

Ionic Requirements for Entry of Shiga Toxin from *Shigella dysenteriae* 1 into Cells

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The ionic requirements for entry of Shiga toxin into cells were examined by measuring inhibition of protein synthesis after short-term incubations with toxin. The sensitivity of Vero cells and HeLa cells to Shiga toxin was strongly dependent on the divalent cation present. Vero cells were most sensitive in the presence of CaCl_2 and SrCl_2 , whereas HeLa cells were equally sensitive in the presence of MgCl_2 , SrCl_2 , and CaCl_2 . Both cell lines were protected by BaCl_2 , CoCl_2 , and MnCl_2 . Inhibitors of Ca^{2+} transport, like verapamil, D600, and Co^{2+} as well as the calcium-ionophores A23187 and ionomycin, protected both cell lines. HEp-2 cells were protected against Shiga toxin by a high concentration of potassium in the medium as well as by potassium depletion of the cells. Substitution of chloride in the medium with slowly permeable anions, like SO_4^{2-} and SCN^- , protected the cells against Shiga toxin. High concentrations of the ionophore nigericin that increase pH of acidic intracellular vesicles did not protect Vero cells against Shiga toxin. Shiga toxin bound Triton X-114 at pH values below 4.5. This binding was shifted to higher pH values after pretreatment of the toxin with dithiothreitol. The results indicate that Ca^{2+} transport through physiologically occurring Ca^{2+} channels is required for entry of Shiga toxin into cells. Furthermore, the sensitivity of cells of Shiga toxin is strongly dependent on the anions present.

Shigella dysenteriae 1 produces a protein cytotoxin, Shiga toxin, which inhibits protein synthesis in sensitive cell lines (4, 8) and in primary cultures of human colonic and ileal epithelial cells (M. P. Moyer, P. S. Dixon, S. W. Rothman, and J. E. Brown, submitted for publication). Toxic action of Shiga toxin on cells involves binding of the toxin molecule to cell surface receptors containing galactose- α (1-4)galactosedisaccharides (3; A. A. Lindberg, J. E. Brown, N. Stromberg, M. Westling-Ryd, J. E. Schultz, and K. A. Karlsson, submitted for publication), and subsequent entry of the enzymatically active A fragment into the cytosol, where it inactivates 60S ribosomal subunits. Protein synthesis then stops (5, 22) due to inhibition of the aminoacyl-tRNA binding step of peptide elongation (3a). Shiga toxin has one enzymatically active chain and five to seven B chains that presumably mediate toxin binding (7, 17).

Recently cytotoxins similar to Shiga toxin have been detected in various enteropathogenic *Escherichia coli* (14), in *Vibrio cholerae* and *Vibrio parahaemolyticus* (13), and in *Escherichia coli* isolates associated with hemolytic uremic syndrome (6, 10). Since Shiga toxin itself or Shiga-like cytotoxins appear to play a role in the pathogenesis of several diarrheal diseases, investigation of the process of Shiga toxin uptake has been undertaken. In the present paper we have examined the ionic requirements for entry of Shiga toxin into cells and compared these requirements with those of the protein toxins abrin, ricin, modeccin, and diphtheria toxin. Like Shiga toxin these toxins enter the cytosol and inhibit protein synthesis (for a review, see reference 18).

MATERIALS AND METHODS

Chemicals. Valinomycin, monensin, ouabain, L-1-tosylamide-2-phenylethylchloromethyl ketone, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid, and 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid were from Sigma Chemical Co., St. Louis, Mo. A23187 and ionomycin were from Calbiochem-Behring Corp., La Jolla, Calif. Triton X-114 was obtained from Fluka AG, Buchs, Switzerland. Material from the Triton X-114 that remained in the water phase at 37°C was removed by dissolving 40 g of Triton X-114 in 1 liter of 10 mM sodium phosphate (pH 7.5)-0.14 M NaCl, stirring overnight at 4°C, and then incubating at 37°C until two phases appeared. The upper phase, which consisted of buffer, was discarded, and new buffer was added. This procedure was repeated three times. Finally this pretreated Triton X-114 was made up to 200 ml by the addition of the buffer used for extraction. Amiloride was a gift from Merck, Sharp & Dohme, West Point, Pa. Verapamil and D600 were kindly provided by Knoll AG, Ludwigshafen.

