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Action of Diphtheria Toxin Does Not Depend on the Induction of Large, Stable Pores Across Biological Membranes

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Summary. Vero cells exposed to diphtheria toxin at pH 4.5 leak monovalent cations but not amino acids or phosphorylated metabolites; affected cells do not take up trypan blue. Monovalent cation leakage is inhibited by 1 mM Cd^{2+}, but not by 1 mM Zn^{2+} or Ca^{2+}. Cd^{2+} blocks calcein leakage from liposomes and closes diphtheria toxin-induced channels in lipid bilayers. It is concluded that translocation of the A fragment of diphtheria toxin across biological membranes does not depend on the formation of large stable pores, but that small Cd^{2+}-sensitive pores may play a role.

Key Words diphtheria toxin · planar bilayers · liposomes · Vero cells · pores · cadmium (Cd^{2+})

Introduction

Bacterial and plant toxins such as diphtheria (Collier, 1975; Pappenheimer, 1977), cholera (Holmgren, 1981), tetanus (Van Heyningen, 1980; Mellanby & Green, 1981), botulinum (Sugiyama, 1980), abrin or ricin (Olsnes & Sandvig, 1983) kill cells by inhibiting key intracellular processes. Each toxin consists of an A polypeptide chain linked through an S-S bond to a B chain: the A chain is the toxic component; the B chain, which binds to, and becomes inserted into, the plasma membrane of susceptible cells, somehow enables the A chain to cross the membrane and gain entry to the cytoplasm. The best-studied of these toxins is diphtheria toxin, and in that case it has been shown that insertion of the B chain, and hence transfer of the A chain, requires a low pH (Sandvig & Olsnes, 1980).

In order to explore the mechanism of transfer, studies aimed at elucidating the kind of membrane lesion induced by the B chain have been carried out. The results have been paradoxical. On the one hand conductivity measurements with planar lipid bilayers have shown the formation of narrow channels by the B chain (Donovan et al., 1981) or by a fragment thereof (Kagan, Finkelstein & Colombini, 1981; Misler 1983); on the other hand experiments on leakage of variously sized molecules across liposomal membranes induced by cleaved toxin (Zalman & Wisnieski, 1984) or by a B chain (Kagan et al., 1981) have indicated a pore size large enough to allow molecules of up to 1500 Da to cross, the implication being that such a channel would allow the A chain (21,000 Da) to cross the membrane. A channel of those dimensions would be comparable in size to the pores formed by another class of toxin, namely one that damages cells directly by the creation of pores large enough to allow leakage of ions, metabolic intermediates and, in some instances, proteins, out of cells (Bhakdi & Tranum-Jensen, 1987). The functional sizes of the lesions that are formed by such agents, which include the terminal membrane attack complex of complement (Mayer, 1972; Bhakdi & Tranum-Jensen, 1984; Muller-Eberhard, 1984) and the cytolysin or perforin of cytotoxic lymphocytes (Henkart, 1985; Young, Cohn & Podack, 1986) are not fixed but vary both with the amount of agent inserted into the membrane (e.g., Sims & Lauf, 1980) and with time (e.g., Bashford, Micklem & Pasternak, 1985). Nevertheless pore sizes similar to that proposed for diphtheria toxin (diam. > 1.8 nm, Kagan et al., 1981; diam. 2.4 nm, Zalman & Wisnieski, 1984) have been reported in the case of haemolytic Sendai virus (diam. ≈1 nm; Wyke et al., 1980), *Staphylococcus aureus* α toxin (diam. 1–2 nm; Bhakdi & Tranum-Jensen, 1988) and low doses of complement (diam. ≈2.3 nm; Sims & Lauf, 1978). Lesions induced by these agents have certain properties in common. These include the leakage of phosphorylated metabolites (Pasternak & Micklem, 1973; Thelestam & Mollby, 1979) from cells and a characteristic sensitivity towards divalent cations (Impraim, Micklem & Pasternak, 1979; Thelestam &

Mollby, 1980) such as Ca^{2+} (Bashford et al., 1984) or Zn^{2+} (Bashford et al., 1986). We have used these criteria to examine the membrane-damaging action of diphtheria toxin in Vero cells, liposomes and lipid bilayers and conclude that the formation of stable pores of diam. > 1.8 nm, similar to those formed by 'pore-forming' agents, is not a prerequisite for the inhibition of protein synthesis by diphtheria toxin.

Materials and Methods

Cells

Vero (African green monkey kidney) cells were grown as a monolayer in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum (Flow Laboratories), 50 μg/ml penicillin, 50 IU/ml streptomycin, 1.7 g/l $NaHCO_3$, pH 7.3 in a 5% CO_2 air incubator.

Liposomes and Planar Lipid Bilayers

Asolectin, purified as described by Kagawa and Racker (1971) was from Sigma. Liposomes were prepared by sonicating (Braunsonic 1510 with a needle probe operated intermittently for a total of 5 min at 100 W) 12.5 mg/ml asolectin in 80 mM calcein in 50 mM NaCl pH 7.0 (NaOH). The sonicate, which contained mainly small, unilamellar vesicles, was applied to a column of Sephadex G50 and eluted with 160 mM NaCl, 10 mM HEPES, pH 7.0 (NaOH); the fractions used for leakage experiments contained >80% entrapped calcein. Planar lipid bilayers of asolectin were prepared as previously described (Bashford et al., 1988*a*).

