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Gliotoxin: Inhibitor of Poliovirus RNA Synthesis That Blocks the Viral RNA Polymerase $3D^{pol}$

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The mode of action of gliotoxin against poliovirus has been analyzed in detail. This fungal metabolite inhibits the appearance of poliovirus proteins when present from the beginning of infection but has no effect on viral translation when added at late times. In agreement with previous findings, this toxin potently inhibited the incorporation of [^{3}H]uridine into poliovirus RNA soon after its addition to the culture medium. Analysis of the synthesis of poliovirus plus- or minus-stranded RNA in the presence of gliotoxin suggests that this compound effectively hampered both processes. This result contrasts with the mode of action of other inhibitors of poliovirus RNA synthesis, such as guanidine or flavones, that selectively block plus-stranded RNA synthesis and suggests that the target of gliotoxin differs from the target of guanidine, i.e., poliovirus protein 2C. Indeed, gliotoxin was found to be a potent inhibitor of poliovirus RNA synthesis in cell-free systems, using membranous crude replication complexes, a reaction that is not blocked by guanidine or Ro 09-0179. Moreover, in vitro activity of the purified poliovirus polymerase $3D^{pol}$ was efficiently inhibited by gliotoxin. These results indicate that this toxin acts on the poliovirus polymerase $3D^{pol}$, providing the first description of an inhibitor of this viral enzyme.

Picornaviruses are among the simplest and best-known animal viruses in molecular terms (10). Despite their simplicity, several aspects of their replicative cycle remain obscure (3, 11, 16, 25). Hopefully, detailed studies of the mode of action of selective inhibitors of picornavirus replication will provide a better understanding of those processes. Although the main steps in the replication of poliovirus RNA have been delineated, there are still some reactions that remain puzzling (25). Thus, it is known that once the genomic RNA is free in the cytoplasm, it is first translated to give rise to an array of viral proteins (3, 11, 16). An unknown number of these viral polypeptides use the plus-stranded genomic RNA as a template to make a complementary minus-stranded RNA (19, 25). This negative-stranded RNA is used, in turn, as a template to synthesize more copies of positive-stranded RNA molecules that can fulfill several requirements, i.e., they can be used as mRNAs to make viral proteins or as templates to make more copies of negative-stranded RNA, or they can be encapsidated to generate mature virions (19, 25).

The virus-encoded RNA-dependent RNA polymerase, known as protein $3D^{pol}$, is able to elongate already initiated plus- or minus-stranded RNA molecules (24, 25). It was suggested that the genome-linked protein VPg (viral protein 3B), was used as a primer to initiate new chains of viral RNA (19, 20). However, recent evidence indicates that this protein is covalently linked to the RNA well after the initiation event (22). Therefore, the exact mechanism of initiation of picornavirus RNA synthesis still remains controversial. Moreover, viral RNA replication complexes are tightly bound to membranes, and the inhibition of membrane formation leads to immediate arrest of poliovirus RNA synthesis (8). Perhaps viral protein 2C or its precursor 2BC plays a part in the coupling between the RNA replication complex and the membranous vesicles that proliferate throughout infection (1). A role for protein 2C in poliovirus RNA replication was suspected on the basis that the selective inhibitor of poliovirus RNA synthesis, guanidine, acted on protein 2C (17, 25). Apart from that finding, the exact role of protein 2C in RNA synthesis is obscure. It is even possible that other viral proteins, such as 2B or 3A, are also involved in the replication of viral genomes (19, 25). Therefore, a better understanding of the mode of action of inhibitors of poliovirus RNA replication can help to unravel this process.

