Studies on the Antiviral Mechanisms of Protein Kinase Inhibitors K-252a and KT5926 against the Replication of Vesicular Stomatitis Virus

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We investigated the antiviral mechanisms of K-252a, a broad non-specific protein kinase inhibitor which was isolated from Nocardiopsis sp. and its derivative (KT5926), against vesicular stomatitis virus (VSV) replication in BHK-21 cells. Although K-252a (5 µM) and KT5926 (15 µM) similarly suppressed the viral primary and secondary transcriptions and genomic RNA synthesis in vivo, the inhibitory mechanisms did not seem to be the same: phosphorylation of the viral NS protein was suppressed by K-252a, which might account for the decreased viral RNA synthesis caused by K-252a. On the other hand, KT5926, being known to preferentially inhibit myosin light chain kinase (MLCK), had little effect on NS protein phosphorylation. Cellular casein kinase II, which is believed to be involved in the phosphorylation of the N-terminal side (domain I) of NS protein, was not inhibited at all by KT5926 even at 15 µM under in vitro assay conditions, and was only weakly inhibited by K-252a at 1 to 10 µM. Neither inhibitor seemed to directly affect viral protein synthesis, but affected it indirectly as a secondary effect of reduced viral RNA synthesis. These results suggest that both the KT5926-sensitive and the KT5926-resistant but K-252a-sensitive functions are involved in the essential processes of viral RNA synthesis. The KT5926-sensitive function(s) might not be involved in the NS protein phosphorylation, but may participate in some other way in the process of virus replication. On the other hand, the KT5926-resistant, K-252a-sensitive function(s) are probably involved in NS protein phosphorylation. The possible nature of those functions is discussed.

Key words vesicular stomatitis virus; VSV; viral RNA synthesis; protein kinase inhibitor; K-252a; KT5926

In our previous studies,[11] we investigated the effect of K-252a (a metabolite of Nocardiopsis sp., which is known to inhibit a broad spectrum of protein kinases)[12] and its derivatives of different inhibitory spectra (i.e., K-252b, KT5720, KT5823, and KT5926)[13,14] in order to search for inhibitory agents which might affect the replication of rhabdoviruses. We found that K-252a and a derivative (KT5926, which preferentially inhibits the cellular myosin light chain kinase; MLCK) displayed antiviral activities against the replication of vesicular stomatitis virus (VSV) in animal cell cultures, while the other derivatives did not. [15] None of these agents affected the Sindbis virus replication in culture.

VSV is a prototype of the Rhabdoviridae, to which also belong the rabies virus, a fearful agent capable of causing acute fatal disease in the brain of human and most mammals, and which is usually transmitted through the bites of rabid dogs. VSV, which causes only slight vesicular lesions in the mouth and rarely causes fatal diseases, has been studied extensively, especially in terms of its replicative mechanism as a model system of the rhabdovirus replication in culture.

The negative-stranded rhabdovirus genomic RNA encodes at least five species of viral proteins (N, NS, M, G and L), two of which, L and NS, serve as catalytic and regulatory subunits of the viral RNA polymerase, respectively, and are involved in the synthetic process of both viral mRNAs (transcription) and genomic RNA. [16] Only the NS protein is a common phosphoprotein of rhabdoviruses, and is thought to play, in addition to a role as a stoichiometric subunit of the viral RNA polymerase, [17] a role as a molecular chaperone probably in helping the free viral N proteins to specifically and correctly bind to the newly synthesized viral genome-sized RNA. [18] And, the phosphorylation and dephosphorylation of NS protein are believed to be essential processes for the regulation of those NS protein functions. [9–11]

Although processes as well as the enzymes involved have long been poorly understood, recent studies on the roles and mechanisms of NS protein phosphorylation have provided some insight. Sánchez et al.[19] first suggested that protein kinase activity is closely associated with the L protein. Chat-topadhyay et al.[10] also showed that L protein-associated protein kinase activity catalyzes NS protein phosphorylation, which might result in the enhanced binding of L protein to the RNP template and stimulate the transcription in vitro. On the other hand, Ikeuchi et al.[12] suggested the involvement of heparin-insensitive kinase III in NS protein phosphorylation. Massey et al.[13] suggested that the phosphorylation of NS protein is catalyzed by certain cellular protein kinase(s) before being incorporated into the virion.