Toxin and Other Chemicals

Nicked diphtheria toxin (Drazin, Kandel & Collier, 1971) was a gift from Dr. S. Olsnes. Partially-purified cytolysin (2 protein bands by SDS gels) from cytotoxic LGL tumors (Blumenthal et al., 1984) was a gift from Dr. P.A. Henkart. [^{3}H]choline, [^{3}H]2 deoxy-D-glucose (dGlc), [^{14}C]2-amino-iso-butyrate (AIB), [^{14}C]cycloleucine, [^{35}S]L-methionine and sodium [^{35}S]sulphate were from Amersham International. DIDS was from Sigma. HEPES-buffered saline (HBS) was prepared as previously described (Bashford et al., 1988*a*).

Leakage From Cells

Vero cell monolayers were washed, exposed to [^{3}H]dGlc and either [^{14}C]AIB or [^{14}C]cycloleucine in HBS for 1 hr at 37°C. Under these conditions, much of the [^{3}H]choline and [^{3}H] dGlc is converted to phosphoryl[^{3}H]choline and [^{3}H]dGlc 6-P, respectively (Impraim et al., 1980; Wohlheuter & Plagemann, 1980; Pasternak, Whitaker-Dowling & Widnell, 1988), which only leak out of damaged cells; [^{14}C]AIB and [^{14}C]cycloleucine, which are concentrated but not metabolized by cells, leak out irrespective of cell damage. Isotope-containing solutions were replaced by diphtheria toxin in HBS (or HBS alone) (0.5 ml per 35-mm dish) and the cells processed as follows. After 15 min at 37°C, toxin was removed and replaced by low pH medium (150 mM NaCl, 5 mM KCl, 10 mM MES, pH 4.5; MES-BS) and incubated at 37°C for 5 min; under these conditions, bound diphtheria toxin enters the cells (Sandvig & Olsnes, 1980). The low pH medium was then replaced by HBS and leakage at 37°C of radioactively labeled metabolites out of cells, or entry of sodium [^{35}S]sulphate into cells, assessed.

Assay of Intracellular Cations

Vero cell monolayers during the low pH treatment, or during the subsequent incubation in HBS, or during the subsequent exposure to [^{35}S]methionine (*see below*) were washed three times with 150 mM choline chloride and extracted with 0.1% triton X-100 in 0.1 M $LiNO_3$ in H_2SO_4, and Na^+ and K^+ content measured by atomic absorption. Leakage of K^+ from damaged cells is accompanied by leakage of Na^+ into cells, with little change in the total cellular content of Na^+ plus K^+ (Bashford et al., 1985; 1988*a*). A convenient index of cation leakage is the relative Na^+ content of the cells, namely $Na^+/(Na^+ + K^+)$ which has a value close to 0.13 for control Vero cells (untreated with diphtheria toxin) and which can rise to a maximum value of 0.97 when the intracellular and extracellular pools of Na^+ and K^+ have equilibrated. In the present experiments the Na^+ and K^+ contents of control Vero cells were 81 ± 17 and 564 ± 91 nmol/μg protein, respectively (mean ± SD, n = 20).

Assay of Protein Synthesis

Vero cell monolayers following the low pH treatment were incubated in Eagle's Minimal Essential medium containing 10 mM NH_4Cl for 2 hr at 37°C. The medium was replaced by methionine-free Eagle's Minimal Essential medium containing [^{35}S]L-methionine and incubated at 37°C. At intervals, this medium was removed, the cells washed with 150 mM choline chloride and extracted in ice-cold 5% TCA containing 0.1 M $LiNO_3$; the TCA soluble fraction was assayed for monovalent cations, and the TCA-insoluble pellets were dissolved in 1 N NaOH and assayed for ^{35}S content and protein content by the method of Lowry et al. (1951). [^{35}S]methionine incorporation into TCA-insoluble material was linear over a 30-min time course.

Leakage From Liposomes

Liposomes containing calcein were incubated in 160 mM NaCl, 10 mM HEPES, pH 4.5 (an essentially unbuffered solution) or the same adjusted to pH 7.0 with NaOH at room temperature in a Perkin-Elmer MPF-44A spectrofluorimeter. Calcein fluorescence was excited at 490 nm and recorded at 520 nm; excitation and emission slit-widths were 10 nm. Maximal leakage was assessed by lysis in 0.025% triton X-100.

Conductivity of Lipid Bilayers

Conductivity was measured as described by Bashford et al. (1988*a*). Diphtheria toxin was added to the bathing solution on one side (*cis* side); that compartment was connected to the virtual ground and voltage signs are referred to it.