When drugs that were dissolved in dimethyl sulfoxide (DMSO) and ethanol were added to the medium, the concentration of the organic solvent was below 0.2% (vol/vol). At this concentration, these solvents have no effect on the cytotoxicity of Shiga toxin. However, at higher concentrations, these solvents will protect (Fig. 1). Similarly, DMSO protects against other protein toxins (23).

Toxins. Shiga toxin (2) and abrin (16) were purified as previously described. Diphtheria toxin was obtained from Connaught Laboratories and purified as previously described (24).

Cells. Vero cells (from African green monkey kidney) were obtained from Flow Laboratories, Herts, England; HeLa S₃ cells are from a HeLa strain and have been growing at the Norwegian Radium Hospital for years. HEp-2 cells were kindly provided by P. Boquet, Pasteur Institute, Paris.

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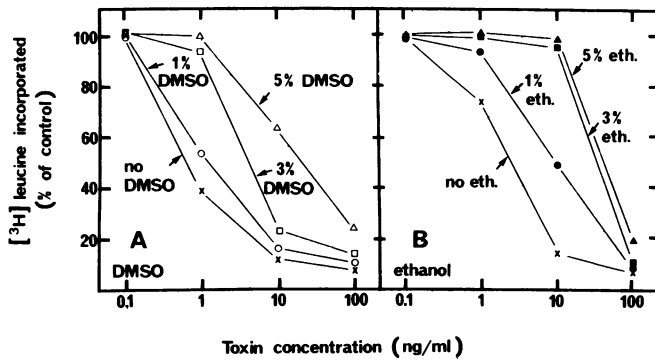


FIG. 1. Ability of organic solvents to protect Vero cells against Shiga toxin. Vero cells growing in 24-well disposable trays were incubated in growth medium containing HEPES (20 mM) instead of bicarbonate and the indicated concentrations of DMSO (A) and ethanol (eth.) (B) for 10 min at 37°C. Then increasing concentrations of toxin were added, and the incubation was continued for 2 h at 37°C before protein synthesis was measured as described in Materials and Methods.

All three cell lines were grown in minimum essential medium with Earle salts, 2 g of sodium bicarbonate per liter, and 10% (vol/vol) fetal calf serum in an atmosphere with 5% CO₂. In these experiments cells were seeded in 24-well disposable trays (Falcon, Oxnard, Calif.) at a density of 10⁵ cells per well. After overnight incubation the cells were transferred to the indicated medium or buffers.

Measurement of cytotoxic effect. After incubation of cells with toxin as described in the legend to each figure, the medium or buffer was removed, and the cells were incubated in the same medium or buffer (no unlabeled leucine) for 15 min at 37°C with 1 μCi of [³H]leucine per ml. Then the medium was removed, the cells were treated twice with 5% (wt/vol) trichloroacetic acid and solubilized in KOH (0.1 M), and acid-precipitable radioactivity was measured.

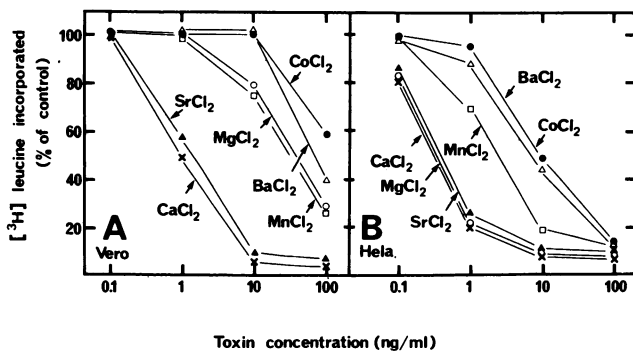


FIG. 2. Effect of different divalent cations on the sensitivity of cells to Shiga toxin. Vero cells (A) and HeLa cells (B) growing in 24-well disposable trays were washed twice with buffer containing 0.14 M NaCl, 20 mM HEPES (pH 7.2), 1 mg of glucose per ml, and 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid]. The cells were then incubated in the same buffer without EGTA, but with 2 mM of the different divalent cations as indicated for 10 min at 37°C. Increasing concentrations of toxin were added, and after 2 h of incubation at 37°C protein synthesis was measured as described in Materials and Methods.

