Monensin Blocks the Transport of Diphtheria Toxin to the Cell Cytoplasm

MARY H. MARNELL, MARGARET STOOKEY, AND ROCKFORD K. DRAPER
Biology Program, The University of Texas at Dallas, Richardson, Texas 75080

ABSTRACT Lysosomotropic amines are believed to inhibit the transport of diphtheria toxin to the cell cytoplasm by raising the pH within intracellular vesicles. If so, then other drugs that dissipate intracellular proton gradients should have a similar effect on toxin transport. We found that monensin, a proton ionophore unrelated to lysosomotropic amines, is a potent inhibitor of the cytotoxic effect of diphtheria toxin. Monensin appears to block the escape of endocytosed toxin from a vesicle to the cytoplasm. Monensin fails to protect cells from the effects of diphtheria toxin that is bound to the cell surface and exposed to acidic medium, suggesting that the step normally blocked by the drug is circumvented under these conditions. The inhibition of toxin transport caused by monensin could not be relieved when monensin was replaced by ammonium chloride, nor when ammonium chloride was again replaced by monensin. This suggests that both drugs block the same step of toxin transport. The effect of monensin on the transport of diphtheria toxin to the cytoplasm is consistent with the proposal (Draper and Simon, 1980. J. Cell Biol. 87:849–854; Sandvig and Olsnes, 1980. J. Cell Biol. 87:828–832) that the toxin is endocytosed and then, in response to an acidic environment, penetrates through the membrane of an intracellular vesicle to reach the cytoplasm.

Diphtheria toxin (63,000 mol wt) arrests protein synthesis in sensitive mammalian cells by enzymatically inactivating elongation factor 2 in the cell cytoplasm (3, 14). A question of interest is the mechanism by which the toxin is transported from a receptor at the cell surface to the cytoplasm. This pathway, which is theoretically available to biologically relevant proteins, is an interesting mechanism of transmembrane signaling because an extracellular protein is physically admitted to the cytoplasm.

It was recently proposed that diphtheria toxin destined to inhibit protein synthesis is endocytosed and encounters an acidic environment within the vacuolar system of the cell, where, in response to the low pH, the toxin inserts into the vesicle membrane (4, 6, 17, 18). Fragment A, which contains the catalytic center of the toxin, presumably translocates to the cytoplasmic side of the vesicle membrane as a consequence of insertion. The evidence for this is based, in part, on the following observations: (a) Lysosomotropic amines protect cells from the cytotoxic effects of the toxin (9, 10). These drugs dissipate intracellular proton gradients and could prevent the acid-dependent penetration of the toxin through a vesicle membrane. (b) In the presence of lysosomotropic amines, the toxin is trapped in a location that is inaccessible to antitoxin added to cells at 4°C, probably within an intracellular vesicle (6). (c) The protective effect of lysosomotropic amines is lost if toxin bound to the cell surface is exposed to medium <pH 4.7 (6, 17, 18). This can be explained if the toxin were induced to penetrate the plasma membrane by the acidic environment. (d) Diphtheria toxin forms ion-conducting channels in planar lipid bilayers <pH 5.0, demonstrating that the toxin can become a transmembrane protein in response to an acidic environment (4). If these data have been correctly interpreted, then other drugs that collapse proton gradients should have effects on toxin transport similar to those of lysosomotropic amines. We report here that the proton ionophore monensin protects cells from the cytotoxic activity of diphtheria toxin. The effects of monensin on toxin transport appear identical to those of ammonium chloride.

