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PKA Inhibitor KT5720, Suppressed Cytoskeletal Components Effect by Vesicular Stomatitis Virus, but did not Affect the Viral Replication

Young Sook Kim
Department of Oriental Medical Food and Nutrition, Asia University, Kyungsan-si, Kyