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 NH_4Cl, 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 NH_4Cl, 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

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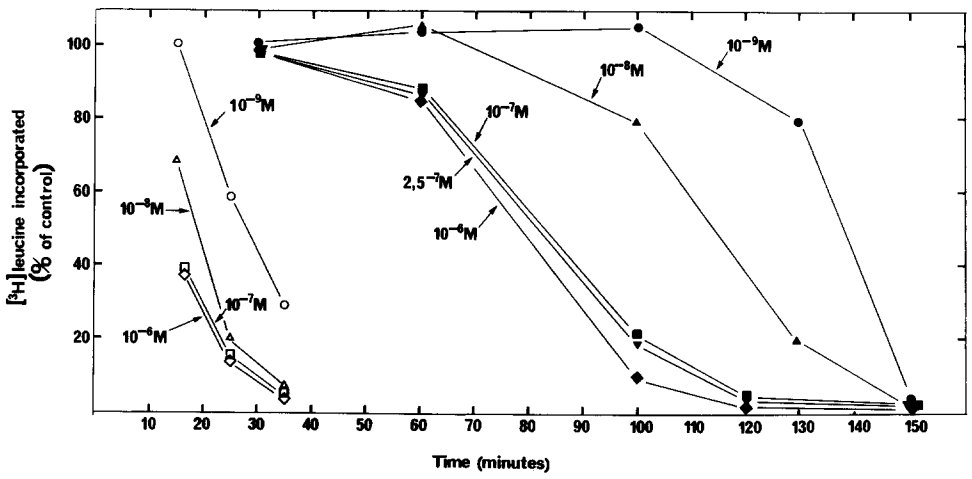

FIGURE 1 Rate of protein synthesis inhibition by diphtheria toxin and modeccin. The indicated amounts of toxin were added to Vero cells growing in 24-well disposable trays as described under Materials and Methods. After different periods of incubation at 37°C, the medium was removed and the cells were incubated for 10 min with [^{3}H]leucine to measure the rate of protein synthesis. Indicated on the abscissa is the time from addition of toxin to the cells until 5 min after addition of [^{3}H]leucine. The toxin concentrations were the following: (○, ●) 10^{-9} M; (△, ▲) 10^{-8} M; (□, ■) 10^{-7} M; (▽, ▼) 2.5×10^{-7} M; (◇, ◆) 10^{-6} M. Open symbols: nicked diphtheria toxin. Filled symbols: modeccin.

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 Drazin 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-phenylethylchloromethyl ketone (TPCK),[1] *N*-α-p-tosyl-L-lysine chloromethyl ketone (TLCK), procaine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), carbonylcyanide p-trifluoro-methoxyphenylhydrazone (FCCP) were obtained from Sigma Chemical Co. (St. Louis, MO).

Cells: Vero cells (African green monkey kidney cells) were obtained from the National Institute for Public Health (Oslo, Norway). The cells were maintained as monolayer cultures in minimum essential medium (Gibco Europe Limited, Paisley, Scotland) with 10% fetal calf serum. In experiments with antitoxins, the serum was incubated at 56°C for 30 min to inactivate complement.

Measurement of Cytotoxicity: Cells were transferred to disposable trays with 24 wells (Falcon, Oxnard, CA) (5×10^4 cells/well in 1 ml of medium), the day before the experiment. Experiments were initiated by removal of the growth medium and addition of medium containing 21 mM HEPES (pH 7.5) instead of bicarbonate (13). In some experiments (see Legends to Figures) buffered salt solutions containing glucose (1 mg/ml) were used instead. Toxin and the compound to be tested were added and the experiments were performed as described in legends to figures. All experiments were terminated by measuring the rate of protein synthesis in the following way: The cells were incubated for 10–30 min in leucine-free medium made up to contain 1 μCi/ml of [^{3}H]leucine, then the medium was removed and 1 ml of 5% (wt/vol) trichloroacetic acid was added per well. After 10 min at room temperature the trichloroacetic acid was removed and another ml of trichloroacetic acid was added to each well. After 5 min this solution was removed and 200 μl KOH (0.1 M) was added to each well to dissolve the cells. The samples were transferred to counting vials, 1 ml of Instagel II (Packard Instruments Co., Downers Grove, IL) was added and the radioactivity was measured. The results are in each case expressed as a percentage of the incorporation in control samples which were treated only with the compound to be tested. The incorporation in the controls varied between 5,000 and 30,000 counts/min in different experiments.

Cell Fractionation: Vero cells grown in 13.5-cm petri dishes for 3 d without change of medium were incubated overnight at 20°C and 37°C in the presence of 1 mg/ml of horseradish peroxidase (HRP). The cells were then washed eight times with medium and incubated for 1 h (without HRP) at 20°C and 37°C. The dishes were then washed twice with 10 mM Na-phosphate, pH 7.3, 0.14 M NaCl containing 0.2% bovine serum albumin and EDTA (1 mM). The cells were scraped off the dishes in a buffer containing 0.25 M sucrose, 1 mM EDTA, 10 mM triethanolamine, 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 β-*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

[1] *Abbreviations used in this paper:* CCCP, carbonyl cyanide m-chlorophenylhydrazone; FCCP, carbonylcyanide p-trifluoro-methoxyphenylhydrazone; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenyl-ethylchloromethyl ketone.

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. 2*A* 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 NH_4Cl 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. 2, *A* 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 NH_4Cl protects cells not only against diphtheria toxin but also against modeccin (8). In medium containing NH_4Cl some free NH_3 is present that can diffuse through the cellular membranes and into the different vesicular compartments. In compartments with low pH, the NH_3 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, NH_4Cl was added after different periods of time. Since under these conditions NH_3 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 NH_4Cl 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 NH_4Cl to protect against modeccin. Thus NH_4Cl protected equally well against modeccin whether it was added simultaneously with the toxin or after 30 min (Fig. 2 *C*), whereas antimodeccin added after 30 min gave little protection (Fig. 2*A*). 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 NH_4Cl than with antitoxin (Fig. 2*C*).

Quite different results were obtained with diphtheria toxin. In this case NH_4Cl protected only to approximately the same extent as antitoxin (Fig. 2*D*). This indicates that diphtheria

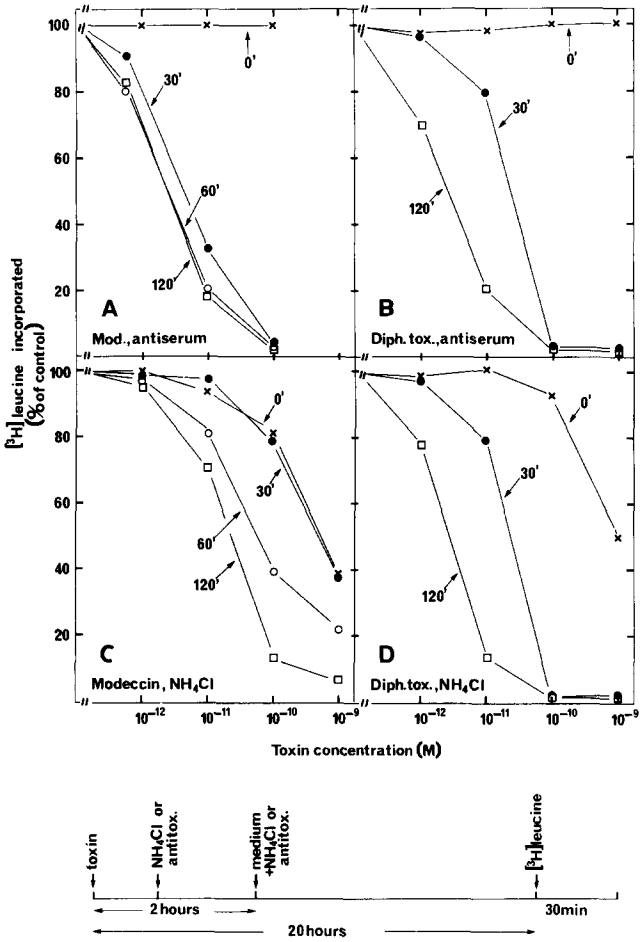

FIGURE 2 Ability of antitoxin (*A* and *B*) and NH_4Cl (*C* and *D*) to protect cells against modeccin and diphtheria toxin. Increasing amounts of toxin were added to Vero cells growing in 24-well disposable trays. After the indicated periods of time 10 μl of antitoxin or NH_4Cl (final concentration 10 mM) was added. 2 h after addition of toxin, the medium was removed from all wells and growth medium containing 10 μl/ml of antitoxin or 10 mM NH_4Cl was added to all wells. After incubation overnight the rate of protein synthesis was measured as described in Materials and Methods. The time period from addition of toxin until the addition of antitoxin or NH_4Cl was 30 (●), 60 (○), and 120 min (□). In one case (×), antiserum or NH_4Cl was added simultaneously with the toxin.

toxin enters the cytosol rapidly after it has been endocytosed. To better demonstrate the difference between the protective effect of NH_4Cl 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 Ca^{2+}-deprivation protects cells against modeccin (24). Co^{2+} cannot replace Ca^{2+} in restoring the sensitivity of the cells to the toxin, but it prevents the cell detachment from the plastic surface otherwise observed in Ca^{2+}-free media (24). As shown in Fig. 4*A*, Ca^{2+} deprivation protected against modeccin almost as well as the presence of NH_4Cl. To test if the step in the entry of modeccin that is inhibited by Ca^{2+} deprivation, is before or after the NH_4Cl sensitive step, we first incubated cells with modeccin in medium containing Co^{2+} instead of Ca^{2+} to allow modeccin to be endocytosed and proceed until the Ca^{2+}-requiring step. Then NH_4Cl was added and after 30 min the buffer was removed and growth medium containing Ca^{2+} and NH_4Cl

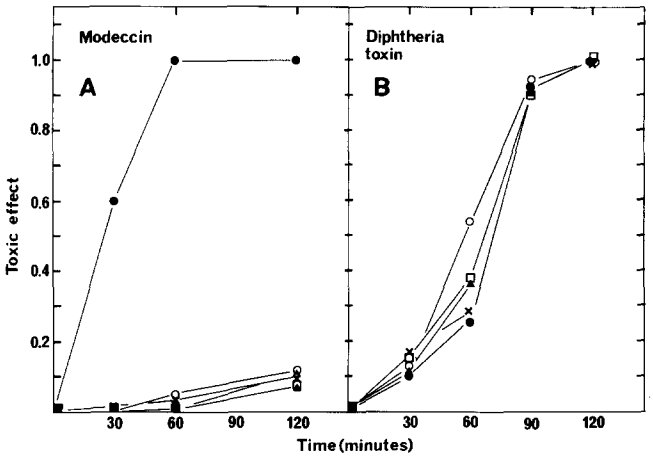

FIGURE 3 Protective effect of various compounds added to cells exposed to modeccin (*A*) and diphtheria toxin (*B*) for different periods of time. The experimental protocol was as in Fig. 2. Toxin was added at time 0 and the compounds to be tested were added to the cells at the times indicated on the abscissa. Dose-response curves as in Fig. 2 were constructed and the toxin concentration required to reduce protein synthesis to 50% of the control value (ID_{50} inhibitor) was measured. In each case the toxin concentration required to induce 50% inhibition in 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: (●) 10 μl/ml antiserum; (○) 10 mM NH_4Cl; (▲) 10 μM FCCP; (×) 10 μM monensin; (□) 10 mM procaine; (△) 15 μM FCCP.

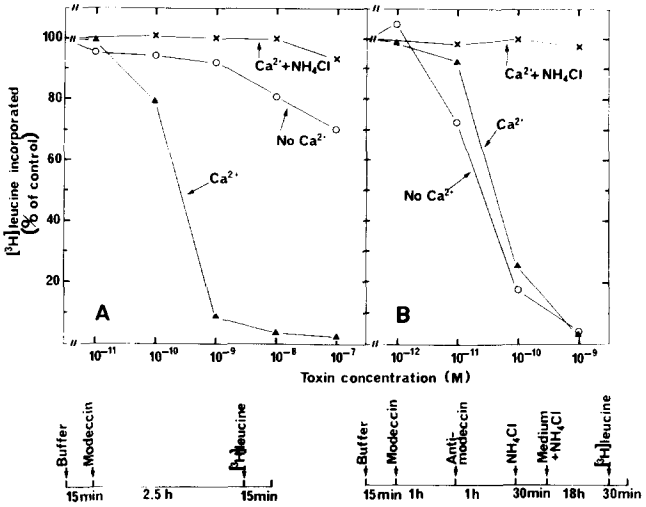

FIGURE 4 Ability of modeccin endocytosed in the absence of Ca^{2+} to intoxicate cells in the presence of NH_4Cl. Cells in 24-well Linbro plates were preincubated in 0.14 M NaCl, 20 mM HEPES, pH 7.2, 1 mg/ml glucose and either 2 mM $CaCl_2$, 2 mM $CoCl_2$ or 2 mM $CaCl_2$, and 10 mM NH_4Cl for 15 min at 37°C. Then increasing amounts of modeccin were added. In *A*, the cells were further incubated for 2.5 h, then the buffer was removed. Medium containing [^{3}H]leucine was added and the incorporation during 15 min was measured. In *B*, the cells were incubated at 37°C for 1 h after toxin addition, then 10 μl antimodeccin was added to each well and, after 1 h more of incubation, NH_4Cl was added to a concentration of 10 mM. After further 30 min the buffer was removed and medium containing 10 mM NH_4Cl was added. The cells were then incubated over night and finally the incorporation during 30 minutes was measured. The buffer contained: (▲) 2 mM $CaCl_2$; (○) 2 mM $CoCl_2$; (×) 2 mM $CaCl_2$ and 10 mM NH_4Cl.

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. 4*B*). This indicates that even in the absence of Ca^{2+}, modeccin had passed the NH_4Cl-sensitive step. If the entire experiment was carried out in the presence of NH_4Cl, the cells were, as expected, protected even after incubation over night (Fig. 4*B*). Unfortunately, the reverse experiment in which toxin was endocytosed in the presence of NH_4Cl and then Ca^{2+} was removed, could not be carried out due to the slow decay of the NH_4Cl-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_4Cl 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_4Cl (Fig. 3*A*). In contrast, monensin protected effectively against diphtheria toxin only when it was added to the cells simultaneously with the toxin (Fig. 3*B*).

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_4Cl. Furthermore, procaine protected against modeccin even when added after the toxin (Fig. 3*A*). 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_4Cl (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_4Cl (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°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 against modeccin while FCCP protected 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).

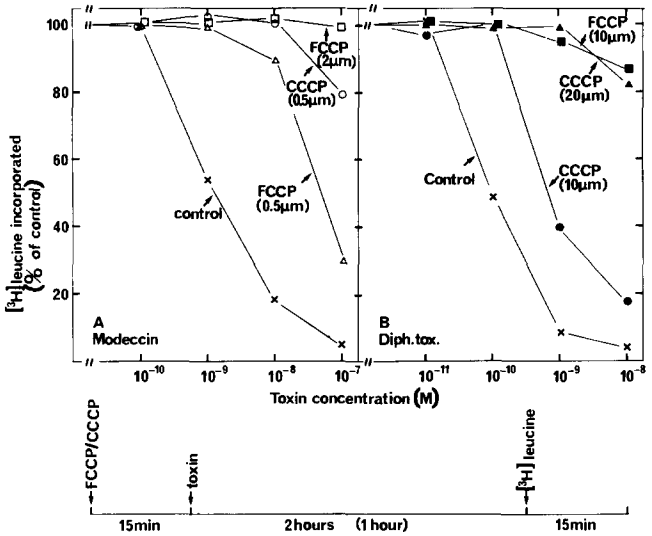

FIGURE 5 Ability of protonophores to protect cells against modeccin and diphtheria toxin. HEPES medium with or without the indicated amounts of protonophores was added to Vero cells growing in 24-well disposable trays and 15 min later increasing amounts of toxins were added. After incubation at 37°C for 2 h in case of modeccin and 1 h in the case of diphtheria toxin, the medium was removed and protein synthesis was measured during 15 minutes as described in Materials and Methods. (×) No protonophore present; (△) 0.5 μM FCCP; (□) 2 μM FCCP; (■) 10 μM FCCP; (○) 0.5 μM CCCP; (●) 10 μM CCCP; (▲) 20 μM CCCP.

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°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°C it was accumulated in intermediary vesicles with a bouyant density of $\rho \approx 1.05$ g/ml in a Percoll gradient. When the incubation was at 37°C a major part of the internalized enzyme was bouyant at $\rho = 1.07$ to 1.08 g/ml indicating that it was present in lysosomes. A large fraction of the lysosomal enzyme β-*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°C for 1 h to permit receptor binding without internalization to occur. Then medium prewarmed to 20°C and 37°C was

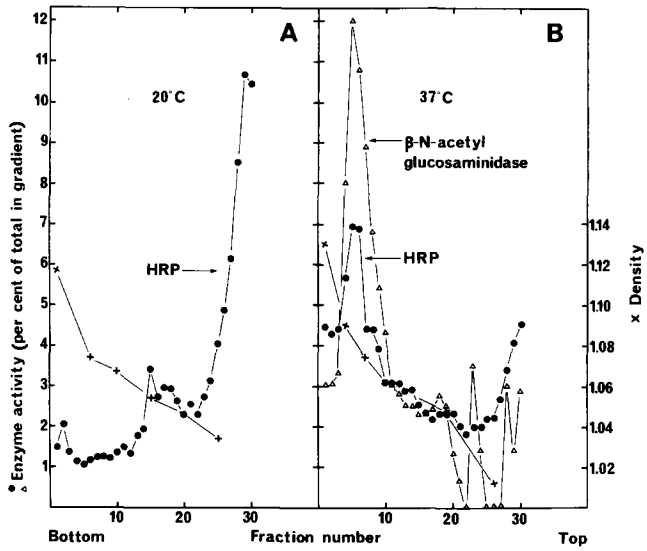

FIGURE 6 Effect of temperature on the transfer of horseradish peroxidase to lysosomes. Confluent Vero cells were incubated with 1 mg/ml of horseradish peroxidase for 16 h at 20°C (*A*) or at 37°C (*B*). The cells were washed eight times with medium. After 1 h more of incubation in medium without peroxidase, the cells were removed by a rubber policeman, and a homogenate was prepared as described in Materials and Methods. The microsome fraction was layered onto 20% Percoll containing 0.25 M sucrose, 1 mM EDTA 10 mM triethanolamine, 10 mM acetic acid, pH 7.5. The tubes were centrifuged at 27,000 rpm in a Ti50 rotor for 50 minutes. Fractions were collected from the bottom of the tubes and aliquots were analyzed for horseradish peroxidase and β-*N*-acetylglucosaminidase.

added to allow internalization to take place. After 30 min (diphtheria toxin) or 4 h (modeccin), NH_4Cl 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 NH_4Cl 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 NH_4Cl, whereas the effect of diphtheria toxin was prevented. The data indicate that the entry of diphtheria toxin at 20°C occurs by the normal NH_4Cl-sensitive way, whereas the entry of modeccin at 20°C is NH_4Cl-insensitive and therefore differs from the highly efficient entry dominating at 37°C. Evidence for the existence of such a NH_4Cl-insensitive entry mechanism

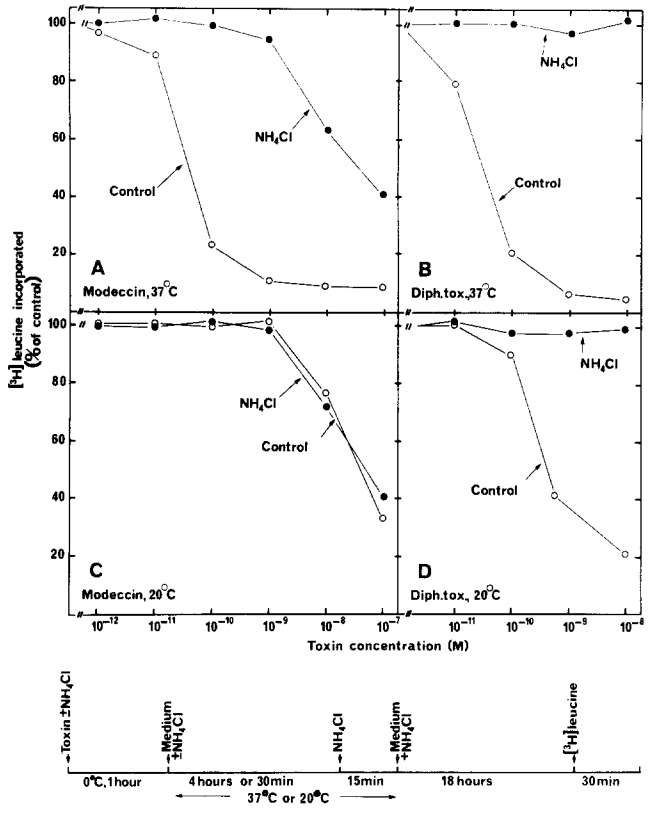

FIGURE 7 Effect of temperature on the NH_4Cl sensitive entry of modeccin and diphtheria toxin. Vero cells growing in 24-well disposable trays were chilled to 0°C and then increasing amounts of toxin were added. When indicated 10 mM NH_4Cl was also added. After binding for 1 h, the medium with unbound toxin was removed and medium prewarmed to 37°C (*A* and *B*) or 20°C (*C* and *D*) with and without 10 mM NH_4Cl was added to the cells. After 30 min (diphtheria toxin) or 4 h (modeccin) incubation at these temperatures, NH_4Cl was added to those wells not already containing NH_4Cl and after 15 min more growth medium containing NH_4Cl was added. After incubation overnight the rate of protein synthesis was measured. (●) 10 mM NH_4Cl present throughout the experiment; (○) 10 mM NH_4Cl only added after 30 min of incubation at 37°C or 20°C.

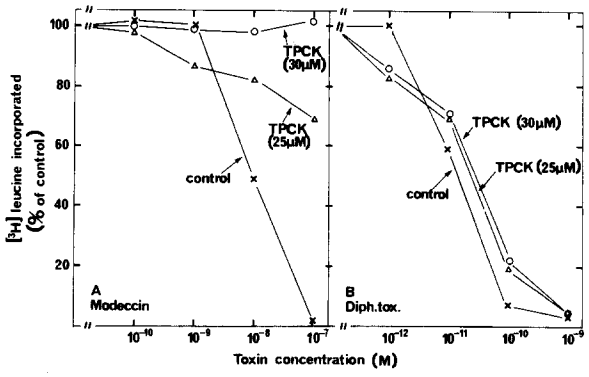

FIGURE 8 Effect of TPCK on the sensitivity of Vero cells to modeccin and diphtheria toxin. HEPES medium with or without the indicated concentrations of TPCK was added to Vero cells and, after 15 min, increasing concentrations of toxin were added. After 2 h further incubation at 37°C the rate of protein synthesis was measured as described in Materials and Methods. (×) control no TPCK; (△) 25 μM TPCK; (○) 30 μM TPCK.

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 Protease Inhibitors on the Toxic Effect of Modeccin and Diphtheria Toxin

If proteolytic cleavage of modeccin A-chain is required for entry, protease inhibitors might protect cells against the toxin. It has earlier been reported that pepstatin and chymostatin have no protective effect against modeccin (8). Also phenylmethylsulfonyl fluoride and soybean trypsin inhibitor added to the medium had no effect. However, as shown in Fig. 8, the protease inhibitor TPCK strongly protected against modeccin. TPCK had little or no protective effect against diphtheria toxin (Fig. 8), although it inhibited the cellular degradation of this toxin (data not shown). A similar protection against modeccin was obtained with another protease inhibitor, TLCK (not shown). Preincubation of modeccin with TPCK did not reduce the toxicity of modeccin as such (not shown). The data therefore are consistent with the possibility that proteolytic cleavage is required for intoxication by modeccin.

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 ^{125}I-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 (NH_4Cl, 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 modeccin where NH_4Cl, monensin, CCCP, and procaine protected almost completely also when added 30 min after the toxin. Even addition as late as 2 h after modeccin had a strong protective effect. This indicates that modeccin 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 modeccin 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 modeccin, but not for diphtheria toxin, is supported by the finding that at 20°C where fusion with lysosomes is prevented, the action of modeccin 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 NH_4Cl while this was not the case with modeccin. This indicates that the limited entry of modeccin 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 NH_4Cl on the pH of acidic vesicles have been shown to rapidly disappear after removal of the compounds (3). The observation that Vero cells are protected against modeccin 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 NH_4Cl, 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 modeccin A-chain is much less active in cell-free systems than abrin and ricin A-chain (16). One possibility is that modeccin 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 modeccin (8). We here 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 modeccin while none of them protected against diphtheria 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 α_2-macroglobulin into cells and these compounds also inhibit the action of various nonproteolytic enzymes (33). It is therefore not clear if the protection against modeccin is due to inhibition of a proteolytic enzyme or to some other effect of the compound.

The finding that the NH_4Cl sensitive step in modeccin entry occurs before the Ca^{2+}-dependent step, indicates that Ca^{2+} is required after the toxin has been exposed to low pH. If the situation is analogous to that with diphtheria toxin, low pH induces insertion of part of the toxin (the B-chain?) into the membrane. Ca^{2+} could then be necessary in the subsequent transfer of the A-chain across the membrane.

Although toxins like diphtheria toxin, modeccin, 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 diphtheria toxin and modeccin 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 diphtheria toxin and modeccin. 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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