Studies on the Antiviral Activity of Protein Kinase Inhibitors against the Replication of Vesicular Stomatitis Virus

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Several derivatives of K-252a, a protein kinase inhibitor isolated from *Nocardiopsis* sp., were investigated for their effects on the replication of vesicular stomatitis virus (VSV) in BHK-21 cell cultures. Among those we tested, KT5926, which preferentially inhibits the myosin light chain kinase (MLCK), suppressed the viral replication by 95–99% at 15 μ M. K-252a, which inhibits a broad spectrum of cellular protein kinase, similarly affected the viral replication. Other derivatives, KT5720 and KT5823, that are known to inhibit the cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), respectively, did not suppress VSV replication even at a high concentration as 15 μ M. None of these inhibitors affected the Sindbis virus replication in BHK-21 cells under similar assay conditions as used for VSV. KT5926 and K-252a seemed to affect the VSV replication at the step(s) after the viral invasion, resulting in decreased viral RNA synthesis. Neither substance inhibited cellular casein kinase (CK) II which is known to be involved in phosphorylation of the nonstructural (NS) protein, a non-catalytic subunit of the viral RNA polymerase. These results suggest that the inhibition of VSV replication by KT5926 and K-252a is not a secondary effect due to generalized suppression of host cell activities, and that the VSV replication requires the KT5926-sensitive function(s) in the cell which would be performed by an enzyme(s) other than CK II.

Key words protein kinase inhibitor; antiviral activity; K-252a derivative; vesicular stomatitis virus; negative-stranded RNA virus

Negative-stranded RNA viruses are comprised of several virus families, including the rhabdoviridae (e.g., rabies virus), paramyxoviridae (e.g., measles virus, mumps virus), orthomyxoviridae (e.g., influenza virus), filoviridae (e.g., Ebola virus). These viruses have a phosphorylated protein called phosphoprotein (P) or “nonstructural” (NS) protein. P (or NS) protein is thought to be involved in viral RNA synthesis in collaboration with a viral large protein (L protein). Viral RNA synthesis of vesicular stomatitis virus (VSV), a prototype of the rhabdovirus, has been extensively studied as a model system of the negative-stranded RNA viruses. The genome of VSV encodes five viral proteins (N, NS, M, G and L). Nucleoproteins (N) associate with the genome RNA covering its entire region to form a viral nucleocapsid (RNP). The virus specific RNA polymerase is comprised of catalytic (L) and regulatory (NS) subunit proteins.¹⁾ Other viral proteins, G (glycoprotein) and M (matrix protein), constitute the viral envelope.

Although the precise role(s) of NS protein in VSV RNA synthesis has not yet been elucidated, its function(s) is thought to be regulated by phosphorylation and dephosphorylation of the protein. Phosphorylation of NS protein has been suggested to be catalyzed by both the cellular and viral kinase activities in a stepwise manner.^{2,3)} Cellular casein kinase (CK) II was shown to be involved in the first step of phosphorylation of newly synthesized NS protein. The second step is thought to be performed by the viral L protein-associated kinase activity.

For expecting the agents to display selective toxicities, they should affect the virus-specific activities. In fact, the targets of many antiviral candidates are the viral enzymes. In developing antiviral drugs, however, we first have to find certain substances with antiviral activity as lead compounds, from which drugs with greater selective toxicity might be developed.

Since the NS protein phosphorylation is an essential step for VSV replication, inhibition of the phosphorylation activity may lead to disturbance of viral RNA synthesis and suppression of the virus replication. Accordingly, we have planned to seek protein kinase inhibitors with antiviral activity using the VSV infection system in cultured cells as a model system of the negative-stranded viruses. Protein kinase inhibitors of a broad spectrum, K-252a and K-252b, were isolated from *Nocardiopsis* sp., and several derivatives were also prepared by chemical modifications of a parent compound K-252a.^{4,5,6)}

In this study, we investigated effects of K-252a derivatives on the VSV replication in BHK-21 cells. We found that an inhibitor (KT5926), which is known to preferentially inhibit the cellular myosin light chain kinase (MLCK), inhibited the VSV replication. This inhibition was not mediated by suppressing the whole cell activity, nor by inhibiting the cellular CK II which is thought to be involved in NS protein phosphorylation in the cell. We will also discuss the KT5926-sensitive function(s) which was shown to be required for VSV replication.

