Antiviral Research, 13 (1990) 81–90
Elsevier

AVR 00400

Inhibition of the RNA polymerase of vesicular stomatitis virus by ppp5′A2′p5′A and related compounds

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(Received 14 July 1989; revision accepted 6 November 1989)

Summary

The diadenylate triphosphates ppp5′A2′p5′A and ppp5′A3′p5′A were found to inhibit the purified RNA polymerase ('nucleocapsid') complex from vesicular stomatitis virus (VSV). The corresponding diadenylate monophosphate p5′A2′p5′A did not inhibit, nor did the triadenylate triphosphate ppp5′A2′p5′A2′p5′A; the diadenylate diphosphate pp5′A2′p5′A had intermediate inhibitory activity. Increasing the concentration of ATP, GTP or CTP in the reaction mixture decreased inhibition by ppp5′A2′p5′A, while UTP had minimal or no protective effect. ppp5′A2′p5′A did not protect the RNA polymerase from inactivation by *N*-ethylmaleimide. This suggests that the action of ppp5′A2′p5′A occurs at a site on the enzyme that is distinct from the *N*-ethylmaleimide-protecting, ATP-binding site characterized previously.

2–5 As; Dinucleotide; Vesicular stomatitis virus; RNA polymerase; Inhibition of RNA synthesis

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Introduction

One well-established effect of interferons on cells is the production of a series of low molecular weight compounds, the 2′,5′-oligoadenylates (2–5A's; Johnston and Torrence, 1984; Lengyel, 1982). These molecules are formed in interferon-treated cells of many types in the presence of double-stranded RNA. The 2–5A's, in turn, activate a latent ribonuclease which is thought to exert antiviral activity by digestion of viral mRNAs. Only the trimer and higher oligomers activate this endonuclease; the dimer, which is the member of the series often produced in greatest amount (Samantha and Lengyel, 1986), is inactive. No other antiviral functions of the 2–5A's are known (Johnston and Torrence, 1984; Lengyel, 1982).

It was therefore especially noteworthy when the smallest member of the 2–5A series, the dimer ppp5′A2′p5′A, was reported to inhibit production of VSV in infected HeLa cells (Alarcon et al., 1984). Inhibition of viral protein synthesis was observed, with concomitant protection from virus-induced cytopathic effects. The compound had to be present during the first hour after viral infection, but did not affect VSV binding to the cell surface or internalization, suggesting an effect on an early step in viral transcription (Alarcon et al., 1984).

In the present study we have examined the inhibitory effects of ppp5′A2′p5′A and several related compounds on the activity of the purified RNA polymerase of VSV (the 'nucleocapsids').

Materials and Methods

Cells, viruses and nucleocapsids

Nucleocapsids were prepared from VSV, Indiana serotype, Glasgow strain, tsG31 (Pringle, 1975), originally provided by Dr Alice Huang. This strain was chosen because its altered M protein was expected to minimize possible inhibition due to small amounts of residual M protein remaining in the nucleocapsid preparation (Morita et al., 1987; Wilson and Lenard, 1981). VSV was grown on BHK 21F cell monolayers infected at MOI=1, harvested after 24 h and purified on sucrose gradients as described previously (Miller and Lenard, 1980). Gradient purified VSV was diluted with Dulbecco's phosphate buffered saline and pelleted. After disruption with detergent, nucleocapsids were purified by centrifugation on a discontinuous glycerol gradient (Carroll and Wagner, 1979). Protein concentrations were determined using a modified Lowry procedure (Bensadoun and Weinstein, 1976).

ppp5′A2′p5′A and related compounds

The oligonucleotides ppp5′A2′p5′A, pp5′A2′p5′A, ppp5′A2′p5′A2′p5′A, and p5′A2′p5′A all were prepared by lead (II) ion-catalyzed oligomerization of adenosine 5′-phosphoroimidazolidate followed, when necessary, by conversion to the 5′-diphosphate or 5′-triphosphate by reaction of the appropriate oligonucleotide

5′-phosphoroimidazolidate with either tri-*n*-butylammonium phosphate or pyrophosphate as required according to methodology reported previously (Imai and Torrence, 1985; Torrence et al., 1984, 1986). Purity was judged to be >95% as determined by HPLC using a Bondapack C18 column (3.9 × 300 mm) and an elution gradient of 0–100% solvent B in solvent A in 30 min followed by 100% solvent B for 10 min [solvent A: 50 mM ammonium phosphate, pH 7.0; solvent B: methanol-water, 1:1; both solvents also contained 0.005 M tetrabutylammonium phosphate (PIC A, Waters Associates, Milford, MS)]. For pp5′A2′p5′A, a different elution program was employed: in this case, solvent A was 0.06 M KH_2PO_4, pH 4.75, 0.005 M PIC A and solvent B was 75% methanol in water containing 0.005 M PIC A. The program was 100–0% solvent A in solvent B in 30 min followed by 10 min at 100% solvent B. In all cases, the flow rate was 1 ml/min.