In addition to guanidine, which has been the classic inhibitor of poliovirus RNA synthesis (21), other agents have been reported to selectively block the step of viral RNA replication. Thus, some natural flavones, such as 3-methylquercetin (4, 5), or Ro 09-0179 (7, 9), selectively block the synthesis of plus-stranded RNA without inhibiting minus-stranded RNA synthesis at concentrations 1,000-fold lower than those for guanidine (7). 2-(α-Hydroxybenzyl)-benzimidazole (HBB) (6), penicillamine (14), and gliotoxin (15) also block viral RNA replication, but the detailed mechanism of action of those agents is still unknown. High concentrations of penicillamine (above 7 mM) or HBB (above 0.1 mM) are required to block poliovirus (6, 14). Gliotoxin and a number of related compounds, either from natural sources or chemically synthesized, are the most potent agents that interfere with poliovirus RNA synthesis (15, 23). We undertook an analysis of the mode of action of this toxin, and the results presented suggest that it differs from the effect of known poliovirus RNA inhibitors, such as guanidine and the flavones.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were grown in Dulbecco modified Eagle's medium supplemented with 10% newborn calf serum (GIBCO, Grand Island, N.Y.) and incubated at 37°C in a 5% CO_2 atmosphere. Poliovirus type 1 (Mahoney strain) (American Type Culture Collection) was grown in HeLa cells. Cell culture and virus titration were as previously described (7).

Compounds. 4'-5-Dihydroxy-3-3',7-trimethoxyflavone (Ro-

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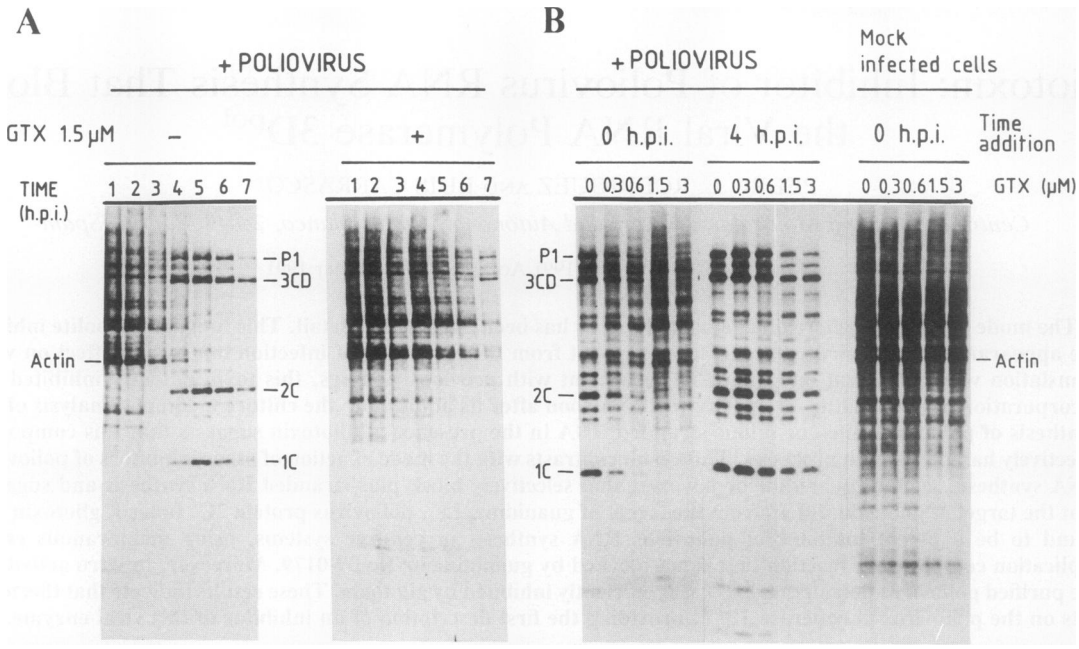

FIG. 1. Effect of gliotoxin on translation. (A) Time course of the proteins synthesized in poliovirus-infected cells minus (−) or plus (+) 1.5 μM gliotoxin (GTX) from zero time. HeLa cells grown in 24-well plates were infected with poliovirus at an MOI of 50 PFU per cell. After a 60-min adsorption period, cells were labeled every hour with 20 μCi of [^{35}S]methionine per ml for 1 h. Proteins were analyzed on a 15% polyacrylamide-SDS gel as indicated in Materials and Methods. (B) Analysis of the proteins synthesized in poliovirus-infected cells or mock-infected cells between 4 to 5 h p.i. Different concentrations of GTX were added at 0 or 4 h p.i.

