Evidence That Diphtheria Toxin and Modeccin Enter the Cytosol from Different Vesicular Compartments

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ABSTRACT

Inhibition of protein synthesis in Vero cells was measured at different periods of time after treatment with diphtheria toxin and the related plant toxin modeccin. Diphtheria toxin acted much more rapidly than modeccin. Cells were protected against both toxins with antiserum as well as with agents like NH₄Cl, procaine, and the ionophores monensin, FCCP, and CCCP, which increase the pH of intracellular vesicles. Antiserum, which is supposed to inactivate toxin only at the cell surface, protected only when it was added within a short period of time after modeccin. Compounds that increase the pH of intracellular vesicles, protected even when added after 2 h, indicating that modeccin remains inside vesicles for a considerable period of time before it enters the cytosol. After addition of diphtheria toxin to the cells, compounds that increase the pH of intracellular vesicles protected only approximately to the same extent as antitoxin. This indicates that after endocytosis diphtheria toxin rapidly enters the cytosol.

At 20°C, the cells were more strongly protected against modeccin than against diphtheria toxin. The residual toxic effect of diphtheria toxin at 20°C could be blocked with NH₄Cl, whereas this was not the case with modeccin. This indicates that at 20°C the uptake of diphtheria toxin occurs by the normal route, whereas the uptake of modeccin occurs by a less efficient route than that dominating at 37°C.

The results indicate that after endocytosis diphtheria toxin rapidly enters the cytosol from early endosomes with low pH (receptosomes). Modeccin enters the cytosol much more slowly, possibly after fusion of the endocytic vesicles with another compartment.

Our knowledge on the binding of proteins to cell surface receptors and on the subsequent internalization and processing of the ligand-receptor complexes has been greatly expanded in recent years. Many ligands are first accumulated in coated pits at the cell surface. They are then internalized into endosomes that may subsequently fuse with lysosomes and other intracellular organelles (1, 2). It has recently been shown that certain early endosomes, also called receptosomes, are rapidly acidified (3, 4). This reduction in intravesicular pH has a number of effects on ligand-receptor complexes. In some cases the ligand is released from the receptor at the low pH. This may allow recycling of the receptor back to the cell surface while the ligand is directed to the lysosomes or elsewhere (5). In the case of many enveloped viruses the low pH induces membrane fusion and injection of the nucleocapsid into the cytosol (6, 7).

Low intravesicular pH is necessary also for the action of diphtheria toxin and probably for the action of modeccin, a toxic plant lectin (8–11). Compounds like amines and ionophores, which increase the pH of intracellular acidic vesicles, strongly protect against these two toxins (8, 12, 13, 14).

Diphtheria toxin and modeccin both consist of two functionally active parts, a B moiety that binds the toxin to sites at the cell surface and an A moiety that has enzymatic properties and that enters the cytosol, where it inactivates in a catalytic way components of the protein synthesizing system (15, 16). Earlier data have indicated that the entry of the A moiety of both toxins occurs from intracellular vesicles rather than from the cell surface (9–11).

Under conditions in which the medium is acidified to pH 4.5, diphtheria toxin appears to enter the cytosol directly through the cell surface membrane (9–11). This is not the case with modeccin (10). Therefore, if low pH is indeed required for the entry of modeccin, the toxin molecule may require some kind of modification before it can pass the membrane. Alternatively, modeccin requires for entry not
only low pH, but also some other condition that the toxin encounters only in certain intracellular vesicles. In the present study we attempted to throw light on these questions.

MATERIALS AND METHODS

Toxins and Antitoxins: Diphtheria toxin was obtained from Connaught Laboratories (Willowdale, Ontario, Canada) and purified as described (11). Nicked diphtheria toxin was used in all experiments. It was prepared from a batch of essentially unnicked toxin by the method of Dräzin et al. (17). Horse antiserum to diphtheria toxin was obtained from the National Institute for Public Health (Oslo, Norway). Modeccin was purified as described earlier (18). Rabbit antitoxin against modeccin was prepared as earlier described (18).