TABLE 1. Effect of Ca²⁺ and compounds that affect Ca²⁺ transport on the sensitivity of cells to Shiga toxin^a

Compound	Compound concn (μM)	CaCl ₂ concn (mM)		ID ₅₀ for Shiga toxin (ng/ml)	
		Vero cells	HeLa cells	Vero cells	HeLa cells
None		2	0.60	0.60	0.35
		0.05	6.0	0.60	
		0.001	6.0		
Verapamil	250	2	3.0	0.7	
		0.025	10.8	2.1	
D600	250	0.1	4.6	2.1	
MgCl ₂	2,000	0.1	0.6		
CoCl ₂	2,000	0.1	37.8		
A23187	0.1	2	1.1	1.4	
		0.5	2	5.7	13.7
		2.0	2	91.2	24.2
		2.0	2	11.8	9.5
Ionomycin	2.0	2			

^a Conditions were as described in legend to Fig. 2. The concentration of toxin required to reduce protein synthesis to 50% (ID₅₀) was determined.

RESULTS

Effect of different divalent cations on the sensitivity of cells to Shiga toxin. The toxic proteins abrin, ricin, and modeccin intoxicate cells only when Ca²⁺ is present in the medium (25). We therefore examined whether divalent cations in the medium are important for toxic action of Shiga toxin on cells. For this purpose we added increasing concentrations of toxin to Vero cells and HeLa cells that were incubated in buffers containing various divalent cations (2 mM) as described in the legend to Fig. 2. The sensitivity of both Vero

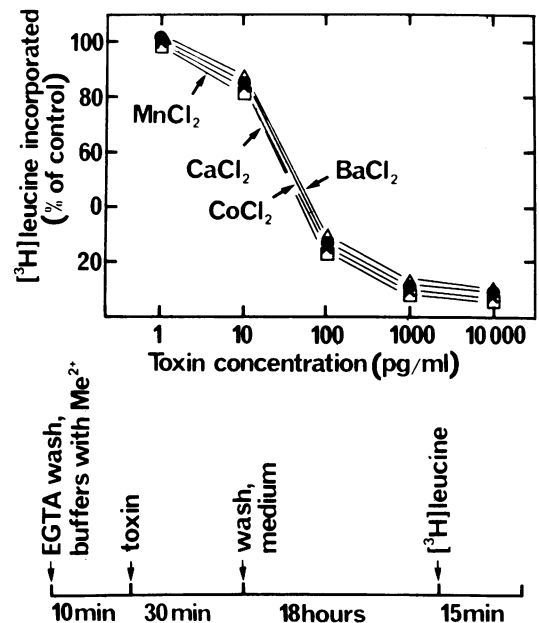


FIG. 3. Effect of divalent cations on the effective binding of Shiga toxins to cells. Vero cells growing in 24-well disposable trays were washed twice in buffer containing EGTA as described in the legend to Fig. 1 and then incubated in buffers containing different divalent cations (2 mM) for 10 min at 37°C. Increasing concentrations of toxin were then added, and after 30 min of incubation at 37°C the cells were washed twice with buffer to remove unbound toxin, growth medium was added, and the cells were incubated overnight before protein synthesis was measured.

cells and HeLa cells to Shiga toxin was dependent on the divalent cation present (Fig. 2). The cells were protected when Co^{2+} , Ba^{2+} , and Mn^{2+} were added instead of Ca^{2+} . There was a difference between the two cell lines with regard to Mg^{2+} . HeLa cells remained sensitive in the presence of Mg^{2+} , whereas Vero cells did not. Substitution of Ca^{2+} with Sr^{2+} did not protect either cell line. The concentration of Ca^{2+} required to give maximal toxic effect on Vero cells was low. The toxic effect was similar in the presence of 0.05 mM Ca^{2+} as when the buffer contained 2 mM Ca^{2+} (Table 1). Lower concentrations of Ca^{2+} reduced the cytotoxicity.