MATERIALS AND METHODS

Materials

Diphtheria toxin was obtained from Connaught Laboratories (Willowdale, Ontario, Canada) and prepared as described previously (6). Monensin was purchased from Calbiochem-Behring Corp., American Hoescht Corp. (La Jolla, CA) and nigericin was a gift from Hoffman-LaRoche (Nutley, NJ). [3H]-L-leucine (45 Ci/mM) was purchased from Schwarz/Mann Div., Becton, Dickinson Co., (Spring Valley, NY). Other chemicals, reagent grade or better, were purchased from either Sigma (St. Louis, MO) or Matheson, Coleman, and Bell (East Rutherford, NJ).
Methods

All experiments were done with VERO cells (African Green Monkey Cells, ATCC registry number CCL81) maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum as previously described (6). Protein synthesis assays for the cytotoxic activity of diphtheria toxin were as described by Draper and Simon (6), except that the radioactive amino acid was [4-5H]-l-leucine. The assay medium was Dulbecco's modified Eagle's medium with 5% fetal bovine serum buffered to pH 7.0 with 0.02 M HEPES instead of CO2 and containing one-fiftieth the normal amount of l-leucine. Monensin was dissolved in ethanol before dilution with the assay medium. The concentration of ethanol never exceeded 0.1% and did not interfere with protein synthesis or the cytotoxic effect of diphtheria toxin. All assays were done in triplicate. The standard deviation of the mean of triplicate samples is indicated by the error bars in all figures, except when the symbols were larger than the standard deviation. Rabbit antitoxin serum was prepared with formaldehyde-treated diphtheria toxin as previously described (6).

RESULTS

Monensin Arrests the Transport of Diphtheria Toxin to the Cytoplasm

Cells preincubated with 10 μM monensin did not respond to 10 nM diphtheria toxin within 3 h (Fig. 1, top). The effect was diminished at 1 μM monensin and absent at 0.1 μM monensin. Cells challenged with up to 100 nM diphtheria toxin synthesized protein normally in the presence of 10 μM monensin (Fig. 1, bottom). Monensin itself had little effect on protein synthesis. Nigericin, an ionophore closely related to monensin, partially protected cells from 10 nM toxin at 1 μM (data not shown). Higher concentrations of nigericin alone abolished protein synthesis, making it impossible to determine the dose of nigericin that would give complete protection. Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), a proton transporter unrelated to monensin or nigericin, did not inhibit the effect of the toxin on protein synthesis. Unlike monensin and nigericin, FCCP is an electrogenic protonophore; the electrical gradient established by proton transport is not balanced by cation transport in the opposite direction. The electrical gradient would oppose continued proton loss and leave a pH gradient intact. The presence of a second cation ionophore, such as valinomycin, should abolish the electrical effect and allow free proton transport; however, a combination of both FCCP (1.0 μM) and valinomycin (0.1 μM) inhibited protein synthesis before any effects on the entry of the toxin could be seen.

If monensin traps some toxin within an intracellular vesicle, then antitoxin added to the cell exterior should not neutralize the effects of the sequestered toxin. The temperature of the cells when the antitoxin is applied is a critical variable in this experiment. As described in Fig. 2, cells were preincubated with monensin and toxin at 37°C to allow the toxin to proceed to that step of the transport process blocked by monensin. The cultures were then divided into two portions; antitoxin was added to one portion at 37°C and to the other portion at 4°C. After a 1-h incubation, the cells were washed to remove all drugs. Protein synthesis, measured 24 h later, is shown in the graph of Fig. 2. The cells were protected by antitoxin added at 37°C but not at 4°C. The effect of temperature is identical to that seen when ammonium chloride is used instead of monensin (6). The result at 4°C is expected if a fraction of the toxin is trapped within intracellular vesicles inaccessible to antitoxin in the medium. Upon removal of the monensin and antitoxin, the trapped toxin could proceed to the cytoplasm at 37°C and arrest protein synthesis. The same result would also be seen if chilling the cells somehow introduced an artifact that allowed the toxin to enter the cytoplasm. This could occur, for example, if a vesicle containing the toxin broke open within the cell when the temperature was lowered. To test this, cells were preincubated with monensin and toxin, chilled to 4°C, exposed to antitoxin for 0.5 h, and raised to 37°C for 2 h with antitoxin and monensin present. If the temperature change caused the toxin to enter the cytoplasm, then protein synthesis should cease; however, as seen in Fig. 3, protein synthesis was normal 24 h later.
How antitoxin present at 37°C in the experiments of Figs. 2 and 3 could neutralize the effects of toxin already within a vesicle is not clear. Some process active at 37°C, but not at 4°C, is implicated. To explain a similar effect found with ammonium chloride, Draper and Simon (6) suggested either that the antitoxin is endocytosed and delivered to vesicles containing the toxin or that the intracellular vesicles containing the toxin are recycled back to the cell surface, exposing the toxin to medium containing antitoxin. Another explanation is that the half-life of toxin trapped within a vesicle by monensin is very short. The antitoxin could neutralize toxin outside the cell while toxin within intracellular vesicles is inactivated at 37°C, giving the appearance that the antitoxin somehow gained access to intravesicular toxin. The experiment of Fig. 4 suggests that this is not the case. Cells were pretreated with monensin and toxin, chilled, and exposed to antitoxin. After 0.5 h, the antitoxin was removed but not the monensin, and the cells were incubated at 37°C for 1 h. If the toxin was inactivated during this time, then protein synthesis should be normal 24 h after the monensin is removed. Protein synthesis was inhibited as shown in bar A of Fig. 4. It is important in this experiment that the neutralization of toxin by antitoxin be irreversible. To test this, cells were incubated at 4°C with toxin, treated with antitoxin, washed, and then incubated at 37°C. Protein synthesis was normal 24 h later, demonstrating that the inhibitory effect of the antitoxin was irreversible (data not shown).