09-0179) was kindly provided by H. Ishitsuka and K. Yokose, Nippon Roche Research Center (Kamamura, Japan). Gliotoxin, guanidine, and dactinomycin were purchased from Sigma Chemical Co., St. Louis, Mo.

Virus infection of cell monolayers. HeLa cells were infected with poliovirus (−1 h) in Dulbecco modified Eagle's medium supplemented with 2% newborn calf serum. After incubation at 37°C for 1 h, unattached virus was removed and fresh medium was added (zero hours).

Polyacrylamide gel analysis of proteins. At the times indicated, cells were incubated in methionine-free medium in the presence of 20 μCi of [^{35}S]methionine (1,450 Ci/mmol; Amersham) per ml. One hour later the radioactive medium was removed, the cell monolayer was washed with phosphate-buffered saline, and cells were collected in 200 μl of buffer containing 62 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol, 17% glycerol, and 0.0024% bromophenol blue. Each sample was sonicated to reduce viscosity and heated at 90°C for 5 min. Five microliters was applied to a 15% polyacrylamide gel and run overnight at 100 V. Fluorography of the gel was carried out with 2,5-diphenyloxazole–dimethyl sulfoxide (20% wt/vol).

Estimation of uridine incorporation in RNA. HeLa cells grown in 24-well plates were infected with poliovirus at a multiplicity of infection (MOI) of 50 PFU per cell. Dactinomycin (5 μg/ml) was added at zero hours. Every hour after infection, 10 μCi of [^{3}H]uridine (25 to 30 Ci/mmol; Amersham) per ml was added. The labeling medium was removed after 1 h, and the cell monolayer was treated with 0.5 ml of 5% trichloroacetic acid, washed twice with ethanol, dried under an infrared lamp, and dissolved in 200 μl of 0.1 N NaOH–1% SDS. Samples of 150 μl were counted in a liquid scintillation spectrophotometer.

Synthesis of labelled RNA probe. The poliovirus strand-specific RNA probe was generated by in vitro transcription of a fragment of poliovirus cDNA encompassing nucleotides 2099 to 4600, subcloned in a Bluescript vector (Stratagene). The poliovirus cDNA sequence was flanked by T7 and T3 promoters. Using T7 (New England Biolabs) or T3 RNA polymerase (Stratagene), we obtained RNA probes specific to the viral minus- or plus-stranded poliovirus RNA, respectively. The probes were labeled with [^{32}P]CTP (400 Ci/mmol; Amersham).

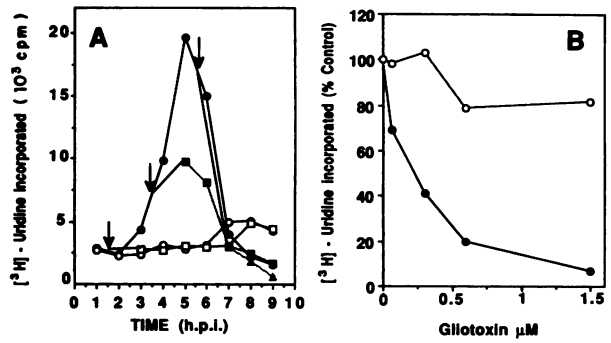

FIG. 2. Inhibition of poliovirus and cellular RNA synthesis by gliotoxin. (A) Time course of poliovirus RNA synthesis. Conditions of infection and analysis of RNA synthesis were as described in Materials and Methods. Poliovirus-infected cells in the absence of inhibitor (●) or in the presence of 1.5 μM gliotoxin from zero time (○) or at 1.5 (□), 3.5 (■), or 5.5 (▲) h p.i. Pulse-labeling with [^{3}H]uridine was carried out every hour p.i. (B) Effect of different concentrations of gliotoxin on RNA synthesis in mock-infected cells in the absence of dactinomycin (○) or in poliovirus-infected cells in the presence of dactinomycin (●). RNA synthesis was determined between 4 to 5 h p.i. Gliotoxin was added at 1.5 h p.i.