The first step in the intoxication process is binding of toxin to cell surface receptors. To study whether Ca^{2+} is necessary for binding of Shiga toxin to functional receptors, toxin was added to Vero cells incubated in buffers containing different divalent cations. After 30 min of incubation at 37°C the cells were washed to remove unbound toxin, and they were then incubated overnight in growth medium to allow bound toxin to intoxicate the cells. The presence of Ca^{2+} , Sr^{2+} , Ba^{2+} , Mn^{2+} , Mg^{2+} , or Co^{2+} during binding of toxin did not alter the intoxication (Fig. 3). Therefore, the protective effect afforded by different cations (Fig. 2) was not due to a reduced binding of toxin to the cells.

Requirement of Ca^{2+} transport for entry of Shiga toxin. Ca^{2+} transport across the membrane appears to be required for entry of the protein toxins abrin, ricin, and modeccin. Compounds like verapamil that block this transport prevented entry of the toxins into the cytosol (25). We therefore tested whether Ca^{2+} transport is required for entry of Shiga toxin. Verapamil and the related compound D600 strongly protected both Vero cells and HeLa cells against Shiga toxin (Table 1). The protection was most effective at low Ca^{2+} concentrations in the medium. Furthermore, Co^{2+} , which inhibits Ca^{2+} transport in Vero cells (25), protected cells against Shiga toxin even in the presence of Ca^{2+} , whereas Mg^{2+} had no protective effect (Table 1).

Since Ca^{2+} transport seemed to be required for the entry of Shiga toxin into cells, we tested the effect of the calcium ionophores A23187 and ionomycin on the intoxication process. These ionophores strongly protected against toxin in a concentration-dependent manner. (Table 1). The addition of these ionophores will induce a Ca^{2+} flux into the cytosol

TABLE 2. Effect of ionophores and inhibitors of ion transport on the sensitivity of cells to Shiga toxin^a

Compound added	Compound concn (μM)	ID ₅₀ for Shiga toxin (ng/ml)	
		Vero cells	HeLa cells
None		0.60	0.35
Amiloride	1,000	0.60	
Monensin	1	7.50	7.0
	0.1	0.60	0.35
Nigericin	10	0.60	
	5	0.60	1.1
	1.80		0.91
	0.1	0.60	
SITS ^b	1,000	17.4	3.2
	100	1.95	0.35
DIDS ^c	1,000	5.4	7.0
	100	0.60	0.35

^a Conditions were as described in the legend to Fig. 2, with the exception that a medium containing HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was used. The concentration of toxin required to reduce protein synthesis to 50% (ID₅₀) was determined.

^b SITS, 4-Acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid.

^c DIDS, 4,4'-Diisothiocyano-stilbene-2,2'-disulfonic acid.