MATERIALS AND METHODS

Chemicals K-252 compounds (K-252a, K-252b) and synthetic derivatives (KT5720, KT5823, KT5926) were purchased from Kyowa Medex Co., Ltd. (Tokyo). [5-³H]uridine-5'-triphosphate (specific activity = 19 Ci/mmol) and [5-³H]uridine (specific activity = 30 Ci/mmol) were products from Moravak Biochemicals Inc. (Brea, CA) and American Radiolabeled Chemicals Inc. (St. Louis, MO), respectively.

Viruses, Cells and Media A BHK-21-adapted clone of VSV (New Jersey serotype)⁷⁾ was used throughout this study. Sindbis virus was the same strain as described

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previously.⁸⁾ BHK-21 cells were usually propagated in Eagle's minimum essential medium (MEM) supplemented with 10% Tryptose phosphate broth (TPB; Difco Laboratories, Detroit) and 5% bovine serum.

Preparation of Virus Stocks and Plaque Assay Practically DI-free stocks of VSV were prepared by one cycle passage of plaque isolates through BHK-21 cell cultures and stored at -20°C until use.⁹⁾ Sindbis virus stocks were also prepared in the same way. Viral infectivity was assayed by plaque formation on BHK-21 cell monolayers as described previously.⁸⁾

Evaluation of Antiviral Activity Monolayer cultures of BHK-21 cells prepared in 24-well plates were infected with VSV at an m.o.i. of about 0.001 p.f.u./cell. After viral adsorption, various amounts of protein kinase inhibitors were added to the culture medium. The cultures were then incubated at 37°C . On the second day, they were observed microscopically at 5 to 6 h-intervals until the untreated infected cultures reached the maximum level of cytopathic effect (CPE; i.e., cell rounding). Culture fluids were harvested and subjected to the infectivity assay by plaque formation.

Studies of RNA Synthetic Activity of the Virus Infected Cells Viral RNA synthesis was investigated by determining the incorporation of radioactive nucleotides into the acid-insoluble fraction of the cell as follows: monolayer cultures of BHK-21 cells were prepared in 35-mm petri dishes and infected with VSV as described above. After incubation for 4.5 h, actinomycin D ($5\text{ }\mu\text{g/ml}$) and $[5\text{-}^3\text{H}]$ -uridine ($1\text{ }\mu\text{Ci/ml}$) were added to the cultures and further incubated for appropriate periods as described for each experiment. After washing with cold phosphate-buffered saline (PBS), the cells were collected and lysed in 0.5 ml of 1% sodium dodecyl sulfate (SDS), and then mixed with the same volume of cold 10% trichloroacetic acid (TCA) and placed on ice for at least 30 min. The TCA-insoluble precipitates were collected on glass micro fiber filters (GF/C, 2.4-cm; Whatman International Ltd., Maidstone), washed several times with cold 5% TCA and, finally, with ethanol. The radioactivity retained on the filter was determined using a liquid scintillation spectrometer (Beckman LS-7500).

In Vitro Test of Protein Kinase Inhibitors against CK II Purified CK II was purchased from a commercial source (human recombinant CK II produced in *Escherichia coli*, 10 mU/50 μl ; Boehringer-Mannheim GmbH). Assay conditions were almost the same as described by Hathaway and Traugh.¹⁰⁾ Reaction mixtures (50 μl) contained 50 mM Tris-HCl (pH 7.1), 100 mM KCl, 10 mM MgCl_2 , 2.5 mM dithiothreitol, 250 μg alpha casein, 20 μU CK II, 10 μM ATP (including 0.5 μCi $[^{32}\text{P}]$ ATP), and various amounts of protein kinase inhibitor (PKI). After incubation for 20 min at 37°C , the reaction mixtures were treated with 10% TCA, and the radioactivities in the precipitates were determined using liquid scintillation spectrometer.