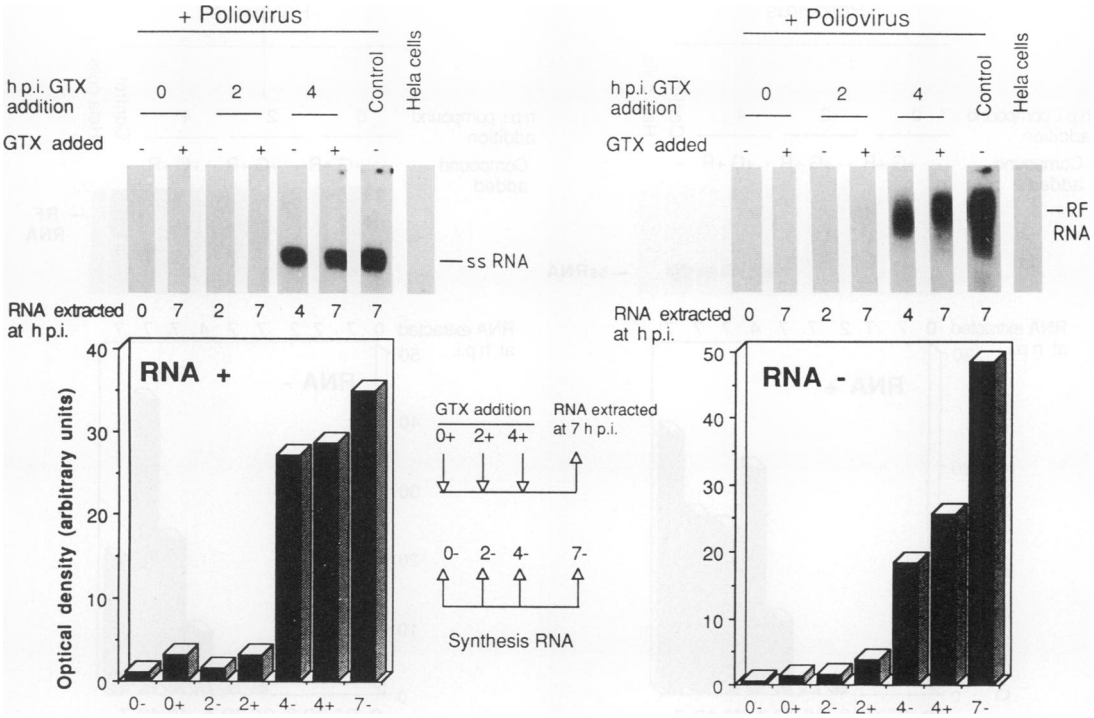

FIG. 3. Effect of gliotoxin (GTX) on the synthesis of poliovirus plus- or minus-stranded RNA. HeLa cells grown in 60-mm-diameter dishes were infected with poliovirus at 50 PFU per cell. Total RNA was extracted from untreated (−) cells at 0, 2, 4, or 7 h p.i. Total RNA from cells treated with 1.5 μM gliotoxin (+) was extracted at 7 h p.i. The inhibitor was added at the indicated times, and incubation continued until the 7th h. The RNA was extracted and analyzed by Northern blotting as indicated in Materials and Methods. The same blot was hybridized to positive-stranded (RNA−) and negative-stranded (RNA+) poliovirus riboprobes.

