Inhibitory effect of protein kinase C inhibitor on the replication of influenza type A virus

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The growth of influenza virus A/PR/8/34 in MDCK cells was inhibited by 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H7) which is a potent inhibitor of protein kinase C, but not by an effective inhibitor of cyclic nucleotide-dependent protein kinases. Analysing the inhibitory effect of H7 during the replication cycle of influenza virus, we found that the primary transcripts were sufficiently synthesized in infected cells exposed to H7. The primary transcripts synthesized in the presence and absence of H7 were active in directing the synthesis of viral polypeptides both in a cell-free system and in the system containing H7. In the system where infected cells were exposed to H7, the viral positive-sense RNAs were also significantly amplified 6 h after infection. However, the synthesis of viral proteins other than nucleoprotein from viral primary or amplified (secondary) mRNAs was extremely restricted. The synthesis of host cellular proteins in mock-infected cells was significantly retained in the presence of H7. These results suggest that the selective inhibition of influenza virus translation following the transcription of viral mRNA was induced by H7 in infected cells.

Methods

Viruses and cells. Influenza virus A/PR/8/34 (H1N1) was propagated in the allantoic cavity of 10-day-old embryonated hen eggs for 48 h at 35 °C. The allantoic fluids were clarified by centrifugation and then stored at -80 °C as described previously (Ochiai et al., 1988). Madin-Darby canine kidney (MDCK) cells were grown in MEM supplemented with 10% heat-inactivated foetal bovine serum (Ochiai et al., 1988).

Viral growth assay. Confluent monolayers of MDCK cells in 24-well plates were infected with 0-05 p.f.u./cell of PR8 virus for 45 min at 20 °C. The infected cells were washed four times with phosphate-buffered saline (PBS) and then incubated in MEM supplemented with 5% foetal bovine serum (maintenance medium) containing various concentrations of the inhibitors H7 or H8 (N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihydrochloride) (Seikagaku Kogyo) at 37 °C. At 24 h post-infection (p.i.), virus yields in culture fluids were determined by plaque titration on MDCK cells as described previously (Ochiai et al., 1988).

Infection and drug treatment for analyses of viral RNAs and proteins. Confluent monolayer cells were infected with the PR8 strain at a multiplicity of 5 p.f.u./cell. After 1 h adsorption the cells were washed with PBS and incubated in maintenance medium containing H7 (40 μM) at 37 °C. To block protein synthesis after infection, the cells were pretreated with medium containing 150 μg/ml cycloheximide (CM) for 30 min at 37 °C and infected in the presence of CM (150 μg/ml) for 1 h. The infected cells were washed briefly with PBS and incubated in maintenance medium containing CM (150 μg/ml) for 3.5 h at 37 °C. When actinomycin D (AD) treatment followed the CM treatment, AD (5 μg/ml) was added 30 min prior to the removal of CM. At 3.5 h p.i. the infected cells were washed with PBS and then further maintained in maintenance medium containing AD (5 μg/ml) for 2 h at 37 °C.

Introduction

Influenza virus particles possess a segmented genome of eight single-stranded negative-sense RNAs that encode at least 10 virus-specific polypeptides (Lamb & Choppin, 1983; McCauley & Mahy, 1983; Palese, 1977). Among these viral polypeptides, nucleoprotein (NP), non-structural protein 1, and matrix protein 1 have been found to be phosphorylated in host cells (Gregoriades et al., 1984; Kistner et al., 1985; Petri & Dimmock, 1981; Privalsky & Penhoet, 1977). To characterize the kinase that phosphorylates viral proteins and to elucidate the role of viral phosphoproteins, we initially examined the effect of several inhibitors of protein kinases on the growth of influenza type A virus. We found that 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H7), a potent and effective inhibitor of protein kinase C (Hidaka et al., 1984), reduced the growth of influenza type A virus in concentrations that are not noticeably toxic for host cells.

In order to clarify the mechanism of the antiviral effect of H7 we examined the synthesis of viral RNAs and proteins in infected cells exposed to H7 and attempted to find a virus-specific function blocked by H7 in a replication cycle of influenza virus. H7 selectively disturbed the translational process of viral mRNAs in the infected cells, and the synthesis of viral proteins other than NP was extremely limited.