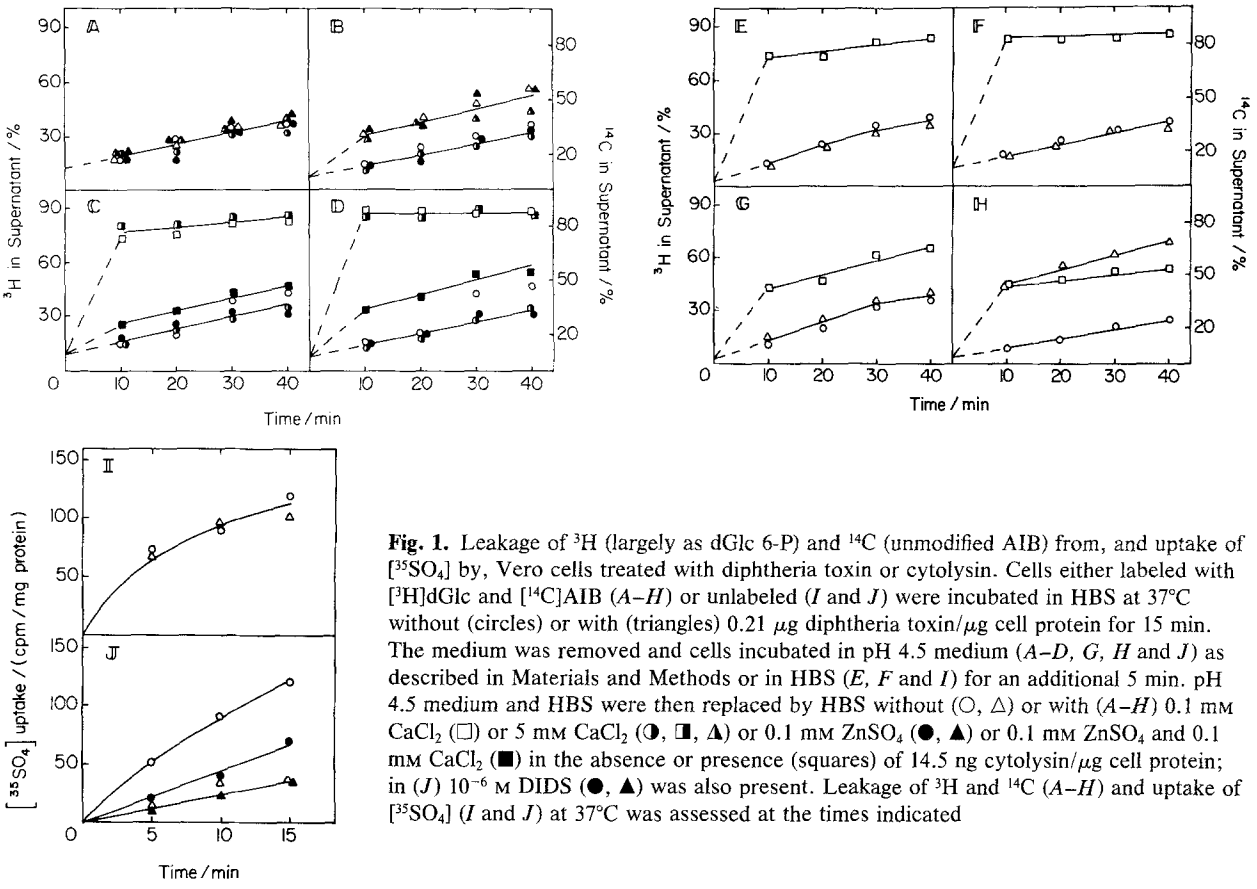

Fig. 1. Leakage of ^{3}H (largely as dGlc 6-P) and ^{14}C (unmodified AIB) from, and uptake of [$^{35}SO_4$] by, Vero cells treated with diphtheria toxin or cytolysin. Cells either labeled with [^{3}H]dGlc and [^{14}C]AIB (*A–H*) or unlabeled (*I* and *J*) were incubated in HBS at 37°C without (circles) or with (triangles) 0.21 μg diphtheria toxin/μg cell protein for 15 min. The medium was removed and cells incubated in pH 4.5 medium (*A–D, G, H* and *J*) as described in Materials and Methods or in HBS (*E, F* and *I*) for an additional 5 min. pH 4.5 medium and HBS were then replaced by HBS without (○, △) or with (*A–H*) 0.1 mM $CaCl_2$ (□) or 5 mM $CaCl_2$ (◑, ◨, ◭) or 0.1 mM $ZnSO_4$ (●, ▲) or 0.1 mM $ZnSO_4$ and 0.1 mM $CaCl_2$ (■) in the absence or presence (squares) of 14.5 ng cytolysin/μg cell protein; in (*J*) 10^{-6} M DIDS (●, ▲) was also present. Leakage of ^{3}H and ^{14}C (*A–H*) and uptake of [$^{35}SO_4$] (*I* and *J*) at 37°C was assessed at the times indicated

Abbreviations

HEPES: N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid
MES: 2-[N-morpholino]ethanesulfonic acid
HBS: HEPES buffered saline
DIDS: 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid
AIB: 2-aminoisobutyrate

Results

Diphtheria Toxin Does Not Induce Metabolite Leakage From Vero Cells

In order to measure the leakage of phosphorylated and unphosphorylated low molecular weight metabolites simultaneously, cells were preincubated with [^{3}H]dGlc and [^{14}C]AIB; most of the [^{3}H]dGlc is converted to [^{3}H]dGlc 6-P under these conditions (Impraim et al., 1980; Wohlheuter & Plagemann, 1980), while the [^{14}C]AIB remains unmodified. When such cells are washed and reincubated, some ^{14}C but little ^{3}H leaks out (e.g., Pasternak et al., 1988); in the presence of a pore-forming agent, leakage of both ^{14}C and ^{3}H is enhanced (Impraim et al., 1980). When Vero cells preincubated with these isotopes are exposed to diphtheria toxin (at pH 4.5), some ^{14}C but no ^{3}H leaks out; the leakage of ^{14}C is unaffected by Ca^{2+} or Zn^{2+} (Fig. 1*A* and *B*). At no concentration of diphtheria toxin tested was any leakage of ^{3}H (i.e., of dGlc6-P) apparent. In contrast, Vero cells exposed to cytolysin isolated from cytotoxic lymphocytes leak both ^{14}C and ^{3}H; leakage is inhibited by 0.1 mM Zn^{2+} (Fig. 1*C* and *D*), as previously demonstrated for Lettre cells (Bashford et al., 1988*b*). In order to confirm that the small amount of [^{14}C]AIB leakage that occurs after treatment with diphtheria toxin is indeed due to toxin-induced membrane damage, the experiment was repeated under conditions in which Vero cells were exposed to toxin at pH 7.4 as well as at pH 4.5. No leakage of ^{3}H occurred after exposure to toxin at either pH (Fig 1*E* and *G*); ^{14}C leakage occurred only after exposure to toxin at pH 4.5 (Fig. 1*F* and *H*). In contrast, leakage of ^{3}H and ^{14}C induced by lymphocyte cytolysin was greater at pH 7.4 than at 4.5 (Fig. 1, *compare E* and *F* with *G* and *H*), since H^+, like Ca^{2+} and Zn^{2+}, inhibits leakage induced by cytolysin (Bashford et al., 1988*b*). Leakage of phosphoryl [^{3}H]choline from Vero cells prelabeled with [^{3}H]choline was not induced by exposure (at pH