Northern (RNA) blot analysis of poliovirus RNA. HeLa cells were grown in 60-mm-diameter dishes and infected with poliovirus at an MOI of 50 PFU per cell. At the indicated times after infection, the cells were lysed in a buffer containing 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 150 mM NaCl, and 0.65% Nonidet P-40. After removing nuclei by low-speed centrifugation, supernatants were mixed with an equal volume of a buffer containing 20 mM Tris-HCl (pH 7.8), 20 mM EDTA, 350 mM NaCl, and 1% SDS. Total RNA was extracted with phenol and precipitated with ethanol. Northern blot analysis with a radiolabeled probe was performed (7). The quantitation was done with a 3,000-A computing densitometer (Molecular Dynamics).

In vitro synthesis of poliovirus RNA. HeLa cells were infected with poliovirus at an MOI of 200 PFU per cell, incubated at 37°C, and harvested at 7 h postinfection (p.i.). Preparation of the crude membrane fraction and its treatment with DEAE cellulose were performed as previously described (20, 25). In vitro RNA synthesis was carried out in a reaction mixture containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 8), 3.5 mM magnesium acetate, 50 mM KCl, 10 μg of dactinomycin per ml, 2.5 mM phosphoenol pyruvate, 2 μg of pyruvate kinase, 10 μg of DEAE-cellulose-treated membranes, and compounds at the concentrations indicated in each experiment. This mixture was incubated for 30 min at 4°C. Then, 50 μCi of [^{3}H]ATP (56 Ci/mmol) per ml–1 mM UTP–1 mM CTP–1 mM GTP–100 μM ATP was added, and incubation continued at 30°C for various periods of time. The reaction was stopped by the addition of 10% trichloroacetic acid at 4°C. The RNA was collected on Whatman GF/C fiber filters, and the radioactivity incorporated in RNA was determined in a scintillation counter.

Enzymatic assay of poliovirus RNA polymerase. Purified poliovirus RNA polymerase was kindly provided by S. J. Plotch (Lederle Laboratories, Pearl River, N.Y.). Poly(A)-dependent oligo(U)-primed poly(U) polymerase activity was measured at 30°C for 60 min as previously described (18), with the modifications that dithiothreitol (DTT) was not added and [^{3}H]UTP was used as the labeled nucleotide (45 Ci/mmol; Amersham).

RESULTS

Effect of gliotoxin on poliovirus protein synthesis. Gliotoxin, a fungal metabolite, inhibits the growth of several animal viruses, including picornaviruses. Earlier evidence indicated that it was a selective inhibitor of picornavirus RNA synthesis and had less effect on protein synthesis when added later during infection (15, 23). However, those studies only relied upon the incorporation of radioactive precursors into trichloroacetic acid-precipitable material. Thus, it was of interest to examine the effects of gliotoxin on cellular and viral protein synthesis and to analyze the proteins synthesized by polyacrylamide gel electrophoresis. Concentrations of gliotoxin above 0.6 μM are effective in the inhibition of viral protein synthesis (Fig. 1B) if present from the beginning of infection, but no effect was observed on viral translation when gliotoxin was added 4 h p.i. Gliotoxin concentrations of 3 μM had almost no effect on cellular protein synthesis. On the other hand, although gliotoxin effectively reduced the synthesis of viral proteins, it was ineffective in preventing