Preparation of pppA3′p5′A

This oligonucleotide triphosphate has not been previously reported, so its synthesis is detailed here.

The dimer A3′p5′A (Sigma, St. Louis, MO) was 5′-phosphorylated with T4-polynucleotide kinase (U.S. Biochemicals, Cleveland, Ohio) according to the general procedure reported earlier (Lesiak et al., 1983) in a reaction mixture containing the following: 275 A_{260} units (10.45 μmol) of A3′p5′A, 240 units (240 μl) of T4 kinase (1000 U/ml in 0.05 M Tris-HCl, pH 7.6, 100 mM KCl, 5 mM dithiothreitol, and 200 μg/ml bovine serum albumin), 1200 μl 0.05 M of $MgCl_2$ (final conc., 0.01 M), 720 μl of 0.5 M Tris, pH 8.5 (final conc., 0.06 M), and 900 μl of 100 mM dithiothreitol (final conc., 0.015 M) and 564 μl of 0.266 M ATP (final conc., 27.5 mM). The total reaction volume was 6000 μl. Incubation was allowed to proceed overnight at 37°C. The progress of the reaction was monitored by HPLC using an Altex Ultrasphere ODS column (4.6 mm × 25 cm) and a 30 min gradient (flow rate, 1.0 ml/min) from 50–0% solvent A in solvent B (B: methanol-water, 1:1 v/v; A: 0.02 M ammonium phosphate, pH 7.0). The eluate was monitored at 260 nm. Retention times were as follows: A3′p5′A, 29.31 min; p5′A3′p5′A, 14.78 min. The reaction was complete after overnight incubation. In order to remove the excess of ATP, the entire reaction mixture was applied to a DEAE-Sephadex column (1 × 40 cm) and the column was eluted with a linear gradient of 0–0.4 M triethylammonium bicarbonate buffer, pH 7.5, total volume 1000 ml. The final separation from ADP was accomplished by means of HPLC using a semi-preparative Altex Ultrasphere ODS column (10 × 25 cm) and a 2 ml/min flow rate.

After HPLC purification, the 3′,5′-dimer 5′-monophosphate was desalted by first employing the barium salt precipitation procedure, followed by application to and elution from a DEAE-Sephadex A-25 column (1 × 40 cm) using a linear gradient of 0.05–0.4 M triethylammonium bicarbonate buffer, pH 7.5, total volume 1000 ml. After repeated in vacuo evaporation to remove buffer, a yield of 214.7 A_{260} units of p5′A3′p5′A was obtained corresponding to 78% of theoretical.

Nuclease P_1 treatment of the above product gave 5′AMP as the sole product. Digestion with alkaline phosphatase gave only one uv-absorbing product identical

with starting A3'p5'A. In all cases the digestion products were identified by comparison with authentic samples by HPLC.

The 5'triphosphate of A3'p5'A was prepared by first converting the 5'-monophosphate to the 5'-phosphoroimidazolidate by a modification of the method of Mukaiyama and Hashimoto (Mukaiyama and Hashimoto, 1971; Torrence et al., 1988). The triethylammonium salt of the 5'-monophosphate of A3'p5'A (78 A_{260} units, 0.003 mmol) was dried by repeated addition and evaporation of dry DMF and the residue was dissolved in a mixture of dry DMF (1600 μl) and dry DMSO (160 μl). To this solution triethylamine (50 μl), imidazole (10 mg, 0.16 mmol), triphenylphosphine (16 mg, 0.06 mmol), and 2,2'-dipyridyldisulfide (10 mg, 0.04 mmol) were added. The resulting yellow solution was kept at room temperature, and the reaction progress was followed by tlc. (Kieselgel, 2-propanol-conc. ammonium hydroxide-water, 11/7/2). In this system, the R_f of the imidazolidate was 0.79 while the R_f of the starting material was 0.27. After the reaction was completed (1.5 h), the entire mixture was poured into 20 ml of a 1% solution of sodium iodide in acetone. The resulting precipitate was centrifuged down, washed with dry acetone (3 × 5 ml) and then dried in vacuo for 2 h.