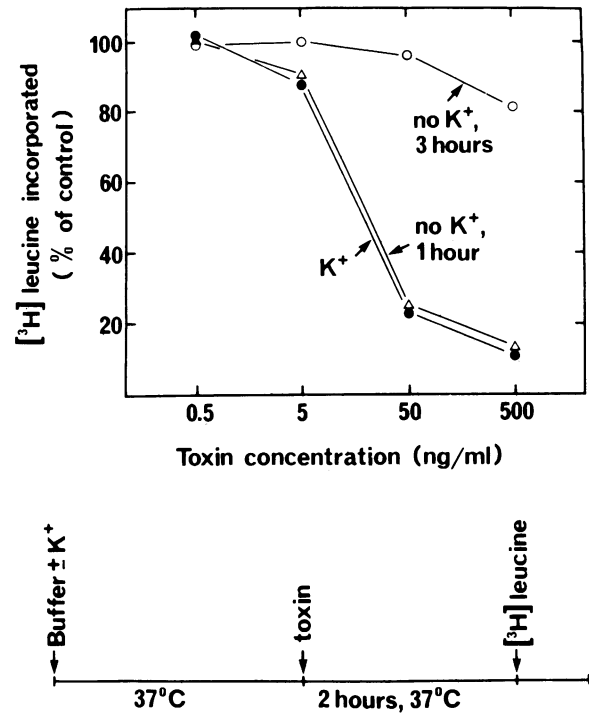


FIG. 4. Effect of potassium depletion on the sensitivity of Hep-2 cells to Shiga toxin. Vero cells growing in 24-well disposable trays were incubated in buffers containing 0.14 M NaCl, 20 mM HEPES (pH 7.2), 2 mM CaCl_2 , 1 mM MgCl_2 , and 5 mM glucose with and without 10 mM KCl for the indicated periods of time. Then increasing concentrations of toxin were added, and the incubation was continued for 2 h at 37°C before protein synthesis was measured as described in Materials and Methods.

both from the medium and from intracellular organelles. To test whether release of Ca^{2+} from intracellular organelles is sufficient to protect cells, the ionophore A23187 was added to HeLa cells in a buffer containing magnesium instead of calcium. The cells were protected against Shiga toxin also under these conditions (data not shown). The calcium ionophores had no effect on the binding of Shiga toxin to cells when tested as in Fig. 3 (data not shown).

Increased cytosolic calcium concentration has been shown to enhance the effect of protein kinase C. This

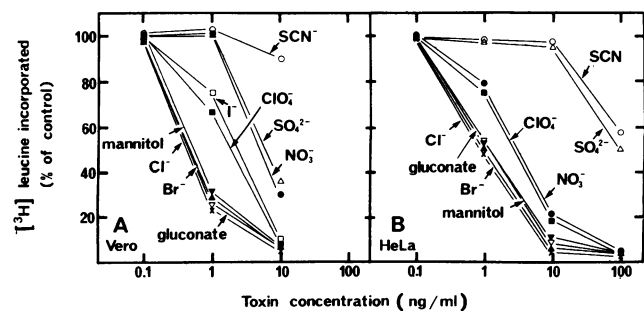


FIG. 5. Effect of different anions on the sensitivity of cells to Shiga toxin. Vero cells (A) and HeLa cells (B) growing in 24-well disposable trays were incubated in buffers containing 20 mM HEPES (pH 7.2), 1 mM $\text{Ca}(\text{OH})_2$, 1 mg of glucose per ml, and the sodium salts indicated (0.14 M) or 260 mM mannitol. Increasing concentrations of toxin were then added, and protein synthesis was measured after 2 h of incubation at 37°C.

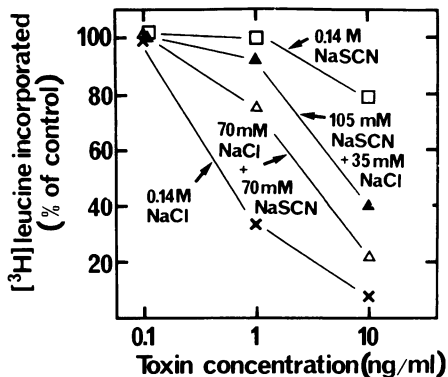


FIG. 6. Effect of NaCl and NaSCN on the sensitivity of Vero cells to Shiga toxin. Vero cells growing in 24-well disposable trays were incubated for 15 min at 37°C in buffers containing 20 mM HEPES (pH 7.2), 1 mM Ca(OH)₂, 1 mg of glucose per ml, and the sodium salt of either Cl⁻ or SCN⁻ as indicated. Increasing concentrations of toxin were then added, and after 2 h of incubation at 37°C protein synthesis was measured as described in Materials and Methods.

enzyme is activated by the phorbol ester TPA (12, 28) and has also been reported to be affected by trifluoperazine and retinoic acid (11, 15). However, none of these compounds had any effect on the sensitivity of the cells to Shiga toxin (data not shown).

It should be noted that a number of the compounds here used were dissolved in DMSO or ethanol, which at higher concentrations protect cells against Shiga toxin (Fig. 1). In the other experiments shown the concentration of these organic solvents was therefore always below 0.2% (vol/vol).