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Labelling of proteins and immunoprecipitation. At the times indicated in the text, the culture medium on the infected cells in 35 mm dishes was replaced with methionine-free maintenance medium containing \[^{[35S]}\text{methionine} (5 \text{ or } 20 \mu \text{Ci/ml})\) and the appropriate drugs for the experiment. After labelling for certain periods of time the cells were washed with cold PBS and lysed in radioimmune precipitation assay (RIPA) buffer (50 mM-Tris-HCl pH 7.0, 150 mM-NaCl, 1% sodium deoxycholate and 1% Triton X-100; van Drunen Littel-van den Hurk et al., 1984). Virus proteins were immunoprecipitated from the lysates by using rabbit antibodies against PR8 as described previously (van Drunen Littel-van den Hurk et al., 1984). The total lysates and immunoprecipitates were analysed by SDS-PAGE followed by fluorography (Bonner & Laskey, 1974; Laemmli, 1970).

Preparation of RNA. Preparation of total RNA from infected cells was carried out according to the method previously described (Glišin et al., 1974; Ullrich et al., 1977). The infected cells in 75 cm\(^2\) culture flasks were washed with cold PBS, lysed in 5 ml guanidine solution (4 M-guanidine thiocyanate, 0.1 M-sodium acetate pH 5.0, 5 mM-EDTA) and homogenized. The homogenate was loaded onto a 4 ml caesium chloride solution (5M-CsCl, 0.1 M-sodium acetate pH 5.0, 5 mM-EDTA) in polyallomer tubes for a Hitachi RPS-40 T rotor and centrifuged at 33 000 rpm for 18 h at 20 °C. The pelleted RNA was stored in 70% ethanol at -80 °C after phenol extraction. Poly(A) RNA was selected from the prepared total RNA by two cycles of oligo(dT) cellulose (Collaborative Research) column chromatography (Aviv & Leder, 1972).

Plasmids and in vitro RNA transcription. The pBR322 plasmids carrying double-stranded influenza virus-specific DNAs, namely the PstI fragments of PBI (Swine/Iowa strain), HA (USSR strain) and NP (Swine/Iowa strain) and the HindIII-XbaI fragments of NS (UDorn strain) were treated with the appropriate restriction enzymes. The excised virus-specific DNAs were subcloned into a multiple cloning site between the T3 and T7 promoters of the Bluescript KS\((+\)) plasmid, generously supplied by Dr Masayuki Yamamoto, Northwestern University, Evanston, Ill., U.S.A. After transfection into MC1061 and screening of each subclone, the plasmids were prepared by alkaline lysis and polyethylene glycol precipitation methods as described by Brush et al. (1985). The subcloned plasmids were linearized by digestion with the appropriate restriction enzymes. Using the linearized plasmids (1 \(\mu\)g), RNA synthesis was performed for 60 min at 37 °C in a 25 \(\mu\)l reaction mixture (40 mM-Tris-HCl pH 8.0, 8 mM-MgCl\(_2\), 2 mM-spermidine, 50 mM-NaCl, 400 \(\mu\)M each of ATP, CTP and GTP, 20 \(\mu\)M-UTP, 30 mM-dithiothreitol, 40 units (U) RNAsin, 100 \(\mu\)Ci \([\alpha-32P]\)UTP (800 Ci/mmol) and 10 U T3 or T7 RNA polymerase). After the addition of DNA digestion buffer (225 \(\mu\)l, 40 mM-Tris-HCl pH 7.5, 6 mM-MgCl\(_2\) and 10 mM-NaCl) and 1 U RNase-free DNase 1 (Pharmacia) the mixture was incubated for 15 min at 37 °C and extracted with phenol/chloroform (1:1, v/v). The labelled RNA was precipitated with ethanol at -80 °C. The precipitated RNA was rinsed with cold 80% ethanol, dried and used for the detection of virus-specific positive- or negative-sense RNA. The polarity of RNA fragments synthesized by T3 or T7 RNA polymerase was determined using poly(A) RNA prepared from infected cells and vRNA prepared from purified virus particles (Palese & Schuman, 1976). Therefore, T3 RNA polymerase was used for the synthesis of negative-sense PBI, HA and NP and positive-sense NS RNA fragments and T7 RNA polymerase was used for the synthesis of positive-sense PBI, HA and NP and negative-sense NS RNA fragments. In RNA blot hybridization, PBI, NP and NS RNAs were simultaneously detected by using a mixture of the probes for their RNAs.