Tri-*n*-butylammonium pyrophosphate (400 μl of a 0.5 M solution in dry dimethylformamide) and 200 μl of dry dimethylsulphoxide were added to the dried imidazolidate and the resulting solution was allowed to remain at ambient temperature for 24 h. Subsequently, the solution was diluted with cold water (6 ml), evaporated, in vacuo, and the residue was taken up in cold water (10 ml). This solution was applied to a DEAE-Sephadex column (1 × 20 cm), and elution was accomplished with a linear gradient of triethylammonium bicarbonate (0.15–0.5 M, pH 7.5, total of 500 ml). Appropriate fractions were combined and evaporated to dryness under reduced pressure at 30°C. The yield of ppp5'A3'p5'A was 45 A_{260} units or 58% of theoretical.

The structure of the 5'-triphosphate was verified by a combination of enzymatic and spectroscopic techniques. When the triphosphate was digested by bacterial alkaline phosphatase, the product was identical to A3'p5'A as determined by HPLC. Snake venom digestion of the putative ppp5'A3'p5'A gave 5'AMP as the sole product by HPLC. ^{31}P nuclear magnetic resonance spectroscopy of ppp5'A3'p5'A was performed in D_2O with 5% H_3PO_4 as external standard. The internucleotide phosphate yielded a resonance at +1.66 ppm integrating to one P, and a typical pattern for the triphosphate was also obtained at −10.85 ppm, −21.84 ppm, and −8.43 ppm, each signal integrating to one P.

RNA polymerase assays

RNA polymerase activity was measured by the production of trichloroacetic acid-precipitable radioactivity as described previously (Massey and Lenard, 1987), except that the concentration of each nucleoside triphosphate (NTP) was varied as indicated in the text and figure legends. Activity was assayed by incubating the following for 2 h at 30°C: 5–10 μg of nucleocapsid protein in a total volume of 50 μl of 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, NTPs

and 2.5 μCi of radiolabeled UTP or CTP. The oligomer ppp5'A2'p5'A and related compounds were added to the assay mixture simultaneously with the NTPs.

Reactions with N-ethylmaleimide (NEM)

Nucleocapsids were incubated in 0.15 mM NEM, 50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl at 0°C for various times in the presence or absence of 220 μM ppp5'A2'p5'A (added simultaneously with the NEM; Massey and Lenard, 1987). The reaction was stopped by addition of excess dithiothreitol, and bovine serum albumin was added to 0.2%. RNA polymerase activity was assayed after passing the treated nucleocapsids through a Bio-Gel P-30 spin column as described (Massey and Lenard, 1987). An average of 85% of nucleocapsid protein was recovered, while ca. 95% of the ppp5'A2'p5'A was retained on the column. Transcriptional activity was corrected for nucleocapsid recovery, based on recovery of trace amounts of added [^{35}S]methionine labeled nucleocapsids.

Results

Fig. 1 shows the effect of several di- and trinucleotide derivatives on the RNA polymerase activity of isolated nucleocapsids. Of the compounds tested, the two diadenylate triphosphates, ppp5'A2'p5'A and ppp5'A3'p5'A are the most effective inhibitors. The diadenylate diphosphate, pp5'A2'p5'A was less effective, while the diadenylate monophosphate p5'A2'p5'A was ineffective, as was the triadenylate triphosphate ppp5'A2'p5'A2'p5'A. The most effective inhibition was thus achieved by a diadenylate triphosphate, regardless of whether the internucleotide linkage was 2'-5' or 3'-5'.

The data shown in Fig. 1 were obtained using 0.1 mM ATP and 0.1 mM GTP

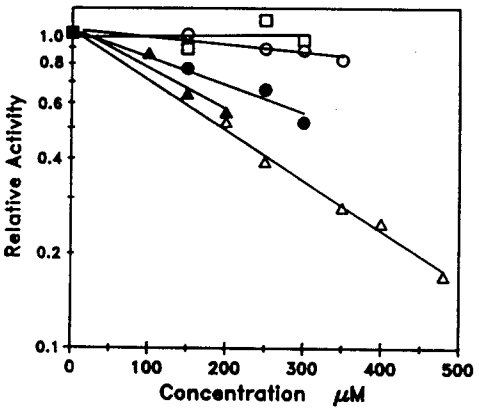

Fig. 1. Inhibition of VSV RNA polymerase activity by: (△), ppp5'A2'p5'A; (▲), ppp5'A3'p5'A; (●), pp5'A2'p5'A; (○), p5'A2'p5'A; (□), ppp5'A2'p5'A2'p5'A. Reaction mixtures contained 0.1 mM ATP, GTP, CTP and UTP.

