0.1 M K^+. An upper bound to k_- is implicit for multiple occupancy to be observed. If two sites are occupied, the extra energy required to bind the second ion is ≤ 0, etc. The single-ion binding energy approximately equals $-$ (total repulsion energy); reaction rate theory then provides an upper bound to k_-. Assuming that the favored tight binding sites are near the end of the constriction (y_1 and y_2 in Fig. 2), and that the third site is in the middle, k_- is given in Table I for $a_0 = 0.25$ nm and various constriction lengths and occupancy possibilities. Because k_- must exceed the capture rate, triple occupancy requires that $L \sim 4$ nm. Double occupancy of the exterior sites is possible if $L \geq 2$ nm. These conclusions are unaltered if another cation is bound externally (at x_+) or if a_0 is decreased to 0.2 nm; the additional repulsion is too small to significantly decrease the bounds to k_-.

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ORIENTATION OF THE DIPHTHERIA TOXIN CHANNEL IN LIPID BILAYERS

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Cytotoxic action of diphtheria toxin (DT; M_r 62,000) requires translocation of its A fragment (DT-A; M_r 21,000) into the cytosolic compartment (Collier, 1975; Pappenheimer, 1977). There DT-A catalyzes transfer of the ADP-ribose moiety of NAD to elongation factor 2, which causes inactivation of the factor and blockage of protein synthesis. The B fragment (M_r 41,000) is responsible for binding DT to the cell surface and is required for transmembrane translocation of DT-A. Various lines of evidence indicate that receptor-bound DT is initially brought into the cell within endosomes and that acidification of the endosomal lumen induces insertion of the toxin into the endosomal membrane and transfer of DT-A to the cytosolic compartment.

Whole toxin has been shown to insert into planar lipid bilayers under acidic conditions, forming ion-conductive channels (Donovan et al., 1981). The amino terminal two-thirds of the B fragment, B45 (M_r 24,000), which alone is capable of forming a channel (d = 18 Å), appears to be necessary for translocation of DT-A across lipid membranes in vitro (Kagan et al., 1981).

Here we report the use of DT ligands to map functional sites of DT with respect to the lipid bilayer. Specifically, we have obtained evidence that a binding site for the dinucleotide ApUp (adenylyl-(3′,5′)-uridine 3′-monophosphate) appears on the *trans* side of the membrane after addition of DT to the *cis* side. Other data suggest that the ApUp site is composed of elements of the NAD site (substrate binding

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site) on DT-A and a cationic site (the P site) on the B fragment (Lory et al., 1980). Exposure of the ApUp site on the *trans* face of the membrane is therefore consistent with the notion that the catalytic center of DT-A is transferred across the membrane during toxin insertion.

RESULTS AND DISCUSSION

We used phospholipid bilayers formed by the union of two monolayers (Montal, 1974) to study the effects of DT and its various ligands. When DT is added to the *cis* aqueous phase of a lipid membrane system at sufficiently low pH (pH < 5.0), channel formation begins rapidly and continues at a rate that is concentration- and voltage-dependent. This is easily measured as the rate of conductance ($g = I/V$ = current/voltage) increase of the membrane. The pH dependence of membrane insertion appears to reflect a conformational change that the toxin undergoes at acidic pH's.

Studies of whole toxin in solution indicate that inositol hexaphosphate (IHP) binds to a cationic site (the P site) on the B moiety with affinity for highly phosphorylated compounds (Lory et al., 1980; Proia et al., 1980). Donovan et al. (1982 *a*) showed that IHP dramatically stimulates the rate of increase of conductance when added to the *trans* side of a lipid bilayer containing DT inserted from the *cis* side. We have confirmed this result for membranes at low pH (4.7 both sides) and extended it to membranes subjected to a pH gradient (*cis* 4.7, *trans* 7.4), mimicking the conditions DT encounters within endosomes. Stimulation can be seen at IHP concentrations as low as 20 μM and is >500-fold at 1 mM IHP. Inositol hexaphosphate has no effect when added to the *cis* side or when added to a lipid bilayer containing no DT. While in most experiments we added IHP to the *trans* compartment only after DT-mediated conductance had been detected, we found that addition of IHP to DT-containing membranes prior to channel opening also stimulated a dramatic rise in conductance.

We reported earlier that DT commonly exists in two forms, depending on the presence or absence of a tightly bound dinucleotide, ApUp (Lory and Collier, 1980; Barbieri et al., 1981). When IHP is added to the *trans* compartment of a membrane containing nucleotide-free diphtheria toxin (DT-nf) the full stimulation is seen. However, prior addition of ApUp (1 μM) to the *trans* side of a DT-nf containing membrane blocks the IHP induced stimulation. Addition of ApUp to the *cis* side does not block the effect of IHP, and ApUp alone has no effect on the rate of rise of conductance. ATP, a weaker DT ligand, produces effects identical to those of ApUp, except that somewhat higher concentrations of ATP are required (25 μM).

CRM-50 (M_r 50,000) is a DT mutant lacking the hydrophilic, 12,000 dalton, COOH-terminal segment that is believed to contain both the P site and the receptor binding site.[1] In membranes, CRM-50 makes channels that behave electrically like DT channels. However, when IHP is added to the *trans* side of a CRM-50 containing membrane, no stimulation of the rate of conductance increase is seen. Another channel-forming DT mutant, CRM-197 (M_r 62,000), which is known to have decreased affinities for P site ligands and dinucleotides, is also not stimulated by IHP. These facts suggest that the action of IHP is mediated via an interaction with the P site.