Requirement of monovalent cations for Shiga toxin entry. Eiklid and Olsnes showed that HeLa cells are protected against Shiga toxin when NaCl in the medium is substituted with KCl (9). We observed similar results here with Vero cells and HEp-2 cells (data not shown). The protection is probably not due to an inhibition of the Na⁺-H⁺ exchanger, since amiloride, which inhibits Na⁺-H⁺ exchange, did not alter the effect of toxin (Table 2). Furthermore, substitution of Na⁺ with choline did not protect the cells (data not shown). In fact, most of the salt in the buffer can be substituted with mannitol without any reduction in toxic effect (see further below).

One effect of a high potassium concentration in the medium is a strong reduction of the membrane potential. The membrane potential can also be reduced by incubating cells in a potassium-free buffer to deplete the cytosol of potassium. In HEp-2 cells we have recently characterized the effect of potassium depletion on the membrane potential as well as on endocytosis (I. H. Madhus, T. I. Tonnessen, S. Olsnes, and K. Sandvig, *J. Cell. Physiol.*, in press). After 3 h in a potassium-free buffer, the membrane potential was reduced by 90%, whereas the endocytosis of transferrin was only reduced to 50% of the control value. A 3-h incubation without potassium before the addition of toxin strongly protected HEp-2 cells against Shiga toxin (Fig. 4). A 1-h incubation without potassium did not protect the cells. The sensitivity of the cells was rapidly restored upon transfer to a buffer containing potassium. The restoration of the sensitivity could be inhibited by ouabain, which inhibits Na⁺-K⁺ exchange, demonstrating that intracellular potassium is required for entry. The protection of the cells obtained by potassium depletion was not due to reduced binding of the toxin (data not shown).

Effect of different anions on entry of Shiga toxin into cells.

Experiments with diphtheria toxin, modeccin, abrin, and ricin have shown that only certain anions support entry of diphtheria toxin and modeccin, whereas abrin and ricin intoxicate cells equally well in the presence of a number of different anions. To test whether various anions in the medium would affect intoxication by Shiga toxin, increasing concentrations of toxin were added to Vero cells and HeLa cells incubated in buffers containing various anions or isotonic concentrations of mannitol (260 mM). Substitution of NaCl with mannitol, gluconate, or with the sodium salt of rapidly permeable anions (19) had essentially no effect on intoxication (Fig. 5). Incubation with slowly permeable anions, like SCN⁻ or SO₄²⁻, protected the cells. Protection was not due to reduced binding of toxin to the cells (data not shown). To test whether the protection afforded by these anions would persist even when chloride was present, Shiga toxin was added to cells incubated in buffers containing various concentrations of chloride and thiocyanate. There was a proportional decrease in the protection obtained as the thiocyanate concentration was decreased and the chloride concentration was increased (Fig. 6).

As shown above, slowly permeable anions known to compete with chloride for transport across the cell membrane protected cells against Shiga toxin. We therefore tested the effect of inhibitors of chloride transport, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid and 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid, on the intoxication. Both of these inhibitors had a protective effect (Table 1), but only at high concentrations (1 mM).

Effect on pH of intracellular compartments on toxin entry.

The ionophore monensin, which is able to increase pH in intracellular compartments, has previously been shown to protect HeLa cells against Shiga toxin (9). As shown in Table 2 Vero cells were also protected by monensin. However, this ionophore may also induce changes in other ion gradients and thereby protect against pH Shiga toxin. We there-