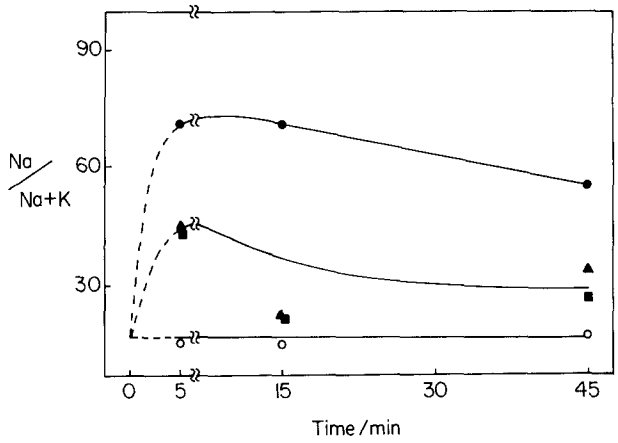

Fig. 2. Leakage of cations from diphtheria toxin treated Vero cells. Cells were incubated at 37°C in MES-BS pH 4.5 without (○) or with 0.2 μg diphtheria toxin/ml in the absence (●) or presence of 1 mM $CdSO_4$ (▲) or 10 mM $CaCl_2$ (■). After 5 min the medium was replaced by HBS, pH 7.4 and incubation at 37°C continued. Intracellular Na^+ and K^+ were assessed after washing cells with 150 mM choline chloride as described in Materials and Methods

4.5) to diphtheria toxin, in contrast to its leakage from cells exposed to lymphocyte cytolysin, *S. aureus* α toxin, melittin or other 'pore-forming' agent. Nor do diphtheria toxin-treated cells take up trypan blue (*results not shown*). Further evidence that diphtheria toxin had indeed been inserted into Vero cell membranes at pH 4.5 was obtained by measurement of DIDS-sensitive $^{35}SO_4^{2-}$ uptake, which is inhibited by toxin (Olsnes & Sandvig, 1986): Fig. 1*I* and *J*, shows that $^{35}SO_4^{2-}$ uptake was indeed inhibited after exposure of Vero cells to diphtheria toxin at pH 4.5, but not at 7.4.

Since [^{14}C]AIB is accumulated by cells partially as a result of an inward-directed Na^+ gradient, any leakage of [^{14}C]AIB caused by diphtheria toxin (Fig. 1*B* and *F*) might be due to collapse of the Na^+ gradient. As shown below, this is a likely possibility, especially as AIB leakage takes place *after* Na^+/K^+ leakage has taken place. Moreover leakage of [^{14}C]cycloleucine, which unlike AIB is not accumulated by linkage to the Na^+ gradient (Le Cam & Freychet, 1977; Christensen, 1984), is not affected by treatment of Vero cells with diphtheria toxin (*data not shown*).

Monovalent Cation Leakage and Inhibition of Protein Synthesis Induced by Diphtheria Toxin in Vero Cells Is Inhibited By Cd^{2+}

Diphtheria toxin (at pH 4.5) induces rapid leakage of K^+ from, and Na^+ into Vero cells (Fig. 2). As soon as the pH is restored to 7.5, leakage stops and some recovery takes place. This is in contrast to the inhibition of $^{35}SO_4^{2-}$ uptake, which is manifest *after* the pH has been restored to 7.5 (Fig. 1*I* and *J*). Cd^{2+} (Sandvig & Olsnes, 1988), and to a lesser extent Ca^{2+} but not Zn^{2+}, prevent leakage (Fig. 2 and Table 1). The efficacy of Cd^{2+} over Ca^{2+} or Zn^{2+} as a protectant is not related to a special property of Vero cells. Vero cells treated with *S. aureus* α toxin, for example, are protected against Na^+/K^+ leakage (and against phosphoryl[^{3}H]choline leakage) by $Zn^{2+} > Cd^{2+} > Ca^{2+}$, just as are Lettre cells and erythrocytes (Bashford et al., 1988*a*).

Inhibition of protein synthesis is likewise relieved, at least partially, by Cd^{2+} (Table 1), provided it is present during the pH 4.5 treatment. Cd^{2+} added after the pH is restored to 7.5, e.g., during the period that [^{35}S]methionine incorporation is measured, is ineffective. Ca^{2+} and Zn^{2+} added during the pH 4.5 treatment are less effective than Cd^{2+}. The data of Table 1 also show that at low doses of diphtheria toxin protein synthesis is inhibited in the absence of an effect on monovalent cation leakage; this confirms another recent report (Papini et al., 1988).

Calcein Leakage From Liposomes, and Conductivity Across Lipid Bilayers, Induced By Diphtheria Toxin Is Inhibited By Cd^{2+}

Asolectin liposomes loaded with calcein leak calcein when diphtheria toxin is added at pH 4.5 (Fig. 3). This implies the creation of relatively large (1–2 nm diam.) lesions in such liposomes. Cd^{2+} reduces the rate and extent of leakage; Ca^{2+} and Zn^{2+} are relatively ineffective (Table 2). Inhibition by Cd^{2+} appears to be reversible, in the sense that if EGTA is added 2 min after diphtheria toxin, leakage begins immediately; ordinarily a short lag is discernible before leakage begins (Fig. 3). This result indicates that the leakage of calcein following removal of Cd^{2+} by chelation is not due to the formation of new lesions by any diphtheria toxin that might have been displaced by Cd^{2+}, but rather to the 'opening' of preformed, or potential, lesions.