RNA analysis. RNA was electrophoresed in 1% to 1.2% agarose formaldehyde gels as described (Maniatis et al., 1982), transferred to nitrocellulose filters and baked for 2 h at 80 °C (Brush et al., 1985). The fixed RNA was prehybridized in 50% formamide, 5 × SSC, 50 mM-Tris-HCl pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% polyvinylpyrrolidone, 0.3% Ficoll and 5 mM-EDTA for 3 h at 65 °C and hybridized to radiolabelled transcripts at 65 °C overnight. The hybridized filters were washed twice in 2 × SSC containing 0.1% SDS for 30 min (each wash), twice in 0.1 × SSC containing 0.1% SDS for 30 min and in 2 × SSC containing RNase A at 4 \(\mu\)g/ml for 30 min at 30 °C (Yamamoto et al., 1985). The dried filters were exposed to Kodak XAR-5 X-ray films at -80 °C. In some experiments, the radioactive fields on the filters were quantitatively scanned with an AMBIS Beta Scanning System (Automated Microbiology Systems).

In vitro translation. Cell-free translation was carried out in 50 \(\mu\)l of reaction mixture containing 0.2 \(\mu\)g of mRNA, 70 \(\mu\)Ci of \([\text{[35S]}\text{methionine} and 40 \(\mu\)l rabbit reticulocyte lysate (Amersham) according to the supplier's specifications. To examine the direct effect of H7 in an in vitro translation system, H7 was added to the reaction mixture to final concentrations of 10, 20, 40 and 60 \(\mu\)M. The mixtures were incubated at 30 °C for 60 min. Virus-specific polypeptides were immunoprecipitated after addition of 50 \(\mu\)l 2 × RIPA buffer as described above. The total radioactive polypeptides and immunoprecipitates were analysed by SDS-gel electrophoresis.

Results

Effect of protein kinase inhibitors on viral growth

H7 and H8 are derivatives of isoquinoline and are potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases respectively (Hidaka et al., 1984). As shown in Table 1, H7 had a much stronger antiviral effect than H8; virus yields were reduced 2200-fold and 1.2-fold by 40 \(\mu\)M of H7 and H8 respectively. At the concentrations used, these inhibitors were not toxic for MDCK cells.

Effect of H7 on the synthesis of viral proteins and RNAs

To clarify the inhibitory effect of H7 in a viral replication cycle, the synthesis of viral proteins and RNAs in infected cells exposed to H7 was examined. For the

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<th>Table 1. Effect of protein kinase inhibitors on the growth of influenza virus PR8 strain in MDCK cells</th>
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<td>Concentration of inhibitor ((\mu)M)</td>
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* Percentage of the virus yield in the absence of inhibitors.
† ND, Analyses not done.
analysis of viral protein synthesis, infected cells maintained in the absence or presence of H7 were labelled with \(^{35}\)Smethionine for 30 min at various times p.i. (Fig. 1a). In the absence of H7, viral proteins were sufficiently synthesized until 6 h p.i. After 8 h p.i., the synthesis of viral proteins declined, probably due to cytopathic effects. By contrast, in H7-treated cells NP was noticeably synthesized with a maximal level at 6 to 8 h p.i. but the synthesis of other viral proteins was markedly restricted. In this case, the synthesis of cellular proteins was shut off (Fig. 1a, B). However, in the mock-infected cells under H7 treatment, the synthesis of cellular proteins was significantly retained at 12 h p.i. although their overall synthesis was reduced to some extent (Fig. 1b).

Subsequently the four kinds of positive- and negative-sense viral RNAs in infected cells were analysed by RNA blot hybridization (Fig. 2). The amount of radioactivity in the area of each positive-sense RNA on nitrocellulose filters (Fig. 2, +RNA) was quantified (Table 2). In H7-untreated cells the amount of positive-sense RNAs decreased gradually after 6 h p.i. whereas the amount of negative-sense RNAs increased. In H7-treated cells, the positive-sense RNAs were significantly detectable after 6 h p.i. (Fig. 2) and their amounts became maximal at 8 h p.i. Although the levels of positive-sense PB1 and NP RNAs were higher in H7-treated cells than in H7-untreated cells, the levels of positive-sense HA and NS RNAs were lower (Table 2). The negative-sense RNAs were synthesized noticeably after 8 h p.i. These results may suggest that the synthesis of viral proteins other than NP was restricted under significant synthesis of viral mRNAs after 6 h p.i.