RESULTS

Antiviral Activity of K252 Derivatives on VSV Infection We first investigated the effects on VSV replication of several synthetic derivatives of the K-252

compound listed in Table 1, which are known to display different inhibitory effects on various kinds of cellular protein kinase.^{4,5,6)} Monolayer cultures of BHK-21 cells were prepared in 24-well plates 1 d before virus infection. VSV was inoculated at a low m.o.i. (about 0.001 p.f.u./cell) and, after the virus adsorption, various amounts of K-252 derivatives were added to the infected cultures. On the second day of virus infection, the cultures were observed under a microscope and, when complete CPE was observed in the untreated infected cultures, the culture fluid was recovered from each well to examine the viral yield.

As shown in Fig. 1, the virus replication was suppressed by K-252a, a derivative which shows inhibitory effect on a broad spectrum of cellular protein kinases. KT5926, that preferentially inhibits the cellular MLCK,⁶⁾ also suppressed the viral replication. KT5926 and K-252a reduced the viral yield by 95–99% at 15 and 5 μM , respectively. Other derivatives (KT5720, KT5823), which are known to inhibit the cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), respectively,^{4,11)} did not display such an inhibitory effect on the VSV infection. Although K-252b is known to inhibit many kinds of protein kinases *in vitro*,⁴⁾ it did not affect the VSV replication.

Time course studies performed with these compounds at the higher m.o.i. of VSV infection demonstrate that both KT5926 and K-252a similarly suppressed the viral replication (Figs. 2A, B). Viral replication was also suppressed when the inhibitors were added to the cultures even 2.5 h after the infection (Fig. 2), indicating that the inhibitory effect was directed to the process after the uncoating step of the virus.

Effect of K-252 Derivatives on the Sindbis Virus Rep-

Fig. 1. Dose-Response Studies on the Effect of K-252 Derivatives on the VSV Replication

Monolayer cultures of BHK-21 cells in 24-well plates were infected with VSV at an m.o.i. of 10 p.f.u./cell. After viral adsorption, the cultures were fed with the maintenance medium containing 1% fetal bovine serum, to which various concentrations of K-252 derivatives were added. They were incubated at 37°C for 8 h when the untreated (control) cultures usually reached the maximum level of progeny virus yield. Then, the culture fluids were collected and the yield of infectivity in the fluids was determined by plaque formation as described in Materials and Methods. Symbols: □, K-252a; ○, K-252b; ■, KT5720; △, KT5823; ▲, KT5926.

Fig. 2. Time Course Studies of the Effects of K-252 Derivatives on the VSV Replication

BHK-21 cell monolayers were prepared in 35-mm dishes. The following day they were infected with VSV at an m.o.i. of 10 p.f.u./cell. After viral adsorption, infected cultures were incubated at 37 °C with the maintenance medium containing 1% fetal bovine serum. K-252a (5 μ M) or KT5926 (15 μ M) was added to the cultures at 0 time or 2.5 h of infection. At one or 2-h intervals, culture fluids were recovered and subjected to the infectivity assay by plaque formation. A: K-252a; B: KT5926. Symbols: ●, untreated control; □, treated from 0 time; ■, treated after initial incubation for 2.5 h.

Table 1. Effects of K-252 Derivatives on the Protein Kinase Activities *in Vitro* and Virus Replication in Cultured Cells

Compound ^{a)}	Inhibition of protein kinase: K_i (nM)					Inhibition of virus replication: IC_{50} (μ M) ^{d)}	
	PKC ^{b)}	PKA ^{b)}	PKG ^{b)}	MLCK ^{b)}	CK II ^{c)}	VSV	Sindbis virus
K-252a	25	16	15	20	16000	0.1–0.2	≥ 10
K-252b	20	90	100	147	3700	> 15	> 15
KT5720	> 2000	56	> 2000	> 2000	15000	> 15	> 15
KT5823	4000	> 10000	234	> 10000	> 20000	> 15	> 15
KT5926	723	1200	158	18	> 20000	0.6–1.0	> 15

a) For chemical structures, see Kase *et al.*⁴⁾ and Nakanishi *et al.*⁶⁾ b) Data were cited from Nakanishi.¹¹⁾ c) Data were obtained from the results shown in Fig. 4. d) IC_{50} against the virus replication was determined as follows: BHK-21 cell monolayers in 24-well plates were infected with VSV or Sindbis virus at about 0.01 p.f.u./cell, and were incubated at 37 °C with various amounts of K-252 derivatives until the infected untreated cultures displayed the maximum level of CPE (cell rounding). The concentration of each compound which displayed 50% inhibition of the CPE was calculated for both viruses.