Becks and Perrault[14,15] more recently described a process of stepwise phosphorylation of VSV NS protein, which was shown to be catalyzed by two kinds of virion-associated protein kinase activities (VSVK1 and VSVK2), although only the former was suggested to be essential. Barik and Banerjee[16,17] also proposed recently that the phosphorylation of phosphate-free NS protein is catalyzed by both the cellular and viral L protein-associated kinase activities in a stepwise manner. They suggested strongly that the cellular casein kinase (CK) II is the enzyme involved in the first step of NS protein phosphorylation. At present, however, the role(s) of these NS protein phosphorylation processes in the viral replicative process remain unclear. Most studies of VSV-related phosphorylation in the cell have been limited to NS protein phosphorylation; however, we cannot eliminate the possibility that some other function(s) or step(s) in the VSV replication process also depends on certain protein kinase activities.

In general, protein kinase inhibitors (PKI) are useful tools for investigating the roles and properties of protein kinases.

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Accordingly, we further investigated the antiviral mechanisms of K-252a and KT5926 against the replication of VSV in order to identify the K-252a and KT5926-sensitive functions in the VSV replicative process. The results suggest that viral protein synthesis did not seem to be affected directly. The inhibitory activities were directed to the steps after the viral invasion, causing a decreased rate of viral RNA synthesis. The phosphorylation of NS protein was greatly suppressed by K-252a, but not by KT5926, suggesting that the suppression of viral transcription by K-252a may partly be due to the impairment of NS protein phosphorylation. On the other hand, KT5926 suppressed VSV RNA synthesis without affecting NS protein phosphorylation, which suggests that VSV RNA synthesis requires certain KT5926-sensitive protein kinase(s) involved in some event(s) other than the NS protein phosphorylation.

MATERIALS AND METHODS

Viruses, Cells and Media A BHK-21 cell-adapted clone of VSV (New Jersey serum type) was used throughout this study. Sindbis virus was the same strain as described previously. BHK-21 cells were propagated in Eagle’s minimum essential medium (MEM) supplemented with 10% Trypsone phosphate broth (TPB; Difco Laboratories, Detroit) and 5% bovine serum.

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

Purification of VS Virions Purified virions of VSV were prepared as described previously. In brief, BHK-21 cells were infected with VSV at a low m.o.i., and culture fluids were harvested when most of the cells in the infected cultures displayed a morphological change (cell rounding), but before the onset of cytolysis. Viruses were concentrated by precipitation with polyethylene glycol (#6000) and then purified by velocity sedimentation through a 10—45% sucrose gradient. Virions were obtained from the band formed in the gradient, concentrated by ultracentrifugation and dissolved in a small amount of NT buffer (0.13 m NaCl, 0.05 m Tris—HCl, pH 7.4) at a concentration of about 1 mg protein/ml. They were divided into several stock tubes and kept at -70°C until use. Estimation of the viral protein content was performed by Lowry’s method using bovine serum albumin (BSA) as a standard of protein concentration.

Metabolic Labeling of the Virus Infected Cells BHK-21 cells sown in plastic Petri dishes were infected with VSV at an m.o.i. of 5 to 10 p.f.u./cell. Mock-infected cultures were also prepared in parallel. The cultures were incubated at 37°C in Eagle’s MEM supplemented with 1% bovine serum. At the time indicated, they were mock-treated or treated with K-252a derivatives. For metabolic labeling of the virus proteins with [35S]methionine, the culture medium was replaced by radio-labeling medium (prepared by mixing 9 parts of methionine-free Eagle’s MEM and one part of normal MEM: i.e., the concentration of l-methionine was reduced to one-tenth that of the ordinary MEM), to which 20 μCi/ml [35S]methionine was added. The cultures were incubated for one more hour. In the case of [32P]ortho-phosphate labeling of viral proteins, the culture medium was replaced by phosphate-free MEM and the cultures were preincubated in the presence of 5 μM actinomycin D for 30 min before the addition of [32P]ortho-phosphate. Then the cultures were labeled with 40 μCi/ml of [32P]ortho-phosphate for 90 min. After the radio-labeling, the cultures were washed three times with PBS, then lysed with either a Laemmli’s sample lysis buffer or a two-dimensional (2-D) gel lysis solution (see below).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Autoradiography For the electrophoresis of viral proteins in an SDS-PAGE gel, the infected cells were lysed with Laemmli’s sample lysis buffer and applied to 10% polyacrylamide gels prepared in a Mini Protein II (Bio-Rad). After electrophoresis at a constant current of 25 to 30 mA, the gels were stained with Coomassie Brilliant blue and dried onto Whatman 3MM filter paper. For autoradiographic analysis, the dried gel was exposed to an imaging plate (type BAS-III; Fuji Photo Film Co., Ltd., Tokyo) for the appropriate numbers of hours before analysis in a Bio-Imaging Analyzer (BAS-2000; Fuji Photo Film Co., Ltd.).

