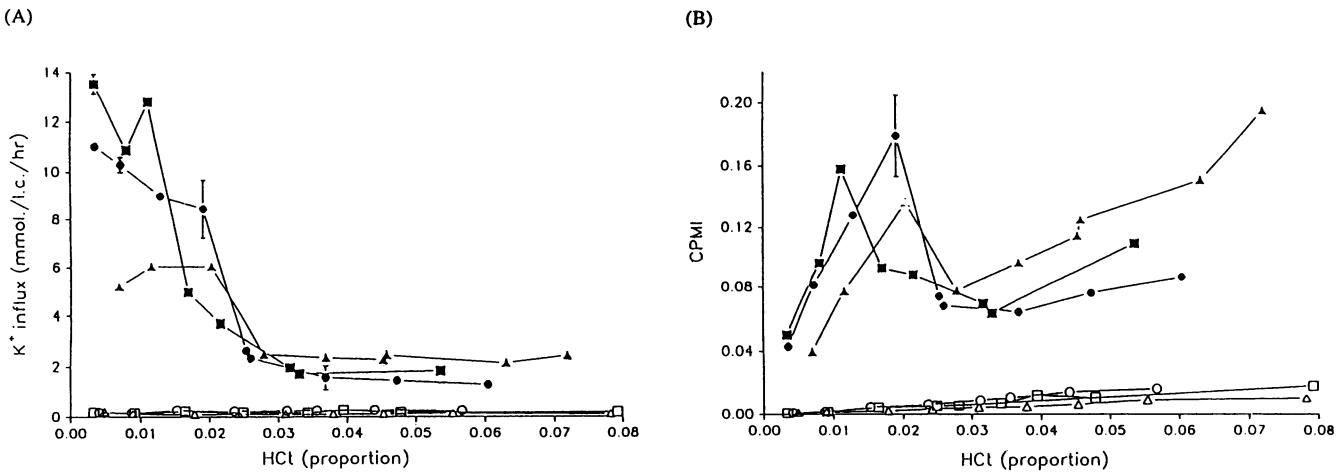

FIG. 4. Multihit analysis. Flux was initiated by the addition of 100 μl of KCl (plus trace ^{86}RbCl) (150 mM) to 0.9 ml of cell suspension (MBS plus ouabain, bumetanide, and nitrendipine) containing (filled symbols) or not containing (open symbols) 12 μl of TDH (twofold dilution of the stock) and showing various HCts, as indicated, such that in the flux tube $[K^+]_o$ = 15 mM and $[Na^+]_o$ = 135 mM. Each symbol shape represents a different donor. l.c., liter of cells. Subscript O indicates extracellular.

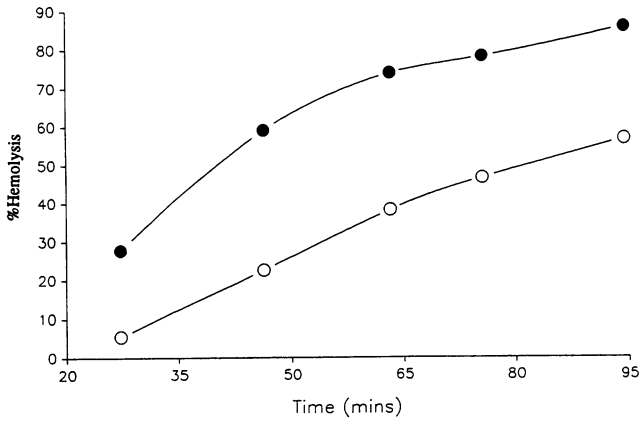

FIG. 5. Comparative hemolytic potencies. At doses at which pure TDH had been shown to produce a higher level of $^{86}Rb^+$ leak activity (see Results), time courses for hemolysis were determined with MBS (100 μl of either a crude [●] or a pure [○] preparation was added to 4.8 ml of MBS containing ouabain, bumetanide, and nitrendipine before the experiment was initiated by the addition of 100 μl of packed cells).

inhibition of TDH-induced influx. Concentrations of Zn^{2+} of >250 μM were noted to cause cell clumping.

Figure 6A shows the time courses of TDH-induced hemolysis in isosmotic solutions of the alkali metal chlorides for a typical experiment, and Fig. 6B shows the Eisenman plot for pooled data from three such experiments, permeability indices being calculated as described in Materials and Methods. On the basis of the assumption that the identity of the extracellular ion does not affect the action of agents that promote hemolysis by a mechanism other than leak induction, the relative rates of TDH-induced hemolysis can be ascribed to the selectivity of the induced leak (46). The selectivity series for the TDH-induced lesion for monovalent cations is $Cs^+ > Li^+ > K^+ > Rb^+ > Na^+$, a series that approximates closely a reversed Eisenman VIII series, with a small K^+ anomaly of debatable significance.