Materials: Dansylcadaverine was obtained from Fluka AG, monensin from Calbiochem-Behring Corp. (La Jolla, CA), and L-1-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK), N-a-tosyl-L-lysinechloromethyl ketone (TLCK), procaine, carbonylcyanide m-chlorophenylhydrazone (CCCP), carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) were obtained from Calbiochem-Behring Corp. (LaJolla, CA), and L-tosylamide-2-phenylacetamide (TPA), N-a-tosylphenylalanine, 10 mM acetic acid, pH 7.5, as described by Marsh et al. (19). The cells were pelleted by centrifuging at 2,000 rpm for 2 min (SS34 rotor, Sorvall centrifuge). The pellet of cells from two dishes was resuspended in 1 ml of the sucrose-containing buffer described above and the cells were homogenized by ten strokes in a Dounce homogenizer with a loose fitting pestle. After centrifugation as above, the supernatant was applied to tubes with 20% Percoll in the sucrose-containing buffer. To form Percoll density gradients, we centrifuged the tubes for 50 min at 27,000 rpm in a TI50 rotor. Fractions (0.4 ml) each were collected and assayed for the lysosomal marker enzyme ß-N-acetylglucosaminidase as described by Hall et al. (20). The content of HRP in each fraction was measured as described by Steinman et al. (21).

RESULTS

Rate of Protein Synthesis Inhibition in Vero Cells after Treatment with Diphtheria Toxin and Modeccin

When the enzymatically active chains or fragments of toxins like abrin, ricin, modeccin, and diphtheria toxin are added to cell-free protein-synthesizing systems, protein synthesis starts to decline immediately. In contrast, when the toxins are given to living cells, protein synthesis is inhibited only after a certain period of time (15, 22). At least part of this lag time must represent the time the toxins require to enter the cytosol. The two toxins diphtheria toxin and modeccin, are structurally related and approximately equally toxic to Vero cells in long-term experiments (11, 16). In spite of this, the lag time from addition of toxin until the rate of protein synthesis starts to decline, was found to be much longer with modeccin than with diphtheria toxin (Fig. 1). Thus, whereas 10-9 M diphtheria toxin reduced protein synthesis to half the control value already after 25 min, ~140 min were required after treatment with the same concentration of modeccin.

With increasing concentrations of both toxins, a limit was reached where the lag time was not further reduced. The minimal time period until protein synthesis was reduced by 50%, was ~15 min in the case of diphtheria toxin, while it was ~75 min with modeccin. However, in experiments where...
cells were exposed to the toxins for different periods of time and then incubated over night to allow the absorbed toxins time to express their full toxic effect, modeccin was at least as efficient as diphtheria toxin (Fig. 2A and B).

We have earlier provided evidence that both toxins enter the cytosol from endocytic vesicles (9-11, 13). The reason for the large difference in lag time could therefore be that diphtheria toxin and modeccin enter the cytosol from different kinds of vesicles which the two toxins reach after different periods of time. The experiments described below were performed to test this possibility.

**Protective Effect of Antitoxin and NH₄Cl Added Different Periods of Time after Exposure of Cells to Modeccin and Diphtheria Toxin**

When antitoxins were added to cells before the toxins, the cells were protected even against the highest concentrations of toxin used (Fig. 2A and B). However, when they were added 30 min or more after the toxins, their ability to protect was strongly reduced. It therefore appears that both diphtheria toxin and modeccin are rapidly transferred to compartments, probably intracellular vesicles, where they are inaccessible to the antibodies. The long lag time observed with modeccin can therefore neither be due to slow binding of the toxin to cell surface receptors, nor to slow endocytosis of the bound toxin.

If modeccin remains inside vesicles for a considerable period of time before it can enter the cytosol, this would explain the long lag-time. To test this possibility, we took advantage of the observation that NH₄Cl protects cells not only against diphtheria toxin but also against modeccin (8). In medium containing NH₄Cl some free NH₃ is present that can diffuse through the cellular membranes and into the different vesicular compartments. In compartments with low pH, the NH₃ becomes protonized, and thus it increases the pH inside the vesicles (23). It is well established that diphtheria toxin requires low pH for transfer across the membrane (9-11). For reasons to be discussed below, this is probably also the case with modeccin.