2-D Gel Electrophoresis of Viral Proteins Cell lysates prepared with a 2-D gel lysis solution [2% Nonidet P-40 (NP-40), 5% 2-mercaptoethanol, 9.5 μM urea, and 2% Ampholine (LKB)] were applied to the two-dimensional non-equilibrium pH gradient gel electrophoresis (2-D NEPHGE) described previously. After the electrophoresis, 2-D NEPHGE gels were dried onto Whatman 3MM filter paper and exposed to an imaging plate for autoradiography as described above.

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: monolayer cultures of BHK-21 cells were prepared in 35 mm plastic Petri dishes, then infected with VSV at an m.o.i. of 5 to 10 p.f.u./cell. Actinomycin D (5 μg/ml) and [5-3H]uridine (1 to 10 μCi/ml) were added to the cultures mostly at 4.5 and 5 h, respectively, and incubated for another hour for radio-labeling of the viral RNA. After being washed with cold PBS, the cells were collected and lysed in 0.5 ml of 1% SDS, then mixed with the same volume of cold 10% trichloroacetic acid (TCA) and placed on ice for at least 30 min. TCA-insoluble precipitates were collected on glass filters (GF/C, 2.4-cm; Whatman International Ltd., Maidstone), and washed several times with cold 5% TCA and finally with ethanol. The radioactivity retained on the filters was determined in a liquid scintillation spectrometer (Beckman LS-7500).

For estimation of the radioactivity in the RNase-resistant and RNase-sensitive fractions in the cell, the recovered cells for each sample were lysed with 0.5 ml of 0.5% Triton X-100 in 50 mM Tris—HCl (pH 7.4), then divided into two parts. They were mock-treated or treated with 25 μg RNase A for 30 min at 30°C. The RNase-treated and untreated samples were then mixed with the same volume of 10% TCA on ice to determine the radioactivity remaining in the acid-insoluble fraction as described above.

Primary Transcription in Vivo Monolayer cultures of
BH-21 cells were infected with VSV at an m.o.i. of 40 p.f.u./cell in the presence of cycloheximide (100 μg/ml). After the viral adsorption at 15 °C, fresh maintenance medium was added to the cultures; the medium contained 100 μg/ml cycloheximide, 5 μg/ml actinomycin D and the PKI as noted in the text. After the initial incubation for 30 min at 37 °C, 10 μCi/ml [5-3H]uridine was added to the cultures, which were then incubated at 37 °C for 3 h. At 30 to 60 min intervals, pairs of dishes were taken out and subjected to the determination of [3H]uridine incorporation into the acid-insoluble fraction as described above.

**Assay of the Transcription in Vitro** Standard reaction mixtures (200 μl) of the in vitro transcription assay contained: 70 mM NaCl, 100 mM Tris–HCl (pH 7.4), 5 mM dithiothreitol (DTT), 0.1% NP-40, 0.5 mM ATP, 1 mM CTP, 1 mM GTP, 0.1 mM UTP, 1 μCi of [5-3H]UTP, 5 mM MgCl₂, and purified viornos (about 10 μg protein). The reaction mixtures were incubated at 30 °C for the time indicated in the text. Transcription activity was determined by quantitating the incorporated radioactive UMP into the TCA-insoluble fraction as follows: samples were mixed with 500 μl of cold 10% TCA and 100 μg BSA, and the precipitates were collected onto 2.4 cm GF/C filters. Radioactivity on the filters was determined as described above. The zero-time samples were determined for the background counts, which usually ranged from 50 to 100 cpm.

**In Vitro Assay of Inhibitors against CK II** Purified CK II was purchased from a commercial source (human recombinant CK II, 10 μM/50 μl; Boehringer-Mannheim GmbH). Assay conditions were almost the same as those described by Hathaway and Traugh.25 Reaction mixtures (50 μl) contained 50 mM Tris–HCl (pH 7.1), 100 mM KCl, 10 mM MgCl₂, 2.5 mM DTT, 250 μg alpha-casein, 20 μg CK II, 10 μM ATP (including 0.5 μCi [14C]ATP), and various amount of 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 by using a liquid scintillation spectrometer (Beckman LS-7500).