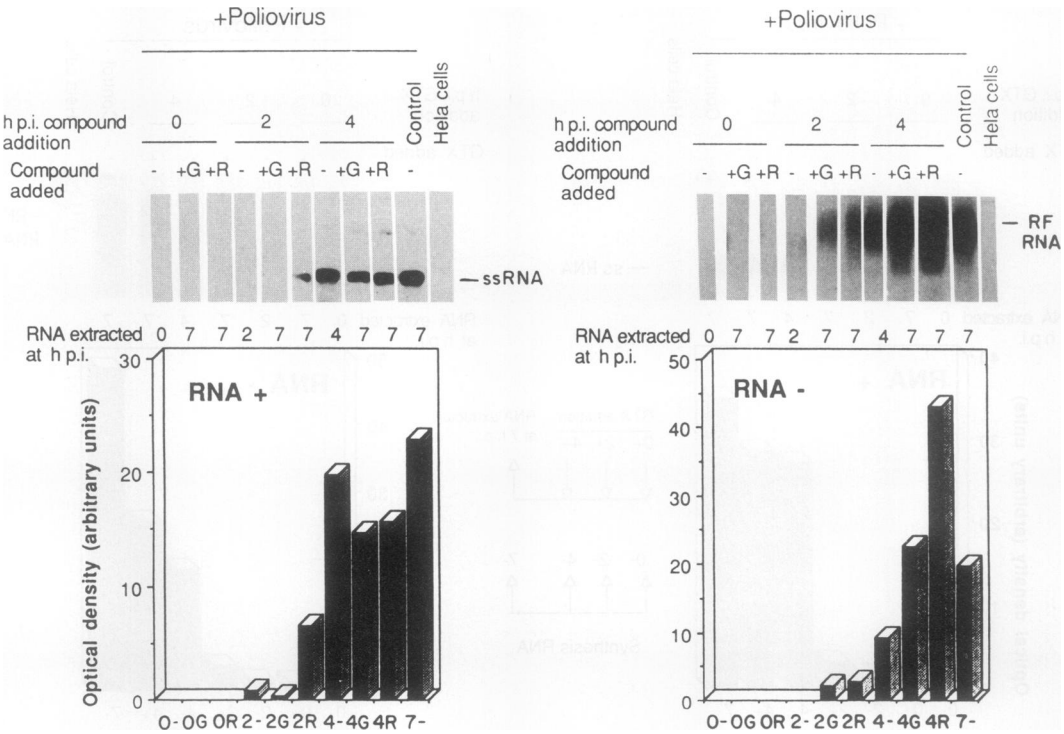

FIG. 4. Effect of guanidine and Ro 09-0179 on the synthesis of poliovirus plus- or minus-stranded RNA. The experiment was carried out as described in the legend to Fig. 3, except that 3 mM guanidine (+G) or 3 μM Ro 09-0179 (+R) was added instead of gliotoxin.

the shutoff of host translation induced by the infection of poliovirus at high multiplicities (Fig. 1A). These results are similar to those found for other inhibitors of poliovirus RNA replication (5, 7).

Action of gliotoxin on viral RNA synthesis. Figure 2 shows the ability of gliotoxin to block poliovirus RNA synthesis when added at different times p.i. When gliotoxin (1.5 μM) was added at zero time or 1.5 h after infection, viral RNA synthesis was completely blocked. Addition of the drug at 3.5 h p.i. significantly reduced the peak of viral RNA synthesis observed at 5 h (Fig. 2A). A concentration of gliotoxin of 0.6 μM inhibited poliovirus RNA synthesis in infected cells by 80%, whereas cellular RNA synthesis was only slightly affected by 1.5 μM gliotoxin. These results agree well with previous findings on gliotoxin activity. In a similar assay, the flavone Ro 09-0179 was twofold less efficacious, whereas concentrations of 200 μg of HBB per ml or 1.5 mM guanidine were required to block poliovirus RNA synthesis by 80% (results not shown).

We found recently that flavones, such as 3-methylquercetin or Ro 09-0179, were selective inhibitors of poliovirus plus-stranded RNA synthesis, whereas the formation of minus-stranded RNA was even stimulated in the presence of Ro 09-0179 (7). To test the action of gliotoxin on plus- or minus-stranded RNA synthesis, specific riboprobes were made and the formation of plus- or minus-stranded poliovirus RNA was measured throughout infection. In parallel, the inhibitor gliotoxin was added at different times p.i. and the RNA was extracted at the end of poliovirus infection (7 h p.i.). Figure 3 shows that gliotoxin efficiently interferes with the synthesis of both plus- and minus-stranded RNA chains when present at any time p.i. It is of particular interest to focus on the inhibition induced by gliotoxin at the 4th h p.i. At that time most of the plus-stranded RNAs have already been synthesized, but the increase in plus-stranded RNA from 4 to 7 h is hampered by gliotoxin. On the other hand, most viral minus-stranded RNA is made from the 4th to the 7th h p.i. This increase in negative RNA is also blocked by the toxin. In a similar assay, guanidine had no effect on negative RNA synthesis, whereas Ro 09-0179 increased its formation by 200% (Fig. 4).