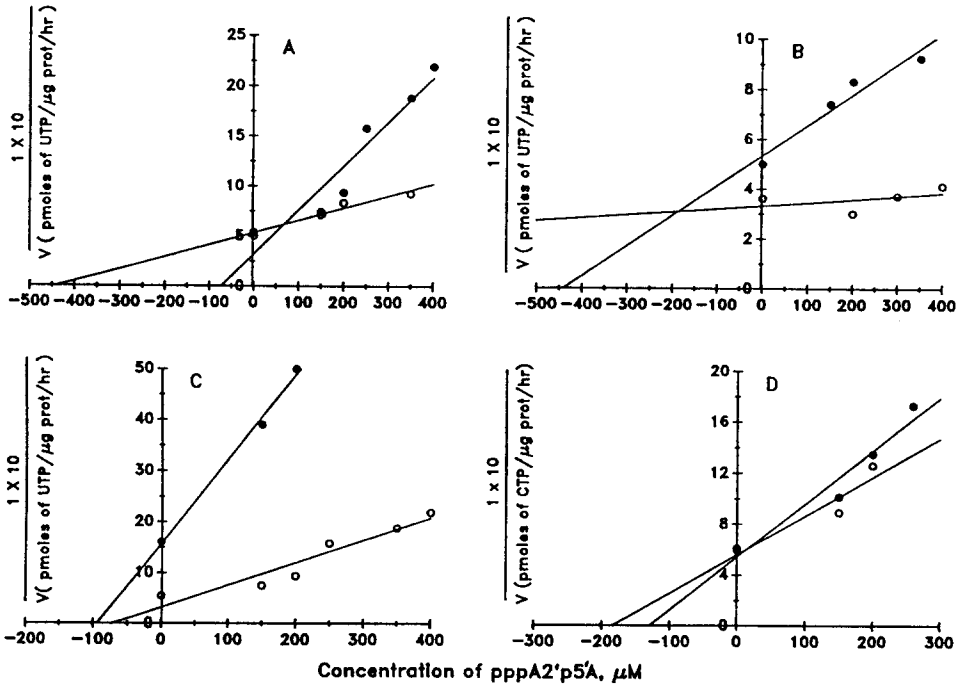

Fig. 2. Protection by NTP's against inhibition by ppp5'A2'p5'A. Reaction mixtures contained: (A), 0.1 mM GTP and UTP, 1 mM CTP and (•) 0.1 mM ATP or (○) 1 mM ATP; (B), 1 mM ATP and CTP, 0.1 mM UTP and (•) 0.1 mM GTP or (○) 1 mM GTP; (C), 0.1 mM ATP, GTP and UTP and (•) 0.1 mM CTP or (○) 1 mM CTP; (D), 0.1 mM ATP, GTP and CTP and (•) 0.1 mM UTP or (○) 1 mM UTP.

in the polymerase reactions, instead of the more usual 1 mM concentrations. Inhibition by ppp5'A2'p5'A was more effective at lower ATP concentrations, as shown by the data in Table 1. In the presence of 0.1 mM ATP and GTP, 50% inhibition required ca. 200 μM ppp5'A2'p5'A (Fig. 1), while 325 μM was required when 1 mM ATP and GTP were used (not shown).

The ability of each NTP to prevent inhibition by ppp5'A2'p5'A was investigated in a series of reactions in which each NTP was varied independently. Results are

TABLE 1

Inhibition of VSV RNA polymerase by 300 μM pppA2'p5'A. Assay mixture contained 1 mM GTP and CTP, 0.1 mM UTP

ATP (mM)	% Inhibition
3	22
1	29
0.3	48
0.1	62

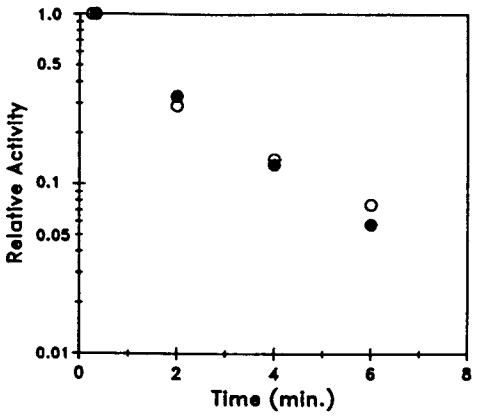

Fig. 3. Covalent inactivation of VSV RNA polymerase by 0.15 mM *N*-ethyl maleimide in the presence (○) or absence (●) of 220 μM ppp5′A2′p5′A.

shown in Fig. 2. It is seen that higher concentrations of ATP (A), GTP (B) and CTP (C) each decrease inhibition, while UTP (D) has little if any protective effect).