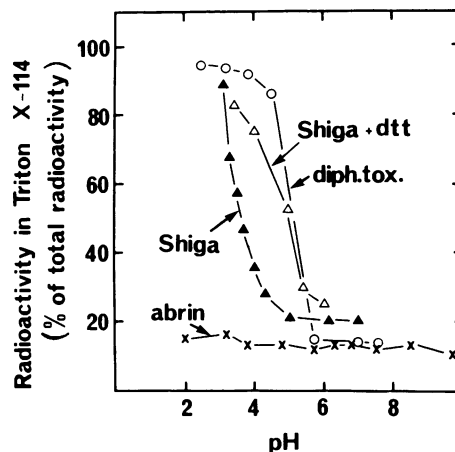


FIG. 7. Effect of pH on the ability of Shiga toxin, abrin, and diphtheria toxin in bind Triton X-114. A mixture of medium (300 μl) and Triton X-114 prepared as described in Materials and Methods (100 μl), adjusted to the indicated pH values, was incubated for 15 min at 4°C with ¹²⁵I-labeled toxins. The samples were then heated to 37°C to induce phase separation, and they were centrifuged for 2 min in an Eppendorf centrifuge model 3200. The radioactivity in the two phases was measured. The amount of radioactivity in Triton X-114, as a percentage of the total radioactivity in each sample is given at the ordinate. dtt, incu-50 mM dithiothreitol.

TABLE 3. Comparison of the requirements for toxic action of abrin, ricin, modeccin, diphtheria toxin, and Shiga toxin on Vero cells

Conditions	Protection of cells from:				
	Abrin	Ricin	Modeccin	Diphtheria toxin	Shiga toxin
Inhibitors of Ca ²⁺ transport	+	+	+	-	+
Inhibitors of Cl ⁻ transport	-	-	+	+	+
Absence of Cl ⁻ , isotonic mannitol	-	-	+	+	-
Nigericin (5 μM)	-	-	+	+	-
Nigericin (0.1 μM)	-	-	+	-	-
Isotonic KCl	-	-	+	-	+
Acidic medium	+	+	+	-	+

fore tested the effect of the ionophore nigericin, which also increases pH of intracellular compartments. Nigericin at 5 to 10 μM did not protect Vero cells, whereas 1 μM of the ionophore had a slight protective effect on both Vero cells and HeLa cells. Furthermore, NH₄Cl (10 mM) had no protective effect against Shiga toxin on either HeLa cells (9) or Vero cells (data not shown).

Of the other protein toxins that enter the cytosol, some require low pH in an intracellular compartments for entry, whereas others do not (18). For example, diphtheria toxin enters the cytosol from early acidic vesicles that are formed after endocytosis. Also *Pseudomonas aeruginosa* exotoxin A and modeccin require a low pH at some stage of the entry. Conversely, abrin and ricin do not seem to require low pH. With diphtheria toxin, low pH induces a conformational change in the molecule, leading to exposure of a hydrophobic domain which allows binding of Triton X-100 (24).

To test whether hydrophobic regions of toxins are exposed at low pH, we used the detergent Triton X-114. This detergent is miscible with water at 0°C, whereas two phases are formed above 20°C (1). Diphtheria toxin became associated with Triton X-114 at low pH, whereas abrin stayed in the water phase at all pH values tested (Fig. 6). With ¹²⁵I-Shiga toxin a certain amount of the radioactivity was in the Triton X-114 phase even at neutral pH. Only at very low pH values did most of the toxin become associated with the Triton X-114 phase (Fig. 7). However, treatment of toxin with dithiothreitol shifted the curve to higher pH values. Binding of detergent is not due to denaturation of Shiga toxin, which is stable even at pH 3 (22). For Shiga toxin to inactivate ribosomes, the A chain has to be proteolytically nicked (5). The preparation of toxin used here was about 95% unnicked. However, similar results were obtained with nicked toxin. Only at pH values below 4 did a significant fraction of nicked toxin bind Triton X-114 (data not shown).

We have shown earlier that direct entry of diphtheria toxin from the cell surface can be induced by exposure of cells to low pH (24). So far we have been unable to induce direct entry of other toxins by manipulations of the medium. Low pH in the medium actually protects cells against abrin, ricin, modeccin, and Shiga toxin (8, 18). The reason for this protection is not clear. Possibly, the other toxins must be modified by intracellular enzymes before entry into the cytosol. To test whether proteolytic cleavage of Shiga toxin is necessary for entry into cells we used a number of compounds that inhibit proteolytic enzymes. Of the different compounds tested, only the comparatively nonspecific inhibitor L-1-tosylamide-2-phenylethylchloromethyl ketone (21) had some protective effect. L-1-Tosylamide-2-phenylethylchloromethyl ketone protected cells both against nicked and unnicked toxin. Other inhibitors of proteolytic enzymes such as phenylmethylsulfonyl fluoride, chymostatin, leupeptin, and pepstatin had no effect at all (data not shown).