Monensin Fails to Protect Cells after Diphtheria Toxin Bound to the Cell Surface Is Exposed to Acidic Medium

The protective effect of monensin, like that of ammonium chloride, should be lost if diphtheria toxin that is bound to the cell surface penetrates the plasma membrane when exposed to acidic medium. As seen in the graph of Fig. 5, monensin failed to protect cells that were preincubated with toxin at 4°C, exposed to medium <pH 4.7, and incubated at 37°C with monensin. A similar effect was seen when the preincubation included both toxin and monensin, suggesting that monensin does not directly inhibit the toxin molecule itself. These results...
that the actual penetration occurs sometime later after endo-
treatment at low pH (perhaps by a conformational change) and
the toxin is only primed for penetration by the membrane in response to the acidic medium. An alternative possibility is that the toxin
is expected if the toxin directly penetrated the plasma mem-
brane. This makes it possible to order the steps in a half cycle of drug exchange would be sufficient to allow the toxin to enter the cytoplasm. This makes it possible to order the steps affected by two drugs with respect to one another. In B, the two drugs inhibit the same step. The toxin should still be blocked at step 3 after any number of drug exchanges. More complicated situations are possible; in C, each drug inhibits two different alternating steps. Two complete cycles of drug exchange would be required to advance the toxin past step 4 and distinguish this example from that in B.

FIGURE 6 The effect of concanavalin A on the cytotoxic activity of diphtheria toxin after cells with toxin on their surface were exposed to acidic medium. The cells were treated as described in Fig. 5 except that monensin was replaced with 50 μg/ml concanavalin A.

FIGURE 7 A model for the analysis of the effects of different inhibitors on the transport of diphtheria toxin to the cell cytoplasm. To reach the cytoplasm, diphtheria toxin negotiates a series of steps beginning at the cell surface and ending when fragment A interacts with elongation factor 2. Four hypothetical steps are shown above. Consider two drugs, x and y, whose effects are reversible. Assume in A that the drugs inhibit two different steps. When cells are incubated with drug x and toxin, the toxin is blocked at step 2 if drug y is then added and x removed, the toxin can proceed past step 2 but is then blocked at step 3. When drug x is added again and y is removed to complete one full cycle of drug exchange, the toxin that had passed step 2 is now free to enter the cytoplasm and inhibit protein synthesis. Note that if drug y were added first, only a half cycle of drug exchange would be sufficient to allow the toxin to enter the cytoplasm. This makes it possible to order the steps affected by two drugs with respect to one another. In B, the two drugs inhibit the same step. The toxin should still be blocked at step 3 after any number of drug exchanges. More complicated situations are possible; in C, each drug inhibits two different alternating steps. Two complete cycles of drug exchange would be required to advance the toxin past step 4 and distinguish this example from that in B.