The data in Fig. 2 are presented as Dixon plots, which were designed to permit the determination of whether an inhibitor functions competitively or non-competitively. If inhibition is competitive, the lines obtained at different substrate concentrations will intersect at a point corresponding to $i=-K_i$, permitting a direct measurement of the competitive inhibition constant (Dixon and Webb, 1964). A suitable intersection is seen only in Fig. 2B; when GTP concentration is varied, an apparent K_i of ca. 190 μM is determined from the point of intersection. However, in Fig. 2A the lines intersect to yield a negative K_i, and the lines in Fig. 2C do not intersect at all. It seems possible, therefore, that each of the NTPs protects against inhibition by a different mechanism. Alternatively, the kinetics of RNA synthesis may be too complex to permit meaningful interpretation using this procedure (see Discussion).

In a previous report from this laboratory, an ATP binding site on the VSV RNA polymerase was identified by analyzing the ability of ATP to protect against irreversible inactivation of the enzyme by NEM, a reagent that forms covalent bonds, generally with free sulphydryl groups (Massay and Lenard, 1987). A binding constant, K_D, of 580 μM for ATP was determined, and it was suggested that this NEM-protective site might correspond to an initiation site for RNA synthesis, for which a K_m of ca. 500 μM was previously reported (Testa and Banerjee, 1979). It was therefore of interest to determine whether the ppp5′A2′p5′A inhibited polymerase activity by binding to the same site. As shown in Fig. 3, however, ppp5′A2′p5′A did not protect against NEM inactivation. The most likely interpretation of this result is that ppp5′A2′p5′A does not bind the putative initiation site, but inhibits by interacting with one (or more) different binding site(s) on the enzyme. It cannot be ruled out from this experiment alone, however, that the ppp5′A2′p5′A might bind to the putative initiation site, but fail to protect against inactivation by NEM.

Discussion

The observations of Alarcon et al. (1984) that ppp5′A2′p5′A could inhibit VSV protein synthesis, replication and cytopathogenicity in VSV infected HeLa cells provided evidence for an antiviral effect of this smallest of the 2–5A's. This paper provides a plausible mechanism for that effect. The antiviral effects reported by Alarcon et al. (1984) were minimal at a concentration of 100 μM ppp5′A2′p5′A, and nearly maximal at a concentration of 500 μM. In the present study, the concentration of ppp5′A2′p5′A required for 50% inhibition of RNA polymerase activity depended upon the concentration of NTPs, but remained in the 200–400 μM range.

It is unlikely, however, that inhibition of transcription by ppp5′A2′p5′A represents a significant anti-VSV effect of interferon. Intracellular levels of 2–5A's have been determined in several studies; they are generally in the 10–40 nM range, with maximum reported levels of ca. 5 μM (although not in VSV-infected cells; Johnston and Torrence, 1984). This maximum level is still nearly 2 orders of magnitude below the levels necessary for effective inhibition of the VSV RNA polymerase as reported here.

The specificity of the inhibition is also quite different from that of the well-studied interferon-related effects involving 2–5A's. The specificity differs in 3 ways: (i) the dinucleotide is the only active species in polymerase inhibition; (ii) the phosphorylation state of the dinucleotide is important, with the 5′ triphosphate being the most active, the 5′ diphosphate somewhat active, and the 5′ monophosphate essentially inactive; (iii) the 2′–5′ linkage is not essential for inhibitory activity, since the corresponding 3′–5′ compound is equally active.

It is noteworthy that 3 of the 4 NTPs (ATP, GTP and CTP, Fig. 2A–C) protected the polymerase against inhibition by ppp5′A2′p5′A, while the inhibitor itself did not protect against irreversible inactivation by NEM (Fig. 3). The ATP binding site that protects against inactivation by NEM has been suggested to correspond to the initiation site (Massey and Lenard, 1987). One simple interpretation of the present results that is consistent with this idea is that ppp5′A2′p5′A (and, equally, ppp5′A3′p5′A) inhibits by binding to the hypothetical NTP binding site for RNA elongation. The complex Dixon plots (Fig. 2) do not rule out such an interpretation, for the following reasons: (i) it is not clear that specific inhibition of elongation would result in a linear decrease in TCA-precipitable material, which is what the assay measures; (ii) each of the 4 NTPs is a substrate, with its own K_m (or multiple K_ms); it may even act as an inhibitor by binding inappropriately to an active site. For example, we have observed that GTP inhibits polymerase activity at low concentrations of ATP (not shown).

Acknowledgements

The technical assistance of Roger Vanderoef is gratefully acknowledged. This work was supported in part by Grant AI-13002 from the National Institutes of Health.

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