Methylamine Stimulates the Action of Ricin Toxin but Inhibits That of Diphtheria Toxin*

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The action of ricin toxin was stimulated by addition of methylamine or some other amines, as shown by measuring the inhibition of protein synthesis of cultured cells by the toxin. Under the same conditions, however, the action of diphtheria toxin was completely inhibited by the amines. In a cell-free protein-synthesizing system, methylamine had no effect on the action of the A chain of ricin toxin and fragment A of diphtheria toxin. Studies on the interactions of 125I-labeled toxins with cells revealed that methylamine did not alter toxin-receptor bindings, but affected the entry of the toxins into the cells. Studies were also made on the effects of methylamine on the actions of two hybrid toxins, formed from a subunit of Wistaria floribunda lectin and fragment A of diphtheria toxin and the A chain of ricin toxin, respectively. Results suggested that the processes of entry of ricin toxin and diphtheria toxin, or at least parts of these processes, are different.

Toxic proteins, such as diphtheria toxin, PA toxin, and ricin toxin, bind to cell-surface receptors and then penetrate the plasma membrane to exert biological activity in the cytoplasm. Diphtheria toxin and ricin toxin have very similar structures and biological activities. Both toxins are constructed from two components: diphtheria toxin is constructed from fragments A and B, and ricin toxin is constructed from the A and B chains (1–3). The polypeptides A (fragment A of diphtheria toxin and A chain of ricin toxin) exert cytotoxicity by inhibiting cellular protein synthesis in the cytoplasm (4–6). In fact, when fragment A of diphtheria toxin was introduced into the cytoplasm by artificial methods, it killed the cell (7, 8). Polypeptides B are known to interact with the membrane receptor of toxin-susceptible cells and to promote the transport of polypeptide A to the cytoplasm (4, 9).

The mechanisms of entry of these toxins into the cytoplasm are interesting in relation to the problem of how biologically active macromolecules enter the cell. One approach to this problem is to determine what functional structures are essential for the entry of proteins into cells. Recently, the synthesis of hybrid toxic molecules was reported (10–14). We previously formed two hybrid toxins by constitution of a subunit of Wistaria floribunda lectin with fragment A of diphtheria toxin and the A chain of ricin toxin, respectively (11, 14). Results with these toxins suggested that the hydrophobic region of the toxin is essential for entry of the toxin into the cell. Another approach to this problem is to investigate the cellular functions and factors responsible for toxin entry.

Although many studies along these lines have been reported, the mechanism is still poorly understood.

Recently, methylamine and some other amines have been reported to inhibit the internalization of various ligands into cells (15). It has also been reported that the action of diphtheria toxin is inhibited by ammonium salts (16) and chloroquine (17). In this work, we compared the effects of amines on the actions of diphtheria toxin and ricin toxin. The results suggest that the processes of entry of these two toxins, or at least parts of these processes, are different.

**EXPERIMENTAL PROCEDURES**

Ammonium chloride and the hydrochloride salts of methylamine, ethylamine, n-propylamine, isopropylamine, pentyamine, and cadavine, were purchased from Nakarai Chemical Co. (Kyoto, Japan). Chloroquine diphosphate was obtained from Sigma Chemical Co. (St. Louis, Mo.). These agents were made up as 1 M stock solutions of pH 7.5 and added to the culture medium. Purified W. floribunda lectin was obtained from Wako Pure Chemical Co. (Osaka, Japan).

*Toxins*—Diphtheria toxin was purified by chromatography on DEAE-cellulose (18). Ricin toxin was extracted from decoctinated ricinus seeds and purified as described previously (19, 20). PA toxin was kindly provided by Dr. B. H. Iglewski (University of Oregon). Fragment A of diphtheria toxin was purified on DEAE-cellulose and Sephadex G-100 from products of the C7hm(23)(22)–carried gene for the A fragment region (21). The A chain of ricin toxin was isolated by reduction of purified toxin with 20 mM dithiothreitol in the presence of 0.5 M galactose at pH 8.5, followed by chromatography on DEAE-cellulose. The crude A chain of ricin toxin was further purified on CM-cellulose (22) and Sepharose 4B.