Leakage of calcein by diphtheria toxin requires (*i*) low pH (Table 2 and Fig. 3) and (*ii*) negatively charged liposomes: liposomes composed of 1:1 1-palmitoyl-2-oleoyl-phosphatidylcholine : cholesterol (neutral) require >20 times as much diphtheria toxin to initiate leakage as do liposomes composed of asolectin (negatively charged). This is in keeping with the observations of Alving et al. (1980) that diphtheria toxin binds better to negatively charged, than to neutral, lipids and of Donovan, Simon and Montal (1982) that bilayers composed of

Table 1. Effect of diphtheria toxin on monovalent cations and protein synthesis in Vero cells

	Monovalent cations[a]		Protein synthesis	
	Na/Na + K	% relative leakage[b]	[^{35}S] incorporation % inhibition of	% inhibition of relative protein synthesis[c]
No toxin	0.13	—	6425 ± 3722	—
+1 mM Cd	0.13	—	5697 ± 2033	—
+1 mM Zn	0.11	—	4476 ± 1932	—
+10 mM Ca	0.13	—	6661 ± 2505	—
0.01 μg toxin/ml	0.13	0	1542 ± 380	76
+1 mM Cd	0.10	0	2474 ± 222	57[d]
+1 mM Zn	0.11	0	996 ± 193	78
+10 mM Ca	0.13	0	2217 ± 851	67
0.1 μg toxin/ml	0.30	20	450 ± 229	93
+1 mM Cd	0.14	1[d]	792 ± 302	86
+1 mM Zn	0.27	19	343 ± 208	92
+10 mM Ca	0.18	6[d]	621 ± 209	91

[a] The values of monovalent cations shown refer to cells assessed during the pH 4.5 treatment. Where leakage occurred, it had returned to control values by the time protein synthesis was measured.
[b] Leakage is expressed relative to the respective control (± divalent cation).
[c] Percent inhibition of protein synthesis is expressed relative to the respective control (± divalent cation).
All values quoted are means (±SD) of two or three separate experiments.
[d] $P < 0.05$ that relative cation leakage or inhibition of relative protein synthesis is unaffected by divalent cations.

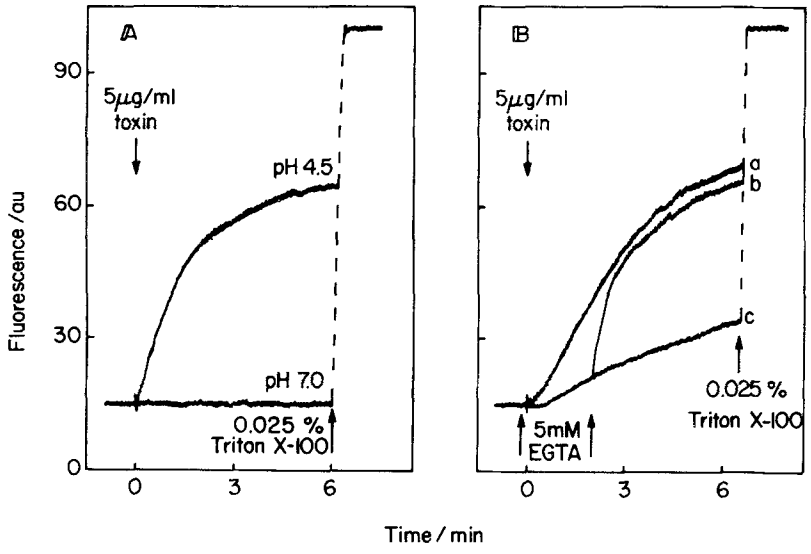

Fig. 3. Leakage of calcein from asolectin liposomes induced by diphtheria toxin. Asolectin liposomes (10 μg lipid/ml), containing calcein, suspended in 160 mM NaCl, 10 mM HEPES, pH 4.5 (*A* and *B*) or pH 7.0 (*A*) without (*A*) or with (*B*) 1 mM $CdSO_4$ at 22°C. Toxin, triton X-100 and EGTA were added to give the final concentrations indicated. In the experiments illustrated in (*B*) EGTA was added prior to toxin (trace *a*) or two min after toxin (trace *b*)

asolectin are more sensitive to diphtheria toxin than those composed of phosphatidylethanolamine or other phospholipids. Even toxins that insert at neutrality, such as tetanus toxin, yield bigger single-channel conductances in negatively charged, than in neutral, phospholipid bilayers (Hoch et al., 1985; Gambale & Montal, 1988).

Phospholipid bilayers composed of asolectin form channels when exposed to diphtheria toxin B chain (Donovan et al., 1981) or a mutant thereof (Kagan et al., 1981). The 'nicked' diphtheria toxin (i.e., containing most of the A and B chain) used in the present experiments gives a similar response. In the presence of 1 mM Cd^{2+}, channels close (Fig. 4). The effect is similar when the Cd^{2+} is present *cis*, or *cis* and *trans*; the latter result rules out an effect of Cd^{2+} resulting mainly from an alteration of surface charge due to binding of Cd^{2+} to the negative asolectin

Table 2. Effect of divalent cations on diphtheria toxin-induced leakage of calcein from liposomes

	Leakage (%)[a]
Diphtheria toxin	56
+1 mM Cd	23
+1 mM Ca	49
+1 mM Zn	52
+10 mM Cd	11
+10 mM Ca	35
+10 mM Zn	52

[a] Asolectin liposomes were incubated at 10 μg/ml at pH 4.5 at 22°C without or with $CdSO_4$, $CaCl_2$ or $ZnSO_4$ at the concentrations indicated. The extent of leakage induced by 5 μg/ml diphtheria toxin was assessed 10 min later.

bilayer (*see* Hille, 1984). Closure by Cd^{2+} appears to be irreversible, in that it was not possible to regain channels at either negative or positive voltage. Ca^{2+} (10 mM) and Zn^{2+} (5 mM) were without significant effect on diphtheria toxin-induced channels.