Inhibition of in vitro poliovirus RNA synthesis. None of the available inhibitors of poliovirus RNA synthesis is effective in cell-free systems. A system that faithfully synthesizes both poliovirus plus- and minus-stranded RNA in vitro has been developed by purifying the replicative complexes bound to membranes, the so-called crude replication complex (CRC) (25). Curiously enough, gliotoxin was a potent inhibitor of this reaction (Fig. 5A). Gliotoxin (750 μM) inhibited RNA synthesis in CRC by 60%, whereas guanidine and Ro 09-0179 had little or no effect (Fig. 5A). The RNA made in these systems by crude replicative complexes mainly comes from elongation carried out by the polymerase $3D^{pol}$, although initiation of new chains in such systems has been described previously (19, 25). Thus, although gliotoxin is the first compound shown to inhibit poliovirus RNA synthesis in the CRC, it cannot be concluded that it acts on polymerase $3D^{pol}$. In order to test this possibility, the effect of gliotoxin was assayed in cell-free systems that used purified 3D polymerase (18). Figure 6A, shows that the incorporation of [^{3}H]UTP directed by poly(A) in the presence of oligo(U) is also blocked by gliotoxin. The inhibition of this reaction by gliotoxin is more potent than the blockade of the CRC. Thus, 150 μM gliotoxin blocked the incorporation of [^{3}H]UTP in this assay by 80%. On the other hand, this inhibition was specific for gliotoxin, since other inhibitors of poliovirus RNA synthesis, such as guanidine or R 09-0179, had no effect (Table 1). The presence of a reducing agent

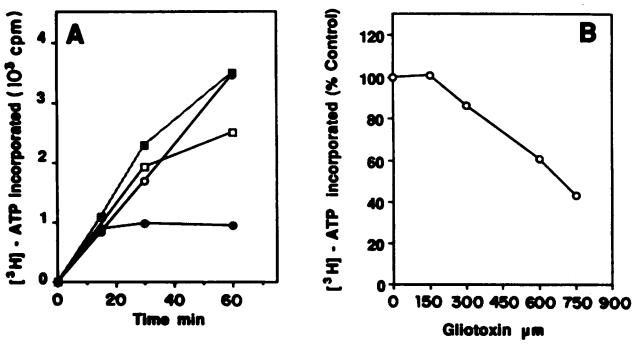

FIG. 5. Effect of gliotoxin, guanidine, and Ro 09-0179 on in vitro synthesis of poliovirus RNA. (A) Preparation of the crude membrane fraction and in vitro RNA synthesis were carried out as described in Materials and Methods. The DEAE-cellulose-treated membranes were incubated at 30°C for various periods of time in the absence of inhibitor (○) or in the presence of 750 µM gliotoxin (●), 30 mM guanidine (□), or 750 µM Ro 09-0179 (■). (B) Effect of different concentrations of gliotoxin on in vitro RNA synthesis.

TABLE 1. Effect of inhibitors of poliovirus RNA synthesis on in vitro 3D$_{pol}$ activity[a]

Incubation conditions	[^{3}H]UTP incorporated	
	cpm	% Control
Time, 0 min	373	0
Time, 60 min + 5 mM DTT	20,063	95
Time, 60 min		
Minus DTT	21,075	100
Minus 3D$_{pol}$	524	0.7
Minus poly(A)	423	0.3
Minus oligo(U)	2,134	9
Plus 2% dimethyl sulfoxide	20,468	97
Plus 30 mM guanidine	16,973	80
Plus 750 µM Ro 09-0179	22,676	107
Plus 600 µM gliotoxin	1,697	6
Plus 600 µM gliotoxin + 5 mM DTT	7,291	33

[a] Poly(U) polymerase activity was measured at 30°C for 60 min as described in the legend to Fig. 6. Inhibitors were added at zero time. Data are the means of duplicate samples.

such as DTT prevents the inhibition of poliovirus RNA synthesis in intact cells (23), suggesting that the blockade by gliotoxin involves the formation of disulfide bridges between gliotoxin and essential sulfhydryl groups on the viral polymerase. The data of Table 1 indicate that the presence of DTT in the cell-free system partially reduced its inhibitory potential, in agreement with the idea that reducing the sulfhydryl groups abolishes the activity of gliotoxin (23).