After exposure of cells to diphtheria toxin and modeccin to allow endocytosis of toxin to take place, NH₄Cl was added after different periods of time. Since under these conditions NH₃ almost immediately enters intracellular vesicles and increases their pH (23), it might be able to protect against toxin that is present within endocytic vesicles. In this respect the protective effect of NH₄Cl is different from that of antibodies, which are only expected to protect against toxin remaining at the cell surface.

We found indeed a striking difference between the ability of antiserum and NH₄Cl to protect against modeccin. Thus NH₄Cl protected equally well against modeccin whether it was added simultaneously with the toxin or after 30 min (Fig. 2C), whereas antimodeccin added after 30 min gave little protection (Fig. 2A). The results indicate that after 30 min a considerable amount of modeccin was located inside endocytic vesicles, but that little toxin had reached the cytosol during this period of time. Even after 60 and 120 min, much better protection was obtained with NH₄Cl than with antitoxin (Fig. 2C).

Quite different results were obtained with diphtheria toxin. In this case NH₄Cl protected only to approximately the same extent as antitoxin (Fig. 2D). This indicates that diphtheria toxin enters the cytosol rapidly after it has been endocytosed.

To better demonstrate the difference between the protective effect of NH₄Cl and antitoxin on modeccin and diphtheria toxin, we replotted the toxic effect measured from the data in Fig. 2 in Fig. 3. In the same figure the effect of several compounds to be discussed below is shown for comparison.

We reported earlier that Ca²⁺-deprivation protects cells against modeccin (24). Ca²⁺ cannot replace Ca²⁺ in restoring the sensitivity of the cells to the toxin, but it prevents the cell detachment from the plastic surface otherwise observed in Ca²⁺-free media (24). As shown in Fig. 4A, Ca²⁺ deprivation protected against modeccin almost as well as the presence of NH₄Cl. To test if the step in the entry of modeccin that is inhibited by Ca²⁺ deprivation, is before or after the NH₄Cl sensitive step, we first incubated cells with modeccin in medium containing Co²⁺ instead of Ca²⁺ to allow modeccin to be endocytosed and proceed until the Ca²⁺-requiring step. Then NH₄Cl was added and after 30 min the buffer was removed and growth medium containing Ca²⁺ and NH₄Cl...
and Dansylcadaverine. Toxin by Monensin, Procaine, Protection against Modeccin and Diphtheria

The slow decay of the NH$_4$Cl-induced protection against modeccin then Ca$^{2+}$ was removed, could not be carried out due to the absence of the protective compound was also measured (ID$_{50}$ control). The toxic effect given at the ordinate was calculated as ID$_{50}$ control/ID$_{50}$ inhibitor. The additions were the following: (O) 10 μl ml antiserum; (C) 10 mM NH$_4$Cl; (Δ) 10 μM FCCP; (x) 10 μM monensin; (□) 10 mM procaine; (Δ) 15 μM FCCP.

was added. The cells were incubated over night and then the rate of protein synthesis was measured. The results showed that cells thus treated were intoxicated to approximately the same extent as cells exposed to modeccin in the presence of Ca$^{2+}$ (Fig. 4B). This indicates that even in the absence of Ca$^{2+}$, modeccin had passed the NH$_4$Cl-sensitive step. If the entire experiment was carried out in the presence of NH$_4$Cl, the cells were, as expected, protected even after incubation over night (Fig. 4B). Unfortunately, the reverse experiment in which toxin was endocytosed in the presence of NH$_4$Cl and then Ca$^{2+}$ was removed, could not be carried out due to the slow decay of the NH$_4$Cl-induced protection against modeccin (13).

Protection against Modeccin and Diphtheria Toxin by Monensin, Procaine, and Dansylcadaverine

Since entry of modeccin from the cell surface cannot be induced by a brief lowering of the pH, it is not certain that it indeed requires low pH. The protective effect of NH$_4$Cl and chloroquine (8) might be due to other effects of these compounds. However, since a series of other compounds that increase the pH of acidic vesicles also protect against modeccin, we believe that low pH is indeed required for the action of this toxin. Earlier experiments have shown that carboxylic ionophores such as Br-X-537A, nigericin, and monensin, which dissipate proton gradients across membranes due to electroneutral exchange of protons for monovalent cations, protect strongly against both diphtheria toxin and modeccin (13, 14). When monensin was added to cells after they had been exposed to modeccin, the cells were protected to approximately the same extent as with NH$_4$Cl (Fig. 3A). In contrast, monensin protected effectively against diphtheria toxin only when it was added to the cells simultaneously with the toxin (Fig. 3B).