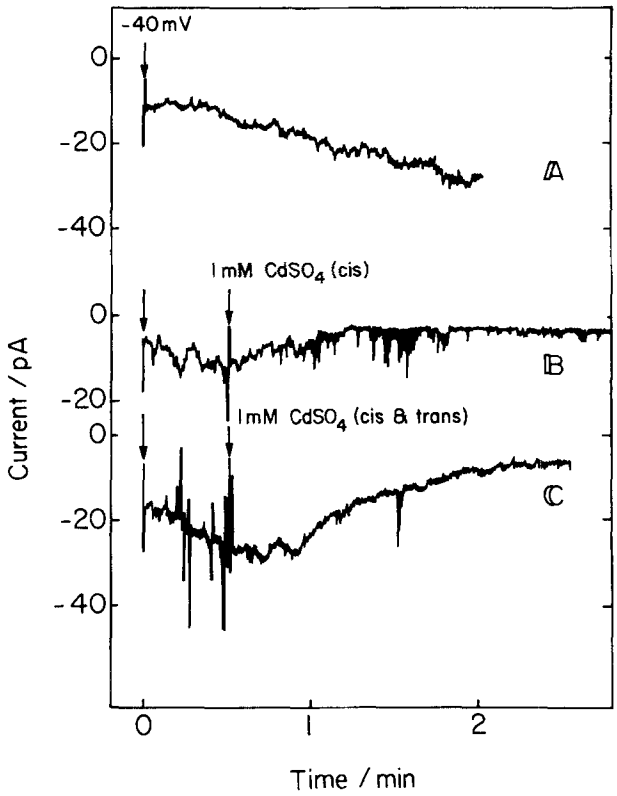

Fig. 4. Effect of Cd^{2+} on diphtheria toxin-induced currents in planar bilayers. Asolectin bilayers in 0.1 M KCl, 0.005 M MES, pH 5.5 at 23°C with 25 ng diphtheria toxin/ml (*cis*). Voltage (−40 mV) and $CdSO_4$ (1 mM) applied as indicated. (*A*) No Cd^{2+}; (*B*) Cd^{2+} *cis*; (*C*) Cd^{2+} *cis* and *trans*

Discussion

The aim of this work has been to compare the membrane lesions induced by diphtheria toxin with those induced by 'pore-forming' agents such as *S. aureus* α toxin, melittin, activated complement or the cytolysin of cytotoxic lymphocytes. Since membrane insertion by diphtheria toxin requires a low pH (Sandvig & Olsnes, 1980), all experiments were conducted at pH 4.5, with a 'nicked' preparation (Drazin et al., 1971) containing both A and B chains. The main conclusion to be drawn is that diphtheria toxin differs from the other agents we have studied in one regard (apart from the requirement for low pH) but resembles them in another.

Unlike classical 'pore-forming' agents, diphtheria toxin does not lead to the leakage of phosphorylated metabolites such as 2-deoxyglucose 6-P or phosphoryl choline from cells (Fig. 1); this is in keeping with the observation that anions such as sulphate or chloride do not leak out either (Sandvig & Olsnes, 1988); the selectivity for transport across phospholipid bilayers is complicated (Hoch et al., 1985). Rapid leakage of Na^+ and K^+ does occur (Fig. 2; Papinini et al., 1988; Sandvig & Olsnes, 1988), which indicates that failure to observe metabolite or anion leakage is not merely the result of pores being shut at the low pH (Bashford et al., 1986, 1988*a*). Hence there is no evidence for a stable channel of >1.8 nm in diameter being formed in cells. The fact that liposomes form such channels (Kagan et al., 1981; Zalman & Wisnieski, 1984; Fig. 3) indicates that caution has to be exercised in extrapolating from purely lipidic vesicles to natural membranes. The same situation has been found in regard to lesions induced by *C. perfringens* θ toxin: this induces the leakage of molecules as large as fluorescein dextran (20 kDa) from liposomes, but no leakage at all from Lettre cells (Menestrina, Bashford & Pasternak, 1990). Surprisingly, the results of bilayer experiments with diphtheria toxin (Fig. 4) are more in keeping with the situation in whole cells, in that they indicate a rather small channel (≈6 pS at 0.2 M NaCl: Donovan et al., 1981; ≈10 pS at 0.1 M KCl: Kagan et al., 1981; 7 pS at 0.1 M KCl: Misler, 1983).

As with classical 'pore-forming' agents, diphtheria toxin induces divalent cation-sensitive pores in cells (Fig. 2; Sandvig & Olsnes, 1988), liposomes (Fig. 3) and bilayers (Fig. 4). In every case, Cd^{2+} is more potent than Zn^{2+} or Ca^{2+} (Tables 1 and 2). This is not the situation with regard to *S. aureus* α toxin, Sendai virus, melittin, polylysine or triton X-100 in Lettre cells or erythrocytes, in which Zn^{2+} is as active as Cd^{2+}, and in some instances actually more so (Bashford et al., 1988*a*). On the other hand,

we have recently found that leakage of calcein from liposomes exposed to *S. aureus* α toxin, melittin or triton X-100, at pH 4.5 or pH 7.0, is also more sensitive to Cd^{2+} than to Zn^{2+} (C.L. Bashford & C.A. Pasternak, *unpublished observation*). Further work on the extent to which different divalent cations 'gate' (Menestrina, 1986) or block agent-induced channels should lead to a better understanding of the nature of the pores in specific instances.