DISCUSSION

These data show that relatively straightforward osmotic fragility and membrane transport experiments with erythrocytes (6) can provide useful information about the nature of the membrane lesion induced by TDH. TDH action on K^+ influx is rapid in onset and constant in magnitude; these observations are in marked contrast to those defining the cation leak induced by other so-called pore-forming agents,

TABLE 1. Effects of Zn^{2+} on TDH-induced K^+ influx[a]

Cells	Flux (mmol/liter of cells/h, mean ± SEM) in the presence of treatment:		
	A	B	A − B
Control	0.21 ± 0.00675	0.30 ± 0.024	−0.09 ± 0.0258
TDH treated	18.89 ± 1.09	11.31 ± 2.93	+7.58 ± 3.13

[a] Flux was initiated by the addition of 100 μl of KCl (plus trace $^{86}RbCl$) (150 mM) to 0.9 ml of cell suspension (MBS plus ouabain, bumetanide, and nitrendipine) not containing (A) or containing (B) Zn^{2+} at 100 μM and TDH, as indicated, such that in the flux tube $[K^+]_o$ = 15 mM, $[Na^+]_o$ = 135 mM, and HCt = 4%. TDH was added at 10 μl/ml. Values are from four experiments. Subscript O indicates extracellular.

such as melittin (44), which causes a leak of only a transient nature, and palytoxin (43), which generates increased membrane cation permeability that increases with time. The hemolytic action of TDH can be attenuated osmotically, as has been shown by Honda et al. (14). Although these data do not necessarily imply that TDH is the only leak-inducing agent in the culture supernatants of pathogenic strains, we interpret the data presented here as good evidence that the induced leak is likely to be the result of nonenzymatic action directly on the cell membrane, rather than either enzymatic or intracellular action. Were these latter mechanisms involved, it is likely that the magnitude of the influx would have increased with time after a more pronounced lag period. Osmotic protection against hemolysis and the rapidity of onset and constancy of magnitude of the K^+ influx pathway, as described here for pure TDH, have been reported elsewhere for crude TDH (17).

Having established the independence of influx and time after addition of the toxin, we examined the effect of erythrocyte concentration on influx to clarify the nature of the lesion. The results were interpreted as follows. (i) Over the lower HCt range (a relatively constant high level of flux and an increasing CPMI), the sites available for leak formation are close to saturation; i.e., the amount of TDH present in solution is larger than that required for the generation of a leak pathway of maximum magnitude. (ii) Several components of TDH (as it exists in the aqueous phase) are required to form the leak pathway responsible for the high level of K^+ influx. This requirement is because not only flux but also CPMI (an index of total rather than fractional uptake of a cation) decreases after reaching a maximum, rather than simply levelling off. Thus, leak induction is a multihit process, in which TDH components have a proportionately greater effect when they operate in unison as opposed to singly. Interestingly, CPMIs at higher HCts are significantly higher than those corresponding to the basal level of leak in control cells. The increase in CPMI with increasing HCt (in the higher HCt range) may indicate that single components of TDH are capable of raising the cation permeability of the membrane, although not as effectively as several components acting in concert. Previous multihit studies have used hemolysis (expressed both as a percentage and as a total, with various HCts) to assay toxin action (18–20, 23, 31). However, the conclusions that can be drawn from such studies are limited in that they indicate the number of hits required only for cell death rather than lesion formation itself. The experiments of Nishibuchi et al. (28) indicate that TDH is likely to have its enteric effects through a mechanism other than cell lysis; in light of these and our own data indicating osmotic protection by sorbitol and a defined selectivity series for the TDH-induced lesion for monovalent cations, we suggest that the said lesion is capable of a degree of solute discrimination. It was therefore appropriate to examine the effects of target cell concentration on TDH-induced K^+ influx, to allow an examination of the nature of the lesion itself. The fact that the lower HCt range indicates the saturation of sites available for leak formation may relate to previous evidence (39, 40) suggesting the presence of a neuraminidase-sensitive ganglioside receptor (G_{T1} or G_{D1} rather than G_{M1} or G_{M2}) necessary for TDH action.