lication It was of interest to know whether the inhibition of VSV replication by K-252 derivatives was not merely a secondary effect due to suppression of the host cell activities. Accordingly, we investigated this possibility in the BHK-21 cell cultures which were infected with one of the other enveloped RNA viruses, such as the Sindbis virus. If this virus does not require any protein kinase activities in its replicative process, the virus would replicate normally regardless of the presence or absence of the inhibitor. As shown in Tables 1 and 2, Sindbis virus replication was not affected by any of these inhibitors at the concentration of 5 μ M. Although the results in Table 1 show that K-252a significantly inhibited the Sindbis virus replication at 15 μ M, this effect varied from experiment to experiment, and no inhibition was observed at 5 μ M. KT5823 displayed a weak inhibitory effect on the Sindbis virus replication at 15 μ M, and KT5926 had only a slight effect at this dosage. From these results, we assume that the inhibition of VSV replication by 5 μ M K-252a and 15 μ M KT5926 is not a secondary effect due to general suppression of the host cell activities.

Effects of K-252 Derivatives on the Viral RNA Synthesis

Since phosphorylation of the viral NS protein is believed to be essential for its activation and/or regulation to take part in regular viral RNA synthesis, we next examined the effects of the inhibitors on this synthesis. We first in-

Table 2. Effect of K-252a Derivatives on the Sindbis Virus Replication in BHK-21 Cells in Culture

Drug	Concentration (μ M)	Virus yield	
		PFU/ml	(%)
No drug		7.1×10^8	100
K-252a	5	6.9×10^8	97.2
	15	2.1×10^8	29.6
K-252b	5	7.5×10^8	105.6
	15	7.7×10^8	108.5
KT5720	5	9.0×10^8	126.8
	15	6.6×10^8	93.0
KT5823	5	6.0×10^8	84.5
	15	4.1×10^8	57.7
KT5926	5	8.8×10^8	123.9
	15	6.2×10^8	87.3

Monolayer cultures of BHK-21 cells were infected with Sindbis virus at an m.o.i. of 10 p.f.u./cell. After viral adsorption, the cultures were fed with the maintenance medium containing 1% fetal bovine serum, to which K-252 derivatives were added immediately at 5 or 15 μ M. They were incubated at 37 °C for 8 h. Then, the yield of infectivity in the culture fluids was determined by plaque formation as described in Materials and Methods.

vestigated the total viral RNA synthesis in the presence of the inhibitors added to the infected cultures. Actinomycin D-resistant incorporation of [5-³H]uridine into the acid-insoluble fraction was compared between the

Fig. 3. Inhibitory Effect of K-252 Derivatives on the VSV RNA Synthesis in the Infected Cultures

Monolayer cultures of BHK-21 cells were prepared in 35-mm dishes. The second day they were infected with VSV at an m.o.i. of 5 p.f.u./cell. After viral adsorption, various concentrations of K-252 derivatives were added to the cultures. They were incubated at 37°C with the maintenance medium containing 1% fetal bovine serum. Actinomycin D (5 μg/ml) and [³H]uridine (1 μCi/ml) were added to the cultures at 1.5 and 2 h after the infection, respectively. The cultures were further incubated at 37°C until the 6th h of infection, and then treated with 5% TCA on ice as described in Materials and Methods to determine the radioactivity incorporated into the acid-insoluble fraction. For K-252b, KT5720 and KT5823, only three points (0, 5 and 15 μM) were examined. Symbols are the same as described for Fig. 1.

Fig. 4. *In Vitro* Assay of K-252 Derivatives on CK II

K-252 derivatives were added to the reaction mixtures (see Materials and Methods) at various concentrations. After incubation for 20 min at 37°C, the mixtures were treated with 10% TCA. The radioactivity in the precipitates was determined as described in Materials and Methods. The residual CK II activity was expressed as % of the control (radioactivity of the control was usually 10000 to 12000 c.p.m.), and plotted against the concentration of the inhibitor. Symbols: □, K-252a; ●, K-252b; ▲, KT5720; ○, KT5823; ■, KT5926.

treated and untreated infected cultures. KT5926 and K-252a were shown to inhibit the viral RNA synthesis while the other derivatives did not. Figure 3 shows the dose-dependent inhibition of viral RNA synthesis caused by KT5926 and K-252a, which resembled the suppressive effect on the progeny virus production shown in Fig. 1A.