The local anesthetic procaine is accumulated in lysosomes, probably because it is a weak amine (25). It was found that procaine protected strongly against modeccin and, as earlier shown (26), also against diphtheria toxin. In fact, it protected to approximately the same extent as NH$_4$Cl. Furthermore, procaine protected against modeccin even when added after the toxin (Fig. 3A). Also, dansylcadaverine protected against modeccin and diphtheria toxin. The protection was best against modeccin. The compounds did not prevent the inhibition of cell-free protein synthesis by the toxins (data not shown).

Cells preincubated with procaine were protected against modeccin several hours after transfer to medium without procaine. Similar findings were earlier made with NH$_4$Cl (13). In contrast, the protection against diphtheria toxin by both compounds was found to be rapidly reversible. The protective effect of procaine and dansylcadaverine against diphtheria toxin was overcome when the cells were briefly exposed to low pH (data not shown), as has previously been found for NH$_4$Cl (9–11).

The protective effect of procaine and dansylcadaverine cannot be accounted for by inhibition of toxin binding. Thus control experiments showed that 10 mM procaine did not affect the binding of $^{125}$I-labeled modeccin to cells and 500 μM dansylcadaverine only reduced the binding to half the control value. Neither of the compounds inhibited the binding of $^{125}$I-labeled diphtheria. Monensin did not inhibit the binding of either toxin under the conditions used here. The transfer
of $^{125}$I-modeccin to a state where it could not be washed off the cells with lactose, which probably represents endocytosis of the toxin, was not reduced by either compound (data not shown).

These binding studies might be misleading if the functional toxin receptors constitute only a subfraction of the total number of binding sites. Experiments were therefore carried out in which cells were exposed to diphtheria toxin and modeccin in the cold to allow toxin binding, but not entry to occur. Subsequently, the cells were washed and treated at $37^\circ$C with monensin, procaine, or dansylcadaverine for 3 h and finally the rate of protein synthesis was measured. The compounds protected even under these conditions, confirming that the protection cannot be accounted for by inhibition of binding.

Monensin, procaine and dansylcadaverine were all found to inhibit the degradation of $^{125}$I-labeled diphtheria toxin (data not shown).

**Ability of Protonophores to Protect against Modeccin and Diphtheria Toxin**

The protonophores FCCP and CCCP have been shown to increase the pH in acidic vesicles (3, 23), and, as expected, FCCP inhibited the degradation of $^{125}$I-diphtheria toxin in Vero cells (data not shown). The data in Fig. 5 show that FCCP and CCCP strongly protected Vero cells against both modeccin and diphtheria toxin. Lower concentrations of the ionophores were required to protect against modeccin than against diphtheria toxin. Similar results were earlier found with Br-X-537A (13). It should also be noted that CCCP provided the best protection while modeccin and diphtheria toxin protect best against diphtheria toxin. Experiments to test whether the protection against diphtheria toxin by the protonophores was overcome by exposing the cells to low pH, could not be carried out since such treatment was lethal to the cells. The inhibitory effect of the toxins on protein synthesis in a rabbit reticulocyte lysate was not reduced by the ionophores (data not shown).

Control experiments showed that the protonophores had no effect on the binding of the toxins to the cell surface and there was no effect of CCCP on the rate of lactose-resistant uptake of modeccin. Altogether the data indicate that not only diphtheria toxin, but also modeccin must reach intracellular acidic vesicles before they can enter the cytosol and intoxicate the cell.

**Effect of Temperature on Intoxication of Cells by Modeccin and Diphtheria Toxin**

Fusion of endocytic vesicles with lysosomes was shown to be inhibited in hepatocytes by lowering the temperature to 15–20$^\circ$C (27). The data in Fig. 6 show that this is also the case in Vero cells. Thus when the cells were incubated with horseradish peroxidase at 20$^\circ$C it was accumulated in intermediary vesicles with a buoyant density of $p \approx 1.05$ g/ml in a Percoll gradient. When the incubation was at 37$^\circ$C a major part of the internalized enzyme was buoyant at $p = 1.07$ to 1.08 g/ml indicating that it was present in lysosomes. A large fraction of the lysosomal enzyme $\beta$-$N$-acetylglucosaminidase was also present at this position.