Data pertaining to the relative flux-inducing and hemolytic abilities of pure and crude TDH preparations (Fig. 5) indicate that TDH is important in the generation of raised membrane cation permeability but that the crude preparation contains other factors that augment hemolysis, presumably acting to increase the osmotic fragility of the cells rather than

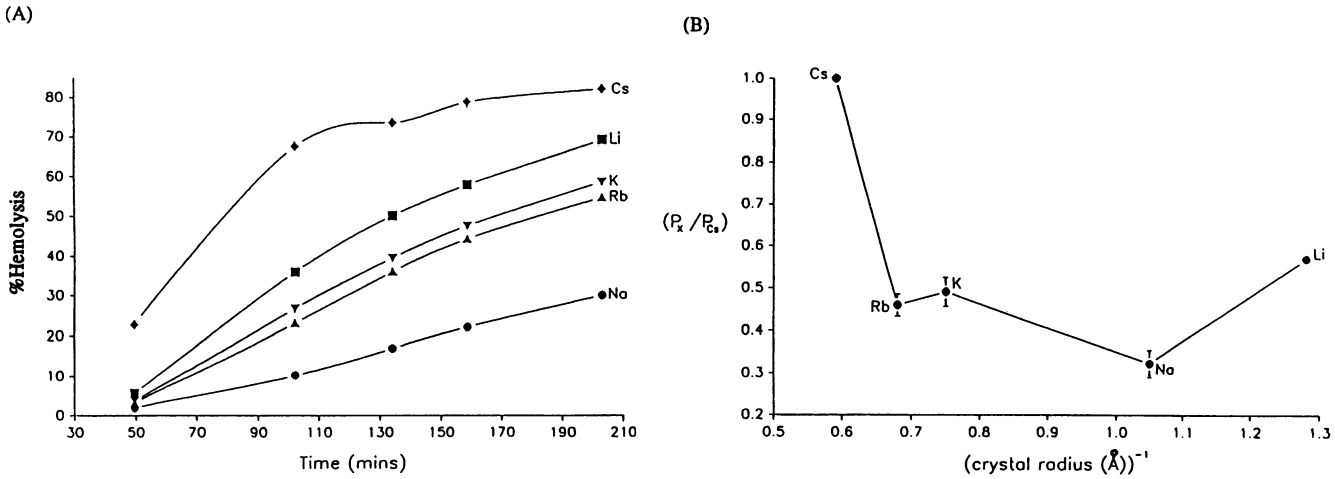

FIG. 6. Monovalent cation selectivity of TDH-induced hemolysis. Isosmotic cell suspensions contained the chlorides of monovalent cations, ouabain, bumetanide, and nitrendipine. TDH (30 μl) was added to each of the cation solutions before the final addition of 100 μl of packed cells (total final volume, 5 ml; $t = 0$). (A) Results of a typical experiment. (B) Pooled results of three separate experiments. P_x, permeability to ion x; P_{Cs}, permeability to Cs.

enhancing membrane cation permeability. This suggestion is consistent with reports that have identified membrane-active enzymes, such as a phospholipase A and a lysophospholipase (47), in the culture supernatants of *V. parahaemolyticus*.

The data shown here indicate that (i) Zn^{2+} significantly inhibits the induced K^+ influx pathway (Table 1) and (ii) the lesion underlying hemolysis is of a defined monovalent cation selectivity series ($Cs^+ > Li^+ > Rb^+ > K^+ > Na^+$), a reversed Eisenman VIII series with a small K^+ anomaly of debatable significance (Fig. 6). These two features of the TDH-induced leak have also been shown for the leak induced by crude culture supernatants of *V. parahaemolyticus* (16, 17). The evidence presented here suggests that the increase in membrane cation permeability induced by culture supernatants of *V. parahaemolyticus* is due to TDH alone, because the fluxes induced by crude and pure TDH preparations cannot be separated on the basis of either Zn^{2+} inhibition or substrate discrimination (reflected by the unusual profile of monovalent cation selectivity). Although the site(s) of Zn^{2+} action remains obscure (3, 4, 32), TDH can be added to a lengthening list of Zn^{2+}-sensitive leak-forming agents.

Diarrhea caused by *V. parahaemolyticus* is usually watery (as opposed to dysenteric), a characteristic taken as evidence that TDH (at "normal" pathophysiological concentrations) perturbs membrane function rather than simply causing lysis. This suggestion is consistent with the results of the study of Nishibuchi et al. (28) implicating TDH as capable of increasing I_{SC} without causing histological damage. For the *V. parahaemolyticus*-associated dysenteric syndrome, it is likely that TDH and other factors (such as the enzymes already mentioned [47]) affecting osmotic fragility cause cell lysis, thus destroying the functional integrity of the intestinal epithelium. Our study shows that TDH is the important cation leak-inducing agent in culture supernatants of *V. parahaemolyticus* and furthermore characterizes several salient features of TDH action. These functional features of the TDH-induced lesion are likely to be important in an analysis of the cellular physiological events that underlie the pathogenesis of *V. parahaemolyticus*-associated diarrhea.

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