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

**RESULTS**

In our previous studies of K-252a derivatives, we observed that an original compound (K-252a) and its derivative (KT5926) displayed antiviral activity against the replication of VSV in BHK-21 cell cultures. K-252a and KT5926 reduced the progeny virus yield by 95 to 99% at final concentrations of 0.5 and 1.5 μM in the culture medium, respectively.1) The anti-VSV activity was also shown not to be due to secondary effects of the generalized suppression of host cell activities, because the Sindbis virus (an enveloped positive-stranded RNA virus) replicated normally in the presence of those agents. Our previous studies also showed that the inhibitors were effective even when they were added at 2.5 h after the infection, suggesting that they affect at least the step(s) after the viral invasion into the cell. In this study, we performed further experiments to identify the function(s) affected by the inhibitors.

**Effect of the Inhibitors on Viral RNA Synthesis in the Cell** We first examined the effect of these inhibitors on the RNA synthesis using ten times the concentration which reduced the yields of progeny viruses by 95 to 99%. The inhibitors were added to the VSV-infected cultures after viral adsorption, and their effect on viral RNA synthesis was monitored by determining the incorporation of [3H]Juridine into the acid-insoluble fraction. As shown in Fig. 1, both inhibitors greatly decreased the accumulation of actinomycin D-resistant [3H]Juridine incorporated into the acid-insoluble fraction from the early phase of the infection.

**Effect of the Inhibitors on the Viral Primary Transcription** We next investigated the effects of the inhibitors on the primary transcription in the cell displayed by the virion-associated transcriptase. After viral adsorption, the infected cultures were mock-treated or treated with K-252a (5 μM) or KT5926 (15 μM), and incubated in the presence of

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Fig. 1. Time Course Study on the Effects of PKI on VSV RNA Synthesis in BHK-21 Cells
Monolayer cultures of BHK-21 cells in 35 mm Petri dishes were infected with VSV at an m.o.i. of 5 p.f.u./cell. Mock-infected control cultures were also prepared in parallel. After viral adsorption, a maintenance medium was added to the cultures, to which K-252a (A) and KT5926 (B) were added at final concentrations of 5 and 15 μM, respectively, before the cultures were incubated at 37 °C. Actinomycin D (5 μg/ml) and [5-3H]Juridine (1 μCi/ml) were added to the cultures at 1.5 and 2 h after the infection, respectively. At 2-h intervals, pairs of infected and mock-infected cultures were taken out and subjected to the procedure for determining the actinomycin D-resistant incorporation of radioactive nucleoside into the acid-insoluble fraction as described in Materials and Methods (at 12 h of infection, portions of infected cells were lost in this experiment due to detachment from the substrate of the dish; accordingly, the curves were somewhat declined at this time). (A) K-252a-treated; (B) KT5926-treated. Symbols: □, mock-infected control; ●, VSV-infected control; □, K-252a-treated; ■, KT5926-treated.
Fig. 2. Effect of K-252a and KT5926 on the Primary and Secondary Transcriptions in VSV Infected Cells

(A) Primary transcription. BHK-21 cell monolayer cultures in 35 mm dishes were infected with VSV at a m.o.i. of 40 p.f.u./cell in the presence of 100 μg/ml cycloheximide. After viral adsorption, the radio-labeling medium (MEM containing 1% bovine serum, 5 μg/ml actinomycin D, 100 μg/ml cycloheximide) was added to the cultures, to which 5 μM K-252a or 15 μM KT5926 was added immediately. The cultures were preincubated for 30 min at 37°C for the viral invasion into the cell, then 10 μCi/ml [3H]uridine was added to the cultures. The cultures were incubated up to 4.5 h after the infection. At 1-h intervals, pairs of the dishes were taken out, and the cells were recovered and subjected to procedures for determination of radioactivity incorporated into the acid-insoluble fraction as described in Materials and Methods. (B) Secondary transcription. VSV-infected BHK-21 cell cultures were prepared as described in Fig. 1. The cultures were preincubated with actinomycin D (5 μg/ml) for 30 min from 4.5 h, then 100 μg/ml cycloheximide, 5 μM K-252a or 15 μM KT5926, and 1 μCi/ml [3H]uridine were added to the cultures, and they were incubated at 37°C for another 5 hours (up to 10 h after the infection). At 1, 3, and 5 h after the addition of the inhibitors, a pair of the cultures were taken out and subjected to the determination of radioactivity incorporated into the acid-insoluble fraction. Symbols: ◆, untreated infected control; ◇, K-252a-treated; ▲, KT5926-treated.