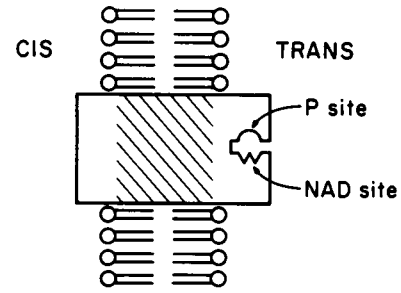

FIGURE 1 Proposed orientation of diphtheria toxin inserted into artificial lipid bilayer. Methods: planar lipid bilayers were formed as described previously (Kagan et al., 1981) from a mixture of purified soybean phospholipids (40% phosphatidylcholine, 40% phosphitidyl ethanolamine, 20% phosphitidyl serine; Avanti Biochemicals, Inc., Birmingham, AL) dissolved in hexane. The aqueous phases contained 100 mM NaCl, 10 mM dimethyl glutaric acid, 2 mM $MgCl_2$, 1 mM EDTA. The pH of the *cis* compartment was adjusted to 4.7 and of the *trans* compartment to 7.2. Voltage-clamp conditions were employed. Calomel electrodes were used to apply voltages and measure resulting currents across the membrane. In a typical experiment 100 ng of DT was added to the *cis* compartment with the voltage clamped to −30 mV (*trans* side negative). After 3–10 min, when a steady, measurable rise in conductance had been present for at least a minute, IHP (0.5–1 mM) was added to the *trans* side, and the rate of rise of conductance was measured 3 min later. In some experiments ApUp or ATP was added to the *trans* side 3 min before adding IHP.

Ligand binding studies have suggested that on toxin in solution the P site, located on the B fragment, is immediately adjacent to the NAD site, on the A fragment (Lory et al., 1980). This is supported by the competitive binding of P site ligands with NAD (and vice versa), and by the fact that ApUp blocks both the P site and the NAD site. We have proposed that the very tight binding of ApUp may result from (*a*) interactions of the nitrogenous bases with the nicotinamide and adenine subsites of the NAD site on the A fragment, augmented by (*b*) electrostatic and hydrogen bonding interactions with the cationic P site on the B fragment (Lory et al., 1980). The results reported here suggest that in the membrane-inserted form of the toxin the entire ApUp site is exposed on the *trans* face of the membrane. If ApUp is present, it binds tightly, blocking access of IHP to the P site. In the absence of ApUp, IHP gains access to the P site and facilitates channel opening.

This model is consistent with the notion that the catalytic center of the A fragment (at least the NAD site) is

[1]Naumovski, L., D. Kaplan, and R. J. Collier, unpublished results.

translocated to the *trans* face of the membrane during insertion of toxin into the bilayer (see Fig. 1). This appears to be an essential step in the intoxication process in vivo and has been mimicked in systems in vitro by two groups, Donovan et al. (1982 *b*) and Kagan et al.[2]

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STUDIES OF PORINS

Spontaneously Transferred from Whole Cells and Reconstituted from Purified Proteins of *Neisseria gonorrhoeae* and *Neisseria meningitidis*

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Many human bacterial pathogens can invade and damage host epithelial surfaces in a process whereby the organism binds to and is transported across the boundary epithelial layer. The factors that define the virulence pattern and the invasive potential of a microbial pathogen are likely to be distinct bacterial products associated with the outer membrane surface or secreted into the extracellular environment. Postulating that invasive pathogens might elicit substances that could affect membrane permeability as a regulatory signal to cause their uptake into the cell, we exposed, under voltage-clamp conditions, artificial planar lipid bilayer membranes composed of phosphatidylethanolamine (PEA) or asolectin (ASO) to whole cells of several strains of invasive gram-negative cocci and noninvasive controls. The cells were maintained in culture under conditions described in reference 1. In bilayers contacted by these organisms, we observed the spontaneous transfer of ion-permeable channels that exhibited voltage-sensitive lifetimes. The ability to spontaneously transfer channel activity into the bilayer was compared in pathogenic strains of *Neisseria*, i.e., *N. meningitidis* and *N. gonorrhoeae* (isolated from patients with systemic and localized diseases) as well as a nonpathogenic strain of *N. sicca*.

Outer membrane proteins from the bacterial pathogens were purified (1) and reconstituted into proteoliposomes that were fused into the planar lipid bilayer (2). In the bilayer, channel-forming activity was determined to be associated with the strain specific protein I, the major outer membrane protein, analagous to the porin channel-forming protein described in *E. coli* (3). Channels transferred into the bilayer from whole cells and from vesicles containing purified protein I exhibited distinct properties that were characteristic of the strain of gonococcus or meningococcus used. For a given strain, the properties of channels reconstituted in the bilayer from whole cells or from the strain specific purified protein I in vesicles were similar.

RESULTS

Fig. 1 *A*, *C*, and *E* demonstrate the channel-forming activity as current jumps that spontaneously entered the model membrane at variable time (10 s–60 min) after the

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