So far as the relevance of pore formation by diphtheria toxin to the mechanism of its entry into susceptible cells is concerned, i.e., to the mechanism whereby the A fragment crosses the membrane from an endocytic (low pH) vesicle to the (neutral) cytoplasm, we draw two conclusions: (*i*) A stable pore of the dimensions induced in liposomes is unlikely to be involved; the possibility that a short-lived pore of this size is transiently formed cannot be excluded, but is rendered unlikely by the fact that 'flickering' between small and large channels in bilayers has not been observed (e.g., Donovan et al., 1981; and our *unpublished observations*). (*ii*) On the other hand, a smaller pore, through which cations but not anions or zwitterionic amino acids like AIB or cycloleucine (Fig. 1) are able to move, does appear to be involved. Even at low doses of diphtheria toxin, at which protein synthesis is inhibited in the absence of measurable cation leakage (Table 1; Papini et al., 1988), Cd^{2+} is still an effective inhibitor (Table 1; Sandvig & Olsnes, 1988); since in these experiments Cd^{2+} was added after any unabsorbed diphtheria toxin was removed, its action cannot be merely to prevent binding of diphtheria toxin. We note that a single pore in a cell, which would be sufficient to allow a molecule of diphtheria toxin to enter and inhibit protein synthesis (Yamaizumi, Mekada & Uchida, 1978), might not lead to measurable cation leakage if the Na^+/K^+ pump continues to operate. If the formation of small Cd^{2+}-sensitive pores does prove to be part of the mechanism whereby diphtheria toxin crosses membranes, it will be instructive to see whether similar pores are involved in the translocation of other 'A-B'-type toxins such as tetanus or botulinum (Hoch et al., 1985; Donovan & Middlebrook, 1986; Gambale & Montal, 1988), or indeed of proteins in general.

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References

Alving, C.R., Iglewski, B.H., Urban, K.A., Moss, J., Richards, R.L., Sadoff, J.C. 1980. Binding of diphtheria toxin to phospholipids in liposomes *Proc. Natl. Acad. Sci. USA* **77**:1986–1990

Bashford, C.L., Alder, G.M., Graham, J.M., Menestrina, G., Pasternak, C.A. 1988*a*. Ion modulation of membrane permeability: Effect of cations on intact cells and on cells and phospholipid bilayers treated with pore-forming agents. *J. Membrane Biol.* **103**:79–94

Bashford, C.L., Alder, G.M., Menestrina, G., Micklem, K.J., Murphy, J.J., Pasternak, C.A. 1986. Membrane damage by haemolytic viruses, toxins, complement and other cytotoxic agents: A common mechanism blocked by divalent cations. *J. Biol. Chem.* **261**:9300–9308

Bashford, C.L., Alder, G.M., Patel, K., Pasternak, C.A. 1984. Common action of certain viruses, toxins, and activated complement: Pore formation and its prevention by extracellular Ca^{2+}. *Biosci. Rep.* **4**:797–805

Bashford, C.L., Menestrina, G., Henkart, P.A., Pasternak, C.A. 1988*b*. Cell damage by cytolysin: Spontaneous recovery and reversible inhibition by divalent cations. *J. Immunol.* **141**:3965–3974

Bashford, C.L., Micklem, K.J., Pasternak, C.A. 1985. Sequential onset of permeability changes in mouse ascites cells induced by Sendai virus. *Biochim. Biophys. Acta* **814**:247–255

Bhakdi, S., Tranum-Jensen, J. 1984. Mechanism of complement cytolysis and the concept of channel-forming proteins. *Philos. Trans. R. Soc. Lond.* **306**:311–324

Bhakdi, S., Tranum-Jensen, J. 1987. Damage to mammalian cells by proteins that form transmembrane pores. *Rev. Biochem. Pharmacol.* **107**:148–223

Bhakdi, J., Tranum-Jensen, J. 1988. Damage to cell membranes by pore-forming bacterial cytolysins. *Prog. Allergy* **40**:1–43

Blumenthal, R., Millard, P.J., Henkart, M.P., Reynolds, C.W., Henkart, P.A. 1984. Liposomes as targets for granule cytolysin from cytotoxic LGL tumors. *Proc. Natl. Acad. Sci. USA* **81**:5551–5555

Christensen, H.N. 1984. Organic ion transport during seven decades. The amino acids. *Biochim. Biophys. Acta.* **779**:255–269

Collier, R.J. 1975. Diphtheria toxin: Mode of action and structure. *Bacteriol. Rev.* **39**:54–79

Donovan, J.J., Middlebrook, J.L. 1986. Ion-conducting channels produced by botulinum toxin in planar lipid membranes. *Biochemistry* **25**:2872–2876

Donovan, J.J., Simon, M.I., Draper, R.K., Montal, M. 1981. Diphtheria toxin forms transmembrane channels in planar lipid bilayers. *Proc. Natl. Acad. Sci. USA* **78**:172–176

Donovan, J.J., Simon, M.I., Montal, M. 1982. Insertion of diphtheria toxin into and across membranes: Role of phosphoinositide asymmetry. *Nature (London)* **298**:669–972

Drazin, R., Kandel, J., Collier, R.J. 1971. Structure and activity of diphtheria toxin. II. Attack by trypsin at a specific site within the intact toxin molecule. *J. Biol. Chem.* **246**:1504–1510

Gambale, F., Montal, M. 1988. Characterization of the channel properties of tetanus toxin in planar lipid bilayers. *Biophys. J.* **53**:771–783

Henkart, P.A. 1985. Mechanism of lymphocyte-mediated cytotoxicity. *Annu. Rev. Immunol.* **3**:31–58

Hille B. 1984. Ionic Channels of Excitable Membranes. pp. 318–320. Sinauer Associates, Sunderland, Massachusetts