Fig. 3. Effect of K-252a and KT5926 on the RNase-Sensitive and RNase-Resistant Viral RNA Synthesis in the Infected Cell

VSV-infected BHK-21 cell cultures were prepared as described in Fig. 1. After an initial incubation for 4.5 h at 37°C, actinomycin D (5 μg/ml) was added to the cultures, which were incubated for another 30 min. At 5 h, 5 μM K-252a or 15 μM KT5926 and 1 μCi/ml [3H]uridine were added to the cultures, which were then incubated for 5 h (up to 10 h after the infection) at 37°C. At each time point indicated (5, 8, and 10 h), pairs of the cultures were taken out and washed three times with PBS (+). The cells were then recovered with rubber scrapers, lysed with 0.5% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.4), and divided into two parts. They were mock-treated or treated with RNase A as described in Materials and Methods. Samples were then subjected to determination of the radioactivity incorporated and retained in the acid-insoluble fraction. A: control (◇); B: K-252a-treated (◇); C: KT5926-treated (◇). The symbols ◇, ◆ and ▲ are for the mock-treated samples, and the symbols ◆, ◇ and ▲ are for the RNase-treated samples.

[3H]uridine for 4 h. 5 μg/ml actinomycin D and 100 μg/ml cycloheximide were also included in the culture medium throughout the period of virus replication (the latter was added to block the genomic RNA synthesis that depends on the continued supply of newly synthesized viral N protein). As shown in Fig. 2A, both inhibitors reduced the rate of [3H]uridine incorporation by the VSV-infected cells. The grade of inhibition, however, was somewhat different between the two inhibitors; the inhibition by KT5926 was stronger than that by K-252a under the tested conditions.

Studies on the Viral Secondary Transcription We next examined the effect of K-252a and KT5926 on the secondary transcription in the VSV infected cells. Experiments were performed in a similar way to that noted for the primary transcription assay, except for the time of inhibitor addition at 5 h of infection (actinomycin D was added to the cultures at 4.5 h, 30 min prior to the addition of the inhibitors and cycloheximide). Figure 2B shows that the secondary transcription was suppressed by K-252a and KT5926 in a similar manner to that seen in the primary transcription experiments.

Next we examined possible effects on viral genome RNA synthesis. After preincubation for 5 h, the infected cultures were treated with 5 μM K-252a or 15 μM KT5926, then labeled with [3H]uridine in the presence of 5 μg/ml of actinomycin D. These cells were then recovered with rubber scrapers, lysed with 1% Triton X-100 in NTE buffer, and split into two equal parts. Each was mock-treated or treated with RNase A as described in Materials and Methods. The radioactivities in the RNase-treated and the untreated samples were determined. As shown in Fig. 3, both the RNase-sensitive (mostly of the viral mRNAs) and RNase-resistant radioactivities (mostly of the nucleoprotein-associated RNA) were greatly decreased in the drug-treated cultures. From these observations, it is suggested that the drugs affected both the viral transcription and genome RNA synthesis.

Studies on the Virion-Associated Transcriptase Next, we examined whether the virion-associated transcriptase is affected by K-252a and KT5926 under in vitro assay condi-
Fig. 4. Studies on the Effect of K-252a and KT5926 on the Virion-Associated Transcriptase

The effect of PKI on virion-associated transcriptase was examined under in vitro assay conditions. In vitro transcription assays were performed as described in Materials and Methods. The reaction mixture (200 μL) contained: 70 mM NaCl, 100 mM Tris- HCl (pH 7.4), 5 mM MgCl₂, 50 mM DTT, 0.1% NP-40, 0.5 mM ATP, 1 mM CTP, 1 mM GTP, 0.1 mM UTP, including 1 μCi of [3H]UTP, and 7.8 μg protein of purified virions, plus the inhibitors tested (the mixture contained 1 or 2% ethanol derived from the inhibitor solution, which did not affect the transcriptase activity. K. Kawa, unpublished observation). The reaction mixtures were incubated at 30°C for the times indicated. Radioactivities incorporated into the acid-insoluble fraction were determined according to the procedures described in Materials and Methods. Zero-time samples were determined for the background counts, which usually ranged from 50 to 100 cpm. (A) Dose-response studies: various concentrations of K-252a or KT5926 were added to the reaction mixtures, which were incubated for 60 min at 30°C. Symbols: square, K-252a; ▲, KT5926. (B and C) Time course studies: 5 μM K-252a (B) or 16 μM KT5926 (C) was added to the reaction mixtures, which were incubated at 30°C for various periods up to 120 min. Symbols: ●, untreated control; □, K-252a; ▲, KT5926.