**Effect of H7 on primary transcription**

In Table 2, the patterns of positive-sense RNA synthesis in H7-treated and -untreated cells were not similar. From the results, it would be difficult to evaluate substantially the effect of H7 on viral mRNA synthesis because in

![Fig. 2. Effect of H7 on viral RNA synthesis. MDCK cells were infected with PR8 strain (5 p.f.u./cell) and incubated in the absence (A) or presence (B) of 40 μM H7. Total RNA was prepared at 6 h, 8 h and 10 h p.i. The total RNA (10 μg) was electrophoresed on 1.2% agarose-formaldehyde gels, transferred to nitrocellulose filters and then hybridized to \(^{32}\)P-labelled probes specific for positive- (+RNA) or negative- (−RNA) sense RNAs for PB1, HA, NP or NS. The blots were washed and exposed to X-ray films for 12 h at −80 °C.](attachment:image)

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<td>A</td>
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* Background (44 c.p.m./cm²) was automatically subtracted from the radioactivity on each +RNA area (Fig. 2) by an AMBIS Beta scanning system.

† Time post-infection of sampling.
H7-untreated cells virus is highly replicated resulting in the effective synthesis of viral RNAs and proteins. In order to clarify the transcriptional effect of H7, we compared the amounts of primary transcripts in H7-treated and -untreated cells during 3-5 h of exposure to CM. As shown in Fig. 3(a), input viral genomes (vRNAs) were detected immediately after 1 h adsorption, but positive-sense RNAs were not. In both the presence and absence of H7 after adsorption, the amounts of input vRNAs remained relatively similar (Fig. 3a; −RNA, lanes 2 and 3). The positive-sense RNAs were not detected in non-poly(A) RNA fractions prepared from the total RNAs by oligo(dT) column chromatography (Fig. 3b). On the other hand, viral mRNAs were detected in both H7-treated and -untreated cells (Fig. 3a; + RNA, lanes 2 and 3). Similar results were also observed on analysis of poly(A) RNA fractions (Fig. 3b). These results indicate that viral transcription was not inhibited by H7.

Fig. 3. Effect of H7 on primary transcription. (a) Analysis of total viral RNA in infected cells under CM treatment. MDCK cells were pretreated with CM for 30 min before and during 1 h adsorption and were maintained thereafter at 150 μg/ml of CM in the presence (lane 2) or absence (lane 3) of H7 for 3-5 h. Total RNA was prepared immediately after adsorption (lane 1) and at 3-5 h p.i. (lanes 2 and 3). The total RNA (10 μg) was electrophoresed on a 1% agarose-formaldehyde gel and analysed by RNA blot hybridization. The blots were exposed to X-ray films for 72 h at -80 °C. (b) Analysis of primary mRNA. A total RNA fraction was isolated from the infected cells in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of H7 and under CM treatment at 3-5 h p.i. From the total RNA, poly(A) RNA (lanes 1 and 2) and non-poly(A) RNA (lanes 3 and 4) were prepared by oligo(dT) column chromatography. The poly(A) RNA (0.2 μg) and non-poly(A) RNA (5 μg) were analysed by RNA blot hybridization. The blots were exposed to X-ray films for a week at -80 °C.

Effect of H7 on in vivo translation of viral primary transcripts

We examined the effect of H7 on the synthesis of viral proteins from primary mRNAs as shown in Fig. 4(a). In infected cells treated continuously with H7 after infection, the synthesis of all viral proteins except NP was markedly inhibited (Fig. 4b, lane 4). This result
Effect of H7 on transcripts observed even in the absence of H7 after CM treatment obviously indicates that H7 selectively inhibited the synthesis of viral proteins from primary mRNAs. We also examined the influence of H7 exposure time on the selective inhibition of viral proteins (Fig. 4a). In the case of the infected cells treated with H7 and CM after infection, the selective inhibition of viral proteins was observed even in the absence of H7 after CM treatment (Fig. 4b, lane 3). On the other hand, in the infected cells treated with CM only, detectable amounts of all viral proteins were observed in both the presence and absence of H7 after CM treatment (Fig. 4b, lanes 1 and 2). With respect to the synthesis of cellular proteins in infected cells treated with and without H7 after CM treatment, there was no significant difference (compare lanes 1 and 2, or lanes 3 and 4 in Fig. 4b). Because CM inhibits translational elongation (Obrig et al., 1971), the initiation complex formed under CM treatment without H7 remains intact (Fig. 4b, lane 1). Thus, it is unlikely that H7 per se interfered directly with translational elongation. It is more likely that some specific event for the selective inhibition of viral proteins was induced by H7 during CM treatment.