Hoch, D.H., Romero-Mira, M., Ehrlich, B.E., Finkelstein, A., Dasgupta, B.R., Simpson, L.L. 1985. Channels formed by botulinum, tetanus and diphtheria toxins in planar lipid bilayers: Relevance to translocation of proteins across membranes. *Proc. Natl. Acad. Sci. USA* **82**:1692–1696

Holmgren, J. 1981. Action of cholera toxin and the prevention and treatment of cholera. *Nature (London)* **292**:413–417

Impraim, C.C., Foster, K.A., Micklem, K.J., Pasternak, C.A. 1980. Nature of virally-mediated changes in membrane permeability to small molecules. *Biochem. J.* **186:**847–860

Kagan, B. L., Finkelstein, A., Colombini, M. 1981. Diphtheria toxin fragment forms large pores in phospholipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **78:**4950–4958

Kagawa, Y., Racker, E. 1971. Partial resolution of the enzymes catalysing oxidative phosphorylation. XXV. Reconstitution of vesicles catalysing $^{32}P_i$-adenosine triphosphate exchange. *J. Biol. Chem.* **246:**5477–5487

Le Cam, A., Freychet, P. 1977. Neutral amino acid transport. Characterisation of the A and L systems in isolated rat hepatocytes. *J. Biol. Chem.* **252:**148–156

Lowry, O.H., Roseborough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the folin reagent. *J. Biol. Chem.* **193:**265–275

Mayer, M.M. 1972. Mechanism of cytolysis by complement. *Proc. Natl. Acad. Sci. USA* **69:**2954–2959

Mellanby, J., Green, J. 1981. How does tetanus toxin act? *Neuroscience* **6:**281–300

Menestrina, G. 1986. Ionic channels formed by *Staphylococcus aureus* alpha-toxin: Voltage-dependent inhibition by divalent and trivalent cations. *J. Membrane Biol.* **90:**177–190

Menestrina, G., Bashford, C.L., Pasternak, C.A. 1990. Pore-forming toxins: Experiments with *Staphylococcus aureus* α toxin, *Clostridium perfringens* θ toxin and *Escherichia coli* haemolysin in lipid bilayers, liposomes and intact cells. *Toxicon (in press)*

Misler, S. 1983. Gating of ion channels made by a diphtheria toxin fragment in phospholipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **80:**4320–4324

Muller-Eberhard, H.J. 1984. The membrane attack complex. *Springer Semin. Immunopathol.* **7:**93–141

Olsnes, S., Sandvig, K. 1983. Entry of toxic proteins into cells. *In:* Receptor-mediated Endocytosis. P. Cuatrecasas and T.F. Roth, editors. Chapman and Hall, London

Olsnes, S., Sandvig, K. 1986. Interactions between diphtheria toxin entry and anion transport in Vero cells. *J. Biol. Chem.* **261:**1553–1561

Papini, E., Sandona, D., Rappuoli, R., Montecucco, C. 1988. On the membrane translocation of diphtheria toxin: At low pH the toxin induces ion channels on cells. *EMBO J.* **7:**3353–3359

Pappenheiner, A.M., Jr. 1977. Diphtheria toxin. *Annu. Rev. Biochem.* **46:**69–94

Pasternak, C.A., Micklem, K.J. 1973. Permeability changes during cell fusion. *J. Membrane Biol.* **14:**293–303

Pasternak, C.A., Whitaker-Dowling, P.A., Widnell, C.C. 1988. Stress-induced increase of hexose transport as a novel index of cytopathic effects in virus-infected cells: Role of the L protein in the action of vesicular stomatitis virus. *Virology* **166:**379–386

Sandvig, K., Olsnes, S. 1980. Diphtheria toxin entry into cells is facilitated by low pH. *J. Cell Biol.* **87:**828–832

Sandvig, K., Olsnes, S. 1988. Diphtheria toxin-induced channels in Vero cells selective for monovalent cations. *J. Biol. Chem.* **263:**12352–12359

Sims, P.J., Lauf, P.K. 1978. Steady-state analysis of tracer exchange across the C5b-9 complement lesion in a biological membrane. *Proc. Natl. Acad. Sci. USA* **75:**5669–5673

Sims, P.J., Lauf, P.K. 1980. Analysis of solute diffusion across the C5b-9 membrane lesion of complement: Evidence that individual C5b-9 complexes do not function as discrete, uniform pores. *J. Immunol.* **125:**2617–2625

Sugiyama, H. 1980. *Clostridium botulinum* neurotoxin. *Microbiol. Rev.* **44:**419–448

Thelestam, M., Mollby, R. 1979. Classification of microbial, plant and animal cytolysins based on their membrane-damaging effects on human fibroblasts. *Biochim. Biophys. Acta.* **557:**156–169

Thelestam, M., Mollby, R. 1980. Interaction of streptolysin O from *Streptococcus pyogenes* and theta-toxin from *Clostridium perfringens* with human fibroblasts. *Infect. Immun.* **29:**863–872

Van Heyningen, S. 1980. Tetanus toxin. *Pharmacol. Ther.* **11:**141–157

Wohlheuter, R.M., Plagemann, P.G.W. 1980. The role of transport and phosphorylation in nutrient uptake in cultured animal cells. *Int. Rev. Cytol.* **64:**171–240

Yamaizumi, M., Mekada, E., Uchida, T. 1978. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* **15:**245–250

Young, J.D., Cohn, Z.A., Podack, E.R. 1986. The ninth component of complement and the pore-forming protein (perforin 1) from cytotoxic T cells: Structural, immunological and functional similarities. *Science* **233:**184–190

Zalman, L.S., Wisnieski, B.J. 1984. Mechanism of insertion of diphtheria toxin: Peptide entry and pore size determinations. *Proc. Natl. Acad. Sci. USA* **81:**3341–3345

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