DISCUSSION

Elucidating the exact steps involved in replication of poliovirus RNA is an important goal. Although the major biochemical events are known, there are still a number of steps that remain obscure (3, 19, 25). Thus, the exact mode of initiation of viral plus- and minus-stranded RNA synthesis, the coupling between membranes and replication complexes, and the identification of host proteins, if any, involved in this process need to be clarified (8, 19, 22). Elucidation of some of these processes at the molecular level will be facilitated by the use of selective inhibitors of viral RNA synthesis. In order to use these inhibitors properly we need first to carry out detailed studies of their mode of action.

Few inhibitors of picornavirus RNA synthesis are available at present (2). Only the modes of action of guanidine and some flavones are partially known (7, 21). These compounds inhibit poliovirus positive-stranded RNA synthesis (7). Flavones stimulate the synthesis of minus-stranded RNA in infected cells by a still unknown mechanism (7). The poliovirus protein target of flavone's action still remains to be elucidated. In fact, the only known target for an inhibitor of poliovirus RNA synthesis is protein 2C, which is the site of action of guanidine (17). This constitutes the major evidence implicating this protein in viral RNA synthesis. Otherwise, the exact role of protein 2C in poliovirus RNA replication is still puzzling (13, 25). This protein may be involved in the physical connection between membranes and the viral replication complexes (1). Of interest in this respect is the recent discovery of helicase activity in a potyvirus protein similar to that of poliovirus 2C (12).

Gliotoxin is certainly the most powerful inhibitor of poliovirus RNA synthesis in cultured cells (15, 23). The action of gliotoxin differs from the effect of guanidine and flavones, since these two agents preferentially inhibit the synthesis of poliovirus positive-stranded RNA (7). On the contrary, gliotoxin does not show this selectivity and interferes with the synthesis of both minus- and plus-stranded RNA synthesis. Our studies of the effect of gliotoxin in cell-free synthesis clearly show that it is a potent and unique inhibitor of poliovirus RNA synthesis in these systems. Moreover, gliotoxin inhibited the activity of purified 3D^{pol}. Therefore, two agents that block two different poliovirus proteins involved in RNA replication are now available: a compound that interferes with the activity of poliovirus protein 2C, guanidine, and another one that blocks protein 3D^{pol}, gliotoxin. Future studies of the action and the characterization of the target for the other inhibitors of poliovirus RNA synthesis will complete our understanding of the effects of these agents.

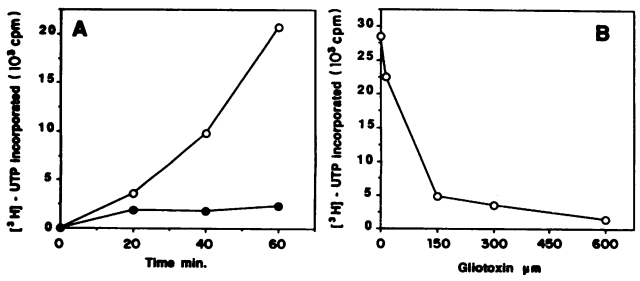

FIG. 6. Effect of gliotoxin on in vitro poly(U) polymerase activity. (A) Enzymatic assay of poliovirus RNA polymerase was carried out as described in Materials and Methods. Poly(U) polymerase activity was measured for various periods of time in the absence (○) or presence (●) of 600 µM gliotoxin. (B) Effect of different concentrations of gliotoxin on poly(U) polymerase activity.

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