DISCUSSION

Entry of Shiga toxin into the cytosol seems to require calcium transport. Verapamil, D600, and Co²⁺, which all block calcium transport across membranes, protected Vero cells and HeLa cells against the toxin. In contrast to Vero cells, HeLa cells remained sensitive to Shiga toxin when Ca²⁺ was substituted with Mg²⁺. Possibly these cells contain enough intracellular calcium to support entry of toxin. As with other protein toxins, entry of Shiga toxin appears to require a Ca²⁺ flux through naturally occurring calcium channels, since the Ca²⁺ ionophores A23187 and ionomycin protected against the toxin. The protection of HeLa cells afforded by A23187 in the presence of Mg²⁺ may be due to fluxes of calcium from intracellular stores. Since binding of Shiga toxin to cell surface receptors appears to be unaffected by the type of divalent cation present, it is likely that intracellular routing of the toxin or transport of A fragment across the membrane requires calcium transport. With abrin, earlier results suggest that Ca²⁺ is required for penetration of A chain through the membrane (25). Interestingly, divalent cations and a membrane potential are required for import of the ADP-ATP carrier into mitochondria (20).

When HeLa cells, Vero cells, and HEp-2 cells were incubated in a buffer containing a high concentration of potassium, they were protected against Shiga toxin, suggesting that a normal membrane potential may be required for entry of toxin. Also, when the membrane potential in HEp-2 cells was reduced by potassium depletion, the cells were protected against Shiga toxin. A reduced membrane potential could affect both the membrane itself and possibly also the interaction between the membrane and the bound ligand. Of the other protein toxins studied, a high potassium concentration in the medium protected Vero cells against modeccin, but not against abrin, ricin, and diphtheria toxin (Table 3).

Several anions known to inhibit Cl⁻ transport in cells, such as SCN⁻ and SO₄²⁻ (19), as well as the inhibitors of anion transport, 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid and 4,4'-diisothiocyano-stilbene-2,2'-disulfonic acid, protected both Vero cells and HeLa cells against Shiga toxin. On the other hand, substitution of Cl⁻ with a rapidly permeable anion such as Br⁻ and even substitution of Cl⁻ with isotonic concentrations of mannitol and gluconate had no effect on the sensitivity of the cells to Shiga toxin. These results differ from those obtained with other protein toxins (Table 3). Vero cells were protected against both modeccin and diphtheria toxin when Cl⁻ was removed or when slowly permeable anions were present. In the case of diphtheria toxin, toxin transport through the outer cell membrane at low pH requires permeable anions (26). On the other hand, entry of abrin and ricin into the cytosol occurs

equally well when Cl^- , mannitol, or SO_4^{2-} is present in the medium.

The fact that nigericin and NH_4Cl do not protect cells against Shiga toxin suggests that an acidic compartment is not required for entry and that the protection obtained after addition of monensin is due to changes other than an increased vesicular pH. Moreover, when we looked at the ability of Shiga toxin to bind Triton X-114 there was no binding of detergent at the pH values present in endosomes. After treatment of the toxin with dithiothreitol the toxin bound Triton X-114 at higher pH values, but it is not clear whether this is of physiological importance. It is not known at what point during entry that the disulfide bond of the protein toxins is reduced. Interestingly, reduction of a disulfide bond in cholera toxin allows penetration of the α -polypeptide chain into Triton X-100 micelles (27).

Some compounds and conditions that affect entry of abrin, ricin, modeccin, diphtheria toxin, and Shiga toxin are summarized in Table 3. Only abrin and ricin cannot be distinguished by the listed criteria. Modeccin entry is inhibited by all of the different conditions listed, whereas Shiga toxin, which is structurally different from the other toxins, and diphtheria toxin each share some similarities with the other toxins. However, in spite of common properties these protein toxins seem to use different mechanisms to get across the membrane and into the cytosol.

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