Fig. 5. SDS-PAGE and Autoradiographic Analysis of the Effects of PKI on Viral Protein Synthesis

BHK-21 cell monolayer cultures were prepared in 35 mm Petri dishes, and were infected on the following day with VSV at a m.o.i. of 5 p.f.u./cell. Mock-infected cultures were also prepared in parallel. The cultures were incubated at 37°C in Eagle's MEM supplemented with 1% bovine serum. PKI were added to the cultures at 0 time (a), 5 h (b) or 4.5 h (c) after the infection. At 5 h, the culture medium was replaced by a labeling medium, to which [35S]methionine (20 μCi/ml) was added, and the cultures were incubated for another hour for radio-labeling. After being washed twice with PBS−/−, the cells were recovered and lysed with a sample lysis buffer for SDS-PAGE. After electrophoresis, the gels were stained with Coomassie Brilliant blue and dried onto Whatman 3MM filter paper, then exposed to an imaging plate (type BAS-III; Fuji Photo Film Co., Ltd.) for autoradiography. Autoradiograms were analyzed in a Bio Imaging Analyzer (BAS-2000) as described in Materials and Methods. Lanes 1, 2 and 3: mock-infected control; lanes 4, 5 and 6: VSV infected. Treatment with inhibitors: no inhibitor (control; lanes 1 and 4); 5 μM K-252a (lanes 2 and 5); 15 μM KT5926 (lanes 3 and 6).

Effects on the Viral Protein Synthesis We next examined whether these inhibitors affect viral protein synthesis. The infected cells were labeled with [35S]methionine and then lysed with Laemmli's sample lysis buffer and subjected to SDS-PAGE and autoradiographic analysis. As shown in Fig. 5a, viral protein synthesis was greatly decreased when the inhibitors were added at the early phase of infection.

When the inhibitors were added to the cultures at 4 to 5 h after the infection (the time when the viral protein synthesis reached almost the maximum level), such profound inhibition was not seen within as short a period as 1 h for the pulse-labeling (Fig. 5b). We observed, however, gradual decreases in viral protein synthesis when the infected cells were preincubated for 30 min in the presence of the inhibitor before starting the pulse-labeling (Fig. 5c). On the other hand, host protein synthesis in uninected cultures was not significantly affected by these inhibitors as shown in Fig. 5 (lanes 1, 2 and 3 of 5a, 5b and 5c). Accordingly, the inhibition seemed to be a secondary effect due to the decreased rates of viral RNA synthesis.

Since NS protein is a unique phosphoprotein of VSV and is known to be composed of multiple forms differing in the extent of phosphorylation, we thought that the inhibitors may affect the phosphorylation of NS protein, which might result in an alteration of viral RNA synthesis. This possibility was examined by 2-D electrophoresis. The infected cells were metabolically labeled with [35S]methionine in the presence or absence of the inhibitors. The cells were then lysed and applied to a 2-D gel for NEPHGE, then subjected to autoradiographic analysis. In the experiments involving 2-D gel electrophoresis, however, we could not differentiate between multiple forms of NS protein because of their poor separa-
Fig. 6. Two-Dimensional Analysis of Phosphorylated Proteins Produced in the Uninfected and VSV-Infected BHK-21 Cells

Monolayer cultures of BHK-21 cells were infected with VSV at an m.o.i. of 10 p.f.u./cell. After incubation for 3 h, the culture medium was replaced by phosphate-free MEM, and the cultures were reincubated for 60 min. Then, the medium was changed again, to which 10 µCi/ml [32P]orthophosphate was added. After incubation for 90 min, the cultures were washed with PBS(−), and the radio-labeled cells were lysed and applied to the 2-D NEPHGE as described in Materials and Methods. The 2-D NEPHGE gels were dried onto Whatman JMM filter paper and exposed to an imaging plate for autoradiographic analysis as described in Materials and Methods. (A) uninfected cells; (B) infected cells. Spots indicated by the letters a, b, c, and d in the photo (B) denote cellular phosphorylated proteins (b corresponds to the location of vimentin), which were used as the endogenous references for the evaluation of the effects of PKI on NS protein phosphorylation as shown in Table 1, while the letter e indicates the NS protein of VSV.

Fig. 7. Effects of K-252a on NS Protein Phosphorylation in the Cell

Infected cultures were prepared and labeled with [32P]orthophosphate as described in Fig. 6. After the addition of 5 µg K-252a at 4 h of infection, [32P]orthophosphate (40 µCi/ml) was added to the cultures, which were then incubated for 90 min. The cells were then recovered with rubber scrapers and subjected to the procedures for 2-D NEPHGE and autoradiography as described in Materials and Methods. (A) infected control; (B) K-252a-treated. Arrow heads indicate the NS protein.