*Formation of Hybrid Toxin*—Hybrid toxin of the A fragment of diphtheria toxin and a subunit of WF lectin was constituted from 0.2 mg of fragment A and 2 mg of WF lectin, as described previously (11). The hybrid was purified on Bio-Gel P-150 and then DEAE-Sephadex 50A. The purified sample gave one major band in a position corresponding to a molecular weight of about 54,000. The purity of the hybrid toxin was 90%. Hybrid toxin of the A chain of ricin toxin and subunit of WF lectin was also constructed and purified as reported previously (14). This hybrid toxin preparation contained 20% hybrid toxin, 20% A chain of ricin toxin, and 60% WF lectin. The toxicity of this hybrid toxin was completely neutralized by antibody against WF lectin or by antibody against purified A chain of ricin toxin, but was not affected by antibody against the B chain of ricin toxin. Therefore, the preparation was not contaminated with intact ricin toxin.

*Assay of the Rate of Protein Synthesis by Cells Cultured with Toxins*—The cells were plated in 24-well tissue culture plates and incubated for 24 h in Eagle’s minimum essential medium (MEM) supplemented with 10% calf serum. The plates were washed with BSS (0.13 M NaCl, 5.4 mM KCl, 0.78 mM KH2PO4, 10 mM Tris-HCl, pH 7.2), and then 0.5 ml of MEM containing 5% calf serum and 20 mM Hepes buffer, pH 7.2, with one-tenth of the normal concentration of leucine added in each well as described previously (23). Then methylamine or another amine was added to the culture medium. After 10 min, various concentrations of the toxins were added and the cells were incubated for 2 to 8 h at 37°C. They were then labeled with 1

*The abbreviations used are: WF lectin, W. floribunda lectin; Hepes, 4(2-hydroxyethyl)laminoethanesulfonic acid.

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Effects of Methylamine on Toxin Entry

mCi/ml of [3H]leucine for 60 min. The medium was removed and 0.1 N NaOH was added. The cells were collected and treated with 1% trichloroacetic acid, and the precipitate was collected on a glass filter, dried, and counted in a liquid scintillation system. The rate of protein synthesis in each culture was expressed as a percentage of the value obtained in the control cultures without toxin. Control values were 10,000 to 20,000 cpm.

Association of Iodinated Diphtheria or Ricin Toxin with Cells—Purified diphtheria toxin (50 μg) was labeled with 125I by the chloramine-T method as reported previously (24); 60 μg of ricin toxin was also labeled with 125I by the chloramine-T method in the presence of 0.2 mM galactose. The labeled diphtheria and ricin toxins had specific activities of 3 × 10⁶ and 9 × 10⁶ cpm/μg, respectively. The associations of iodinated toxins with cells were examined as reported by Middlebrook et al. (25). Briefly, Vero cells were seeded into tissue culture dishes and the cell lysate was counted in a liquid scintillation system.

For washing were combined and then centrifuged at 3,000 rpm for 20 min. The precipitate was collected on a glass filter, dried, and counted in a liquid scintillation system.

RESULTS

Protein synthesis of FL cells was inhibited by ricin toxin. Great inhibition was observed when methylamine was added to the culture medium with ricin toxin (Fig. 1, a and c). Since methylamine itself did not affect cellular protein synthesis, it obviously stimulated the action of ricin toxin. The extent of stimulation depended on the methylamine concentration. In contrast, methylamine inhibited the action of diphtheria toxin (Fig. 1, b and c). Under conditions similar to those used in assays with ricin toxin, the inhibition of protein synthesis by diphtheria toxin was completely prevented by methylamine at up to 10 mM. These effects of methylamine on the two toxins were reversed by washing off the methylamine: the continuous presence of methylamine was required for the effects. We also tested the effect of methylamine on the action of PA toxin. In this experiment, L-cells were used because they are highly sensitive to PA toxin. Methylamine showed contrary effects depending on its concentrations: at low concentrations (2.5 to 5 mM), it inhibited the action of PA toxin, whereas at higher concentrations of up to 10 mM, it inhibited the action of PA toxin (Fig. 1d). Under similar conditions, the action of ricin toxin was stimulated in all concentrations of methylamine. These findings suggest that methylamine acts at two sites on the cell.