Effect of K-252a Derivatives against the Cellular CK II We finally examined whether the cellular CK II activity, which has been suggested to be involved in NS protein phosphorylation,^{2,3)} is inhibited by KT5926 under *in vitro* assay conditions. In our preliminary experiment, the CK II activity was inhibited completely by heparin sulfite at a concentration of 1 μg/ml under usual assay conditions (see Materials and Methods). Under the same

reaction conditions, none of the K-252a derivatives we tested inhibited the CK II activity even at 1 μM (Fig. 4). At higher concentration, however, KT5720, K-252a and K-252b inhibited 50% or more of CK II activity at 10 μM, while KT5926 did not, suggesting that the KT5926-sensitive function(s) is not performed by cellular CK II in the VSV infected cells.

DISCUSSION

1) In this study we found that replication of VSV in BHK-21 cells was suppressed by an inhibitor (KT5926) which is known to inhibit preferentially the MLCK and also by a broad nonspecific PKI (K-252a). Neither of them affected Sindbis virus infection under the same infection conditions at the effective concentration of each inhibitor (5 μM K-252a, 15 μM KT5926). Their effective concentrations in the VSV infected cultures were much higher than those required for inhibiting the protein kinase activities *in vitro*. This requirement of higher concentrations may be due to their inefficient invasion into the cells through the cell membrane. Consistent with this assumption, Usui *et al.*¹²⁾ also reported use of a higher concentration of K-252a in their studies on the cell cycle events in culture, although these events were a little more sensitive to K-252a than VSV replication. K-252b, another derivative of potent non-specific PKI, did not affect the VSV infection, which may also be due to its much reduced efficiency in entering the cell.¹²⁾

2) The experiments with Sindbis virus suggest that the inhibitory effect on VSV replication was not a secondary effect due to reduction of whole cell activities. In other words, the replication of VSV appears to require some KT5926-sensitive function(s) which is not required for Sindbis virus replication. Treatment of the VSV infected cell culture with KT5926 or K-252a resulted in reduction of viral RNA synthesis, which may also account for the reduced progeny virus production. Inhibition of the VSV replication was observed even when the inhibitors were added to the cultures at a later phase of infection. This suggests that the agents affected the step after the viral invasion, although it does not necessarily eliminate a possibility that they might also affect the step before the onset of viral transcription.

3) We can think of at least two possibilities for the KT5926-sensitive function(s) involved in viral RNA synthesis: one is the function(s) involved in NS protein phosphorylation; the other may be a cellular function(s) involved in other than the NS protein phosphorylation. KT5926 did not inhibit cellular CK II under *in vitro* assay conditions, suggesting that the CK II involved in NS protein phosphorylation in the cell is not its target. Our very recent studies on the antiviral mechanisms of KT5926 and K-252a seemed to show that the former did not affect the phosphorylation of NS protein, which also indicate that KT5926 does not affect the viral L protein-associated protein kinase activity. In other words, the KT5926-sensitive function(s) required for VSV RNA synthesis is thought to be cellular origin (Kim *et al.*: manuscript submitted to *J. Gen. Virol.*).

As for the KT5926-sensitive cellular function(s), we have

to consider the possible involvement of cellular MLCK or MLCK-like activity in the replicative process of VSV. Cellular MLCK has to date been thought to be involved in cytoskeleton-associated functions such as platelet aggregation and contraction of vesicular smooth muscles,^{13,14} but its involvement in the virus replication process has not yet been suggested. Using staurosporine, a non-specific protein kinase inhibitor isolated from *Streptomyces staurosporeus*, Rigaut *et al.*¹⁵ suggested that staurosporine-induced suppression of VSV RNA synthesis in the cell may be a secondary effect due to the toxic effects on the host cell activity. Although they did not mention the involvement of cytoskeletal functions, such activity may include the KT5926-sensitive function(s). At present, we can only say that the KT5926-sensitive function(s) is cellular in origin.

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