Accordingly, we changed the radioactive material from [35S]methionine to [32P]orthophosphate for labeling viral proteins. Figure 6 compares the spots of [32P]labeled proteins produced in the virus infected and mock-infected cells. A highly radio-labeled spot seen in Fig. 6B (indicated by a letter c) was identified as NS protein based on its presence only in the infected, but not the uninfected, cells and also on its isoelectric point and migration rate to the second dimension of the 2-D gel.

By using this method, we investigated the effects of PKI on the production of phosphorylated viral proteins. When the infected cells were treated with K-252a, the phosphorylation of NS protein, as well as cellular vimentin (indicated by a letter b in Fig. 6B), was greatly decreased (Fig. 7B). Determination of the radioactivity of NS protein in Figs. 7A and 7B showed that K-252a reduced the phosphorylation of NS protein by more than 80% (Table 1). On the other hand, KT5926 did not significantly affect the NS protein phosphorylation, while the phosphorylation of vimentin was notably suppressed (Fig. 8B and Table 1). These results suggest that the K-252a treatment reduced the production of phosphorylated NS protein, probably by affecting the protein kinase(s) involved in NS protein phosphorylation, while KT5926 did not seem to affect such enzyme(s).

Studies on the CK II

Since cellular CK II is suggested to be involved in the first step of NS protein phosphorylation,17 we checked here whether the enzyme is sensitive to K-252a and KT5926 under in vitro reaction conditions. As shown in our previous study also,11 the CK II activity was not inhibited by KT5926 at any concentrations from
Fig. 8. Effects of KT5926 on NS Protein Phosphorylation in the Cell

Infected cultures were prepared and labeled with [32P]orthophosphate as described in Fig. 6. After the addition of KT5926 (15 μM) at 4 h, [32P]orthophosphate (40 μCi/ml) was added, and the cultures were incubated for 90 min. Then, the cells were recovered and subjected to 2-D NIEHPGE and autoradiographic analysis as described for Fig. 7. (A) infected control; (B) KT5926-treated. Arrow heads denote the NS protein.

Table 1. Comparison of the Effects of PKI K-252a and KT5926 on the Phosphorylation of Cellular and Viral NS Proteins

<table>
<thead>
<tr>
<th>PKI</th>
<th>Spot</th>
<th>Radioactivity (32P)</th>
<th>B/A (%)</th>
</tr>
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<tbody>
<tr>
<td>K-252a</td>
<td>a</td>
<td>907</td>
<td>496</td>
</tr>
<tr>
<td>(5 μM)</td>
<td>b</td>
<td>1401</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>c</td>
<td>717</td>
<td>616</td>
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<td>d</td>
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<td>681</td>
<td>721</td>
</tr>
<tr>
<td></td>
<td>d</td>
<td>845</td>
<td>802</td>
</tr>
<tr>
<td></td>
<td>e (VSV NS)</td>
<td>468</td>
<td>3055</td>
</tr>
</tbody>
</table>

a) Radioactivity of five selected spots on Figs. 7A, B, K, and L was determined by the Bio-Imaging Analyzer BAS2000. Selected spots are indicated by arrows and letters in Fig. 6; spots a, b, c, and d are of cellular phosphorylated proteins (b corresponds to vimentin) selected as the endogenous reference of PKI-sensitive and resistant components, while spot e corresponds to the viral NS protein.

0.1 to 10 μM, while K-252a slightly reduced the activity only at a higher concentration, such as 10 μM, which is several hundred times higher than that required for the sensitive protein kinases (Fig. 9). On the other hand, the activity was strongly suppressed by heparin at concentrations of 1 to 10 μg/ml (data not shown). These results suggest that the CK II is not responsible for either the K-252a-sensitive or KT5926-sensitive activities involved in VSV RNA synthesis.

DISCUSSIONS

1) The present study showed that an MLCK inhibitor (KT5926) and a broad non-specific PKI (K-252a) suppressed the VSV replication by affecting the viral RNA synthesis. These inhibitors did not seem to directly affect the translational process, and the decrease observed in viral protein synthesis seemed to be a secondary effect due to the reduced viral RNA synthesis. Since we have observed that both K-252a and KT5926 did not affect the Sindbis virus replication in culture, it is strongly suggested that the substrate supplies for the viral RNA and protein synthesis were not affected by these agents.