Next, we tested the effects of various other amines on the actions of diphtheria and ricin toxins. Ethylamine, n-propylamine, and isopropanolamine strongly inhibited the action of diphtheria toxin and stimulated that of ricin toxin. The effects of methylamine and these amines showed similar concentration dependence. Cadaverine (10 mM) and putrescine (10 mM) had little or no effect on the action of either toxin. This was probably because these amines do not readily penetrate the cell (15). Chloroquine and ammonium salts have been reported to inhibit the action of diphtheria toxin (16, 17). We also tested these substances. Chloroquine (600 μM) inhibited the action of diphtheria toxin and stimulated that of ricin toxin, as did methylamine. Ammonium chloride (20 mM) inhibited the action of diphtheria toxin, but had little or no effect on that of ricin toxin.

In a cell-free protein-synthesizing system from wheat germ extract, fragment A of diphtheria toxin or the A chain of ricin toxin inhibited the incorporation of [3H]leucine into cellular proteins. Methylamine (10 mM) did not have any influence on the toxic actions in this system. This suggests that methylamine alters either the binding of these toxins to cell-surface receptors or the subsequent process of entry of these toxins into the cell.

The following experiment was carried out to determine whether methylamine alters the binding of diphtheria toxin

![Fig. 1. The effects of methylamine on the toxicities of ricin, diphtheria, and PA toxins.](image-url)
or ricin toxin to their receptors. The cells were incubated with diphtheria or ricin toxin for 1 h at 4°C in the presence or absence of methylamine (20 mM). They were then washed with cold BSS containing 0.2 mM CaCl2 to remove unbound toxin and methylamine, and incubated for 2 h at 37°C in fresh medium. Then, they were labeled with [3H]leucine, and the rate of protein synthesis was measured as described under "Experimental Procedures." The result showed that incubation with diphtheria or ricin toxin at 4°C and then without toxin at 37°C caused a decrease in protein synthesis in the cells. The presence of methylamine during incubation with diphtheria or ricin toxin at 4°C had no effect, suggesting that methylamine does not alter the initial interaction of these toxins with their receptors.

We also studied the associations of 125I-toxins with the cells, using Vero cells. In Vero cells, methylamine inhibited the action of diphtheria toxin and stimulated that of ricin toxin. Experiments were performed both at 4 and 37°C. At 4°C, the toxins are not internalized, and so the initial interactions between toxins and their binding sites on the cell surface can be observed. At 37°C, internalization occurs and then the toxins within the cell are degraded and excreted. Thus, the complete process can be observed.

The interaction of 125I-diphtheria toxin with Vero cells was studied in the presence or absence of methylamine. When the cells were cultured with 125I-diphtheria toxin at 4°C, the radioactivity associated with the cells increased slowly, reaching a maximum in 12 h as reported by Middlebrook et al. (25).

Methylamine had no effect on this association. Scatchard analysis (27) also demonstrated that methylamine did not change the number of diphtheria toxin-binding sites or their affinity for the toxin.

However, when the cells were cultured with 125I-diphtheria toxin at 37°C, the association profile of the toxin was changed by methylamine. In the absence of methylamine, the amount of radioactivity associated with the cells increased for 1 to 2 h and then decreased as described previously (25), while in the presence of methylamine, the amount of radioactivity increased with time, reaching a maximum in about 4 h (Fig. 2). Less than 20% of the total radioactivity associated at 37°C in the presence of methylamine was removed by trypsin treatment of the cells, whereas more than 90% of the radioactivity associated at 4°C was removed by this treatment. These facts suggest that the toxin mainly accumulates in the interior of the cells when they were cultured with diphtheria toxin at 37°C in the presence of methylamine. Similar results were reported on the effect of chloroquine (17). The radioactivity accumulated in the cells during incubation for 4 h with methylamine rapidly decreased when methylamine was removed from the culture medium. These results show that methylamine did not prevent the initial interaction of diphtheria toxin with the receptor, but inhibited part of the following processes, including internalization, degradation, and excretion. The accumulation of radioactivity within cells induced by methylamine could result from new synthesis of toxin receptor and from failure of subsequent processing of the toxin in the cells.

The interaction of 125I-ricin toxin with Vero cells was also studied. The cells were incubated with 125I-ricin toxin at 4°C in the presence or absence of methylamine and then the radioactivity associated with the cells was measured. The association profile was not changed by methylamine. Scatchard analysis again showed that the number and affinity of ricin toxin receptors were not changed by methylamine. Therefore, methylamine seems to act on the process of internalization of ricin toxin. In the case of ricin toxin, the association kinetics of the toxin at 37°C exhibits a simple bimolecular reaction pattern. Addition of methylamine to the cultures resulted in a slight increase in radioactivity associating with
the cells. This increase, however, cannot explain the stimulation of the action of ricin toxin by methylamine as measured by inhibition of protein synthesis.

To examine what is responsible for the difference in the effects of methylamine on the actions of diphtheria and ricin toxins, we formed two kinds of hybrid toxin and studied the effects of methylamine on their toxicities. The hybrids were constructed by combination of a subunit of WF lectin with the A chain of ricin toxin (WFL-RA) and with fragment A of diphtheria toxin (WFL-DA) (14). The toxicities of both hybrid toxins were stimulated by methylamine (Fig. 3). This result confirms that methylamine does not directly affect fragment A of diphtheria toxin. It also suggests that the stimulatory or inhibitory effect of methylamine on the toxic actions is determined, not by polypeptides A, but by polypeptides B.

DISCUSSION

As shown here, methylamine inhibits the action of diphtheria toxin but stimulates that of ricin toxin. Experimenting with a cell-free protein-synthesizing system and studies on toxin-receptor interactions revealed that methylamine alters the process of entry of these toxins into the cytoplasm. This implies that the entry pathways of these toxins, or at least part of these pathways, are different. Studies on hybrid toxins showed that polypeptides B are important for determining whether methylamine stimulates or inhibits the actions of the toxins. This also suggests that the two toxins have different kinds of ligands which are transported into the cell by separate pathways. On the basis of this idea, WF lectin seems to be a kind of ligand which is transported into the cell by separate processes. On the basis of this idea, WF lectin seems to be a kind of ligand which is transported into the cell by separate processes.

Methylamine seems to interact with cells at multiple sites, since it inhibits the clustering of α-macroglobulin and epidermal growth factor (15), the activity of the enzyme transglutaminase (29), and protein degradation (30). Chloroquine has also been reported to cause a variety of cellular changes (31, 32). Therefore, the different effects of methylamine on diphtheria and ricin toxins may be due to separate actions of this drug. The contrary effect of high and low concentrations of methylamine on PA toxin supports this possibility. It has also been suggested that proteolytic cleavage of diphtheria toxin at a specific site in fragment B is necessary for fragment A to reach the cytoplasm (33). Methylamine may inhibit this proteolytic cleavage of diphtheria toxin, although such an effect cannot explain its stimulation of the action of ricin toxin.

Recently, we and others showed that 1 molecule of diphtheria or ricin toxin can kill a cell (7, 34). We also reported that only about 300 molecules of fragment A-176 were actively imported into the cytoplasm when cells were incubated for 2 h with CRM176 at very high concentration (400 μg/ml of medium) (7, 35). Therefore, only small fractions of the toxin bound to cell-surface receptors seem to be imported into the cytoplasm to exert biological activity. This consideration means that the bulk internalization of the toxin should be distinguished from internalization of the small number of molecules that actually inhibit protein synthesis. Although the incorporation of diphtheria and ricin toxins into the cell have been observed ultrastructurally (36) and by tracing radiolabeled toxin (37), these methods cannot distinguish physiologically significant amounts of entry of the toxins from bulk internalization. Thus, another approach is necessary. Hybrid toxic molecules will be helpful in studies on toxin entry.

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