We then studied the effect of such temperatures on the ability of modeccin and diphtheria toxin to intoxicate cells. In these experiments, the cells were incubated with toxins at 0$^\circ$C for 1 h to permit receptor binding without internalization to occur. Then medium prewarmed to 20$^\circ$C and 37$^\circ$C was added.
added to allow internalization to take place. After 30 min (diphtheria toxin) or 4 h (modeccin), NH₄Cl was added to stop the transport of toxin into the cytosol and 15 min later the medium was changed. To allow the toxin that had entered during exposure at the two temperatures sufficient time to express its full toxic effect, we incubated the cells at 37°C overnight in medium containing NH₄Cl and finally protein synthesis was measured. The data in Fig. 7, C and D show that when the exposure was at 20°C, the toxic effect of both toxins was strongly reduced. ~100 times higher concentrations of modeccin were required to obtain the same toxic effect as at 37°C (Fig. 7, A and B). With diphtheria toxin the difference was ~10 times less.

The most interesting observation is, however, that the toxic effect obtained with modeccin at 20°C was not affected by NH₄Cl, whereas the effect of diphtheria toxin was prevented. The data indicate that the entry of diphtheria toxin at 20°C occurs by the normal NH₄Cl-sensitive way, whereas the entry of modeccin at 20°C is NH₄Cl-insensitive and therefore differs from the highly efficient entry dominating at 37°C. Evidence for the existence of such a NH₄Cl-insensitive entry mechanism for modeccin was previously obtained in experiments with a modeccin-resistant cell line (8). Apparently the most efficient entry mechanism for modeccin is only operating at 37°C and may therefore involve some kind of vesicle fusion.

**Effect of Temperature on the NH₄Cl Sensitive Entry of Modeccin and Diphtheria Toxin**

![Figure 7](image)

**FIGURE 7** Effect of temperature on the NH₄Cl sensitive entry of modeccin and diphtheria toxin. Vero cells growing in 24-well disposable trays were chilled to 0°C and then increasing amounts of toxin were added. When indicated 10 mM NH₄Cl was also added. After binding for 1 h, the medium with unbound toxin was removed and medium prewarmed to 37°C (A and B) or 20°C (C and D) with and without 10 mM NH₄Cl was added to the cells. After 30 min (diphtheria toxin) or 4 h (modeccin) incubation at these temperatures, NH₄Cl was added to those wells not already containing NH₄Cl and after 15 min more growth medium containing NH₄Cl was added. After incubation overnight the rate of protein synthesis was measured. (●) 10 mM NH₄Cl present throughout the experiment; (○) 10 mM NH₄Cl only added after 30 min of incubation at 37°C or 20°C.

**DISCUSSION**

The main conclusion emerging from the present work is that the enzymatically active parts of diphtheria toxin and modeccin enter the cytosol by different routes. Diphtheria toxin enters rapidly after endocytosis, apparently from early endosomes (receptosomes) with low internal pH. Modeccin also appears to enter from acidic vesicles, but much later than diphtheria toxin, possibly because the modeccin-containing vesicles must first fuse with other vesicular compartments.

The great difference in lag-time was the first indication that diphtheria toxin reaches the cytosol much more rapidly than modeccin. This difference is unlikely to be due to low endocytic uptake of modeccin, since 125I-modeccin is rapidly transferred to a compartment where it cannot be removed by washing the cells with lactose, and where it cannot be neutralized with antimodeccin added to the medium. This compartment most likely represents endocytic vesicles.

Further evidence for different routes of entry was obtained...
with several compounds that are known to increase the pH in acidic vesicles (NH4Cl, monensin, FCCP, CCCP, procaine, and others) and that protect against both toxins. In the case of diphtheria toxin, these compounds must be added immediately after the toxin to achieve full protection. Since these compounds are expected to protect also against toxin present in endocytic vesicles formed before the compounds were added, the data indicate that endocytosed diphtheria toxin does not remain in endocytic vesicles for a long period of time before it enters the cytosol. Only a small fraction of the total number of endocytosed diphtheria toxin molecules (or their A fragment) is supposed to ever reach the cytosol, whereas most of the toxin is degraded, probably in the lysosomes (28).