are expected if the toxin directly penetrated the plasma membrane in response to the acidic medium. An alternative possibility is that the toxin is only primed for penetration by the treatment at low pH (perhaps by a conformational change) and that the actual penetration occurs sometime later after endo-

cytosis. Concanavalin A arrests the endocytosis of surface-bound diphtheria toxin (11) and protects cells from the effects of the toxin (5). If the lectin fails to protect cells after the toxin is exposed to a low pH at the cell surface, then endocytosis is probably unnecessary under these conditions. The results in Fig. 6 demonstrate that the lectin does not protect cells <pH 4.7.

Ammonium Chloride and Monensin Inhibit the Same Step of the Toxin Transport Process

The effects of monensin on toxin transport appear similar to those of ammonium chloride. Both drugs neutralize intracellular pH gradients, albeit by different mechanisms, and their effects should be similar if they prevent the acid-dependent penetration of the toxin through the membrane of a vesicle. If both drugs actually do inhibit the same step of the transport mechanism, it should be impossible to relieve the inhibition of one drug by replacing it with the other; if two different steps are affected, the inhibition of one drug should be relieved when it is replaced by the other. The rationale for this analysis is described in Fig. 7. In Fig. 8, cells were incubated with monensin and toxin, then ammonium chloride was added and the monensin was removed. The drug exchanges were repeated for two complete cycles until monensin was the last drug present. Protein synthesis was normal 4.5 h later as shown in bar A of Fig. 8. This suggests that monensin and ammonium chloride block the same step of toxin transport. Bar B shows protein synthesis when the toxin was omitted from the protocol. Bar C shows that protein synthesis ceased when the last drug added,
monensin, was removed along with ammonium chloride; this demonstrates that toxin capable of arresting protein synthesis was present throughout the experiment.

DISCUSSION

The proposal that diphtheria toxin is endocytosed and penetrates the membrane of a vesicle with a low internal pH was based, in part, on the assumption that lysosomotropic amines interfered with this process by raising the pH within the vesicle (6, 17). An alternative is that the toxin penetrates the plasma membrane (2) in some manner that can be blocked by these reagents. It is important to verify whether other drugs that abolish intracellular pH gradients have effects on toxin transport that are similar to the effects of lysosomotropic amines. Monensin is an ionophore that collapses proton gradients by the electroneutral exchange of a proton for a monovalent cation (preferably sodium) across a membrane (15). Ray and Wu (16) recently reported that monensin did not alter the sensitivity of cells to diphtheria toxin in plating efficiency tests, but they did not study concentrations of monensin >50 nM. We found that protein synthesis in Vero cells was unaffected by as much as 100 nM diphtheria toxin in the presence of 10 μM monensin. On a concentration basis, monensin is approximately one thousand times more potent than ammonium chloride in protecting cells from the effects of the toxin.

Monensin might inhibit the cytotoxic activity of the toxin by blocking any one, or more, of five basic processes: (a) the binding of the toxin to a receptor; (b) the possible penetration of the toxin through the plasma membrane; (c) the endocytosis of the toxin; (d) the possible penetration of the toxin through the membrane of a vesicle; and (e) the enzymatic activity of fragment A in the cytoplasm. The last possibility cannot be true; toxin that is bound to the cell surface and exposed to acidic medium inactivates elongation factor 2 in the presence of monensin. When cells are incubated with monensin and diphtheria toxin, a lethal amount of the toxin becomes insensitive to neutralization by antitoxin, provided the cells are exposed to the antitoxin at 4°C. It is unlikely that the change in temperature either alters or shelters toxin on the cell surface because the toxin binds to its receptor at 4°C and reacts in this position with antitoxin (6). It appears that monensin either traps the toxin in some intermediate form on its way through the plasma membrane that is beyond inhibition by antitoxin or that the toxin is trapped within a vesicle segregated from the antitoxin; however, when the antitoxin is present at 37°C in this experiment, the toxin is completely neutralized, clearly indicating that monensin does not trap the toxin in some intermediate form unreactive with antitoxin. These observations can be reconciled if, at 37°C, the toxin within vesicles is recycled back to the cell surface or if antitoxin is endocytosed and reacts with toxin already in vesicles. Both possibilities are supported by recent reports directly demonstrating the recycling of endocytosed membrane components (12, 19, 20). Another explanation, that the toxin within vesicles is inactivated, is inconsistent with the results in Fig. 4. These data suggest that, in the presence of monensin, the toxin binds to a receptor and is endocytosed but cannot escape from a vesicle to the cytoplasm. This behavior is identical to that seen in the presence of ammonium chloride (6).

We compared the effects of monensin and ammonium chloride on toxin transport in the experiment of Fig. 8 to see whether both drugs do inhibit the same step of the transport process. This analysis assumes that, if the two drugs inhibit different steps, then enough toxin can be isolated between these steps to inhibit protein synthesis after two full cycles of drug exchange. The toxin failed to enter the cytoplasm, suggesting that both drugs block the same step. Both drugs collapse proton gradients, and the affected step is probably the acid-dependent penetration of the toxin through a vesicle membrane.

Donovan et al. (4) demonstrated that diphtheria toxin inserts into lipid bilayers at a low pH. It is likely that the toxin directly penetrates the plasma membrane when bound to the cell surface and exposed to medium at a low pH. Thus, the step normally blocked by monensin and ammonium chloride is provided outside of the cell, and the drugs no longer arrest toxin entry. It is possible that the toxin must still undergo some processing event that only occurs after endocytosis before it can penetrate a membrane; however, we found that concanavalin A, which inhibits the endocytosis of the toxin (11), also fails to protect cells from toxin treated with acidic medium at the cell surface. This suggests that a postendocytic processing event is not required under these conditions.

The identity of the vesicles from which the toxin escapes is not yet clear. Lysosomes are obvious candidates because their interior pH is about 4.5 (13), but the toxin could escape from some other compartment of the vacuolar system if it encountered a low pH. Sandvig and Olsnes (18) recently suggested that toxin entering the lysosomes was digested before fragment A could pass through a membrane and that the toxin may escape before an endocytic vesicle fuses with the lysosomes. The experiment in Fig. 6 bears on this point: after the antitoxin was removed, the cells were incubated for 1 h at 37°C in the presence of monensin, and the toxin was still active. We have extended this incubation up to 4 h with similar results and also repeated the observation with ammonium chloride (data not shown). This is ample time for the toxin to have entered lysosomes. Normal drug-free toxin transport, however, may be different; the presence of the drug might protect the toxin, perhaps by preventing the fusion of endocytic vesicles with lysosomes, or by inhibiting lysosomal proteolysis.

Diphtheria toxin reaches the cytoplasm by a mechanism similar to that of Semliki Forest virus (8). Other protein toxins, and perhaps some protein hormones, might also use this pathway. Diphtheria toxin should be an interesting model system with which to study endocytosis and the subsequent fate of the vesicles. In response to a low pH, diphtheria toxin undergoes a structural change with important functional consequences. This could also be true for some endocytosed membrane proteins during normal cell metabolism. A possible example of this may be recycled receptors since drugs that neutralize pH gradients appear to arrest the reappearance of these membrane components in the plasma membrane (1, 7, 21, 22).

We thank K. Sandvig and S. Olsnes for discussing their unpublished results with us and for suggesting that toxin within the cell may be inactivated in the presence of monensin. We thank G. Breen for critically reading the manuscript and S. Rahn for typing.

This work was supported by a biomedical research support grant and by grant no. 1 R01 AI 17460-01 from the National Institutes of Health.

Received for publication 30 September 1981, and in revised form 30 November 1981.

REFERENCES

1. Bace, S. K., J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1981. Monensin interrupts the recycling of low density lipoprotein receptors in human fibroblasts. Cell 24: 493-502.
2. Boquet, P., M. S. Silverman, A. M. Pappenheimer, Jr., and W. B. Vernon. 1976. Binding of Triton X-100 to diphtheria toxin, crossreacting material 45, and their fragments. Proc. Natl. Acad. Sci. U. S. A. 73:4449-4453.
3. Collier, J. R. 1975. Diphtheria toxin: mode of action and structure. Bacteriol. Rev. 39:54-85.
4. Donovan, J. J., M. I. Simon, R. K. Draper, and M. Montal. 1981. Diphtheria toxin forms transmembrane channels in planar lipid bilayers. Proc. Natl. Acad. Sci. U. S. A. 78:172-176.
5. Draper, R. K., D. Chun, and M. I. Simon. 1978. Diphtheria toxin has the properties of a lectin. Proc. Natl. Acad. Sci. U. S. A. 75:261-265.
6. Draper, R. K., and M. I. Simon. 1980. The entry of diphtheria toxin into the mammalian cell cytoplasm: evidence for lysosomal involvement. J. Cell Biol. 87:849-854.
7. Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sty. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. J. Cell Biol. 85:839-852.
8. Helenius, A., J. Kartenbeck, K. Simons, and E. Fries. 1979. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. 84:404-420.
9. Kim, K., and N. B. Groeman. 1965. In vitro inhibition of diphtheria toxin action by ammonium salts and amines. J. Bacteriol. 90:1552-1556.
10. Leppla, S. H., R. B. Dorland, and J. L. Middlebrook. 1980. Inhibition of diphtheria toxin degradation and cytotoxic action by chloroquine. J. Biol. Chem. 255:2247-2250.
11. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1979. Effects of lectins on the interaction of diphtheria toxin with mammalian cells. Exp. Cell Res. 121:95-101.
12. Muller, W. A., R. M. Steinman, and Z. A. Cohn. 1980. The membrane proteins of the vacuolar system. II. Bidirectional flow between secondary lysosomes and plasma membrane. J. Cell Biol. 86:304-314.
13. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U. S. A. 75:3327-3331.
14. Pappenheimer, A. M., Jr. 1977. Diphtheria toxin. Ann. Rev. Biochem. 46:63-94.
15. Pressman, B. C. 1976. Biological applications of ionophores. Ann. Rev. Biochem. 45:501-530.
16. Ray, B., and H. C. Wu. 1981. Enhancement of cytotoxicities of ricin and Pseudomonas toxin in Chinese hamster ovary cells by nigericin. Mol. Cell. Biol. 1:552-559.
17. Sandvig, K., and S. Olness. 1980. Diphtheria toxin entry into cells is facilitated by low pH. J. Cell Biol. 87:828-832.
18. Sandvig, K., and S. Olness. 1981. Rapid entry of nicked diphtheria toxin into cells at low pH. J. Biol. Chem. 256:9004-9016.
19. Schneider, Y., P. Tulkens, C. de Duve, and A. Trouet. 1979. Fate of plasma membrane during endocytosis. II. Evidence for recycling (shuttle) of plasma membrane constituents. J. Cell Biol. 82:466-471.
20. Thiol, L., and G. Vogel. 1980. Kinetics of membrane internalization and recycling during pinocytosis in Dictyostelium discoideum. Proc. Natl. Acad. Sci. U. S. A. 77:1015-1019.
21. Tietze, C., P. Schlesinger, and P. Stahl. 1980. Chloroquine and ammonium ion inhibit receptor-mediated endocytosis of mannose-glycoconjugates by macrophages: apparent inhibition of receptor recycling. Biochem. Biophys. Res. Commun. 93:1-8.
22. Tollefsberg, H., and T. Berg. 1979. Chloroquine reduces the number of asialoglycoprotein receptors in the hepatocyte plasma membrane. Biochem. Pharmacol. 28:2912-2922.