Both the viral transcription and genomic RNA synthesis in the cell were suppressed soon after the addition of K-252a or KT5926. The viral RNA replicase involved in genomic RNA synthesis is believed to be the same enzyme that is involved in the viral transcription.27) A major difference between the viral genomic RNA synthesis and the transcription is that the former requires a continuous supply of viral N protein.28,29) As noted above, the N protein supply was not rapidly affected by these inhibitors, but rather decreased gradually due to a reduction in the viral transcription. Based on these results and considerations, we assume that the rapid decrease in genomic RNA synthesis is caused by a mechanism other than the blockade of N protein supply.

2) Although viral RNA synthesis was similarly inhibited by K-252a and KT5926, the inhibitory mechanism may not be the same, because K-252a caused decrease in NS protein phosphorylation, while KT5926 did not. Accordingly, we
suppose that at least two kinds of protein kinases are involved in the VSV replication process; one is K-252a-sensitive but KT5926-resistant, and another is KT5926-sensitive (the latter might also be K-252a-sensitive). From the result shown in Fig. 9, CK II will not be included in such kinases. Since the only NS protein is a phosphorylated protein of VSV, and its activity is believed to be regulated by phosphorylation and dephosphorylation, suppression of the phosphorylation of NS protein by K-252a would account for the reduced rate of viral RNA synthesis.

3) K-252a may directly inhibit the enzyme(s) involved in NS protein phosphorylation. As to which enzymes may be affected, we have to consider at least two protein kinases. Barik and Banerjee\textsuperscript{4,17} proposed that NS protein is phosphorylated in a stepwise manner by two kinds of protein kinases; the first step is assumed to be catalyzed by cellular CK II, and the second step by the viral L protein-associated kinase activity. In this study, we observed that CK II activity was not inhibited by KT5926 and was only weakly inhibited by K-252a at a high concentration, such as 10 \( \mu \text{M} \) under \textit{in vitro} assay conditions, while the enzyme was strongly inhibited by heparin. The effective concentration of K-252a required to inhibit the VSV replication in culture, however, was much higher than the \( K_i \) values determined in the \textit{in vitro} assay conditions against several kinds of protein kinases.\textsuperscript{2-4} The requirement of these higher concentrations for cultured cells may be due to inefficient invasion of the inhibitors into the cell. Usui \textit{et al.}\textsuperscript{30} have also reported the use of higher concentrations of K-252A for the blockade of cell cycle events in culture. Based on these considerations, we think that K-252A does not inhibit CK II activity in the cell, even when added to the culture medium at as high a concentration as 5 \( \mu \text{m} \). Accordingly, we suppose that, in addition to CK II, other K-252a-sensitive but KT5926-insensitive kinase(s) might also be involved in NS protein phosphorylation, in which the L protein-associated kinase might be included. At present, however, our studies have not excluded the possibility that heparin-insensitive kinase III\textsuperscript{19} is involved in NS protein phosphorylation.

4) Since KT5926 did not affect NS protein phosphorylation, the KT5926-sensitive function(s) may be involved in some other event(s) required for the efficient initiation or continuation of viral RNA synthesis. In this study, we observed that the primary transcription \textit{in vivo} was inhibited by K-252a and KT5926, while the virion transcriptase activity was not inhibited under \textit{in vitro} assay conditions, although both activities are thought to be performed by the same virion-associated transcriptase. Rigaut \textit{et al.}\textsuperscript{31} also obtained similar results using staurosporine (a metabolite isolated from \textit{Streptomyces} sp. which is known to be a strong inhibitor against certain protein kinases, including protein kinase C). As suggested by Massey \textit{et al.},\textsuperscript{13} NS proteins in the virion have been phosphorylated sufficiently before being incorporated into the virion. We think, however, that the \textit{in vitro} transcription conditions we used may not be complete for reproducing the complete molecular events which occur in the cell; after viral invasion into the cell, the transcriptional apparatus may be activated or enhanced by some KT5926-sensitive function(s) in the cell.

KT5926 is known to preferentially inhibit cellular MLCK (a kind of protein kinase C but highly substrate-specific), while other types of protein kinase C, as well as protein kinases A and G, are little affected by KT5926 at the low concentrations which were effective against the MLCK under \textit{in vitro} test conditions.\textsuperscript{2-4} MLCK is one of the KT5926-sensitive functions in non-muscle cells, and specifically phosphorylates the myosin light chain. In our preliminary experiments, we also observed the disruption of actin-containing stress fibers in KT5926-treated BHK-21 cells. From these considerations, we suppose that viral RNA synthesis is performed in close association with cytoskeletal structures, and such association depends on KT5926-sensitive function(s). Rigaut \textit{et al.}\textsuperscript{31} also described the possible involvement of cellular function(s) in viral RNA synthesis, which might also be impared similarly by staurosporine.

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REFERENCES