A quite different pattern was found with modecin where NH4Cl, monensin, CCCP, and procaine protected almost completely also when added 30 min after the toxin. Even addition as late as 2 h after modecin had a strong protective effect. This indicates that modecin is present in vesicles for a long period of time before it is transferred to the cytosol.

We and others (8–12) have earlier found that low pH is necessary for entry of diphtheria toxin into cells. Furthermore, diphtheria toxin was found to bind to coated pits (29). Since vesicles formed from coated pits rapidly acquire low pH (3, 4), diphtheria toxin probably enters the cytosol from such vesicles. It is also likely that modecin enters from acidic vesicles of some kind, possibly from lysosomes or acidic compartments in the Golgi apparatus.

The possibility that vesicle fusion is necessary for entry of modecin, but not for diphtheria toxin, is supported by the finding that at 20°C where fusion with lysosomes is prevented, the action of modecin was more strongly inhibited than that of diphtheria toxin. Furthermore, in the case of diphtheria toxin, the moderate toxic effect obtained at 20°C was prevented by NH4Cl while this was not the case with modecin. This indicates that the limited entry of modecin at 20°C is of another kind than that dominating at 37°C. In the case of diphtheria toxin only one uptake mechanism seems to operate at both temperatures.

The effect of monensin, chloroquine, and NH4Cl on the pH of acidic vesicles have been shown to rapidly disappear after removal of the compounds (30). The observation that Vero cells are protected against modecin for hours after removal of the same compounds (13), is therefore surprising. Possibly other effects of these compounds are not as rapidly reversible as the effect on the pH. Both NH4Cl, local anesthetics and dansylcadaverine have been reported to inhibit fusion of endosomes with lysosomes (30, 31), an effect that could be slowly reversible.

It is not clear why modecin A-chain is much less active in cell-free systems than abrin and ricin A-chain (16). One possibility is that modecin is somehow processed by the cells to yield an A-chain with high activity. We have earlier reported that inhibitors of lysosomal enzymes have no effect on the toxicity of modecin (8). We have tested further inhibitors of proteolytic enzymes for ability to protect against the toxins. The two inhibitors of trypsin-like enzymes TPCK and TLCK (32), did in fact protect against modecin while none of them protected against diphertheria toxin. It should be noted, however, that these compounds do not only inhibit proteases. Thus TPCK, but not TLCK, has been reported to inhibit the uptake of α2-macroglobulin into cells and these compounds also inhibit the action of various nonproteolytic enzymes (33). It is therefore not clear if the protection against modecin is due to inhibition of a proteolytic enzyme or to some other effect of the compound.

The finding that the NH4Cl sensitive step in modecin entry occurs before the Ca2+-dependent step, indicates that Ca2+ is required after the toxin has been exposed to low pH. If the situation is analogous to that with diphertheria toxin, low pH induces insertion of part of the toxin (the B-chain?) into the membrane. Ca2+ could then be necessary in the subsequent transfer of the A-chain across the membrane.

Although toxins like diphertheria toxin, modecin, abrin, ricin, viscumin, and others have many properties in common, it is now clear that the mechanism by which they deliver their enzymatically active A-moiety to the cytosol differs to a greater or lesser extent. Thus a variety of compounds that protect against diphertheria toxin and modecin do not protect against abrin and ricin (8). The difference in uptake mechanism could be due to intrinsic differences in the toxin molecules or it could be due to the different binding specificities. It is well known that different cell surface binding sites follow different intracellular routes after endocytosis (1, 34–36). The fact that toxins with a broad binding specificity such as abrin, ricin, and viscumin bind to a variety of surface binding sites suggests why they may enter by several routes. This could be the reason it is more difficult to inhibit completely the entry of these toxins than that of diphertheria toxin and modecin. The latter toxins bind to a comparatively small number of sites, possibly all identical, that may enter by a single route.

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