Effect of H7 on in vitro translation of viral primary transcripts

A possible reason for the defective translation of viral primary mRNAs is that some of the primary mRNAs transcribed from the viral genome in the presence of H7 may not have been able to be translated. To examine this possibility, we prepared mRNAs from the infected cells treated with or without H7 during CM exposure. Using rabbit reticulocyte lysate, the prepared mRNAs were translated in vitro with [35S]methionine. As shown in Fig. 5(a), either mRNA preparation was able to direct viral protein synthesis successfully. In this SDS-gel analysis, we could not observe an HA band which should migrate between the bands of NP and P (see Fig. 1). When the translated polypeptides and non-glycosylated HA in the infected cells treated with tunicamycin were immunoprecipitated by anti-HA monoclonal antibody and analysed by SDS–gel electrophoresis, a detectable band of non-glycosylated HA was observed at a position similar to that of NP (data not shown). Thus, the non-glycosylated HA synthesized in the in vitro translational system probably migrated with NP. To examine the direct effect of H7 on an in vitro translational system, we added H7 to the system. As shown in Fig. 5(b), H7 at concentrations up to 60 μM did not inhibit the synthesis of viral polypeptides. Therefore, these results indicate that the primary mRNAs themselves, synthesized in the presence of H7, were intact and capable of directing viral protein synthesis. There was no direct interference of H7 with the in vitro translational system using rabbit reticulocyte lysates.

Discussion

H7 was found to inhibit the growth of influenza virus A/PR/8/34 (H1N1) strain in MDCK cells (Table 1). We also observed the antiviral effect of H7 against the growth of other type A strains, Adachi/2/57 (H2N2) and Aichi/2/68 (H3N2), but not Newcastle disease and vesicular stomatitis viruses (unpublished data). The antiviral effect of H7 would thus appear to be specific for influenza type A virus. The inhibitory effect of H7 was much stronger than that of H8, a potent inhibitor of cyclic nucleotide-dependent protein kinases (Table 1). This result suggests that the inhibitory effect of H7 may result from the inactivation of cellular protein kinase C. However, when we compared the activities of protein kinase C partially purified from mock-infected MDCK cells treated with and without H7 (40 μM) for 3 h, the enzyme activity in H7-treated cells was only reduced to 83% of the control (unpublished data). It was uncertain whether or not the reduction of protein kinase C activity in H7-treated cells was significant for the inhibition of viral growth in MDCK cells. Love et al. (1989) have recently demonstrated that there is no significant difference in protein kinase C activity extracted from H7- and non-H7-treated murine thymoma or T cell
hybridoma cell lines. Therefore, the inhibitory effect of H7 on viral growth may be related to some unknown action of H7, rather than to protein kinase C inhibition by H7.

In this study we focused on the effect of the H7-blocking site on the replication cycle of influenza type A virus. We demonstrated that H7 selectively inhibited the synthesis of influenza virus proteins other than NP in the infected cells exposed to H7 (Fig. 1). The phenomenon was shown to occur at the translational level of the viral primary mRNAs synthesized in H7-treated cells (Fig. 3 and 4).

Fig. 2 and Fig. 4 show that influenza virus RNAs were amplified in infected cells exposed to H7 after infection despite the defective synthesis of viral proteins other than NP from primary transcripts. It is possible that of the viral proteins translated from primary mRNAs NP is important and indispensable for the progression of the viral replication process in infected cells. It has been demonstrated that viral RNA synthesis after primary transcription is dependent on viral protein synthesis (Inglis & Mahy, 1979; Mark et al., 1979; McCauley & Mahy, 1983). Recently NP molecules have been found to be necessary for viral RNA synthesis (Shapiro & Krug, 1988; Honda et al., 1988). This may indicate a functional role in influenza virus replication. In Fig. 2 the amplification of both positive and negative-sense RNAs was delayed in the presence of H7. This may be due to a somewhat lower rate of NP synthesis in the presence of H7 rather than to its absence as shown in Fig. 4. On the other hand, the selective inhibition of viral protein synthesis was caused by H7 only under CM treatment (Fig. 4, lanes 3 and 4). It is likely that some factor that already exists in infected cells under CM treatment and that participates in the formation of translational machinery for influenza virus was modulated by H7 before the removal of CM so that the translational system for viral mRNAs became defective. In fact, we observed the accumulation of the 80S translational initiation complex with viral mRNAs in polysomal fractions prepared from H7-treated infected cells (unpublished data). It would be interesting to identify the factors on which H7 acts in influenza virus-infected cells. We are currently attempting to identify the factor by analysing the blocking steps of H7 in the translational processes of viral mRNAs and by developing cell-free translational systems from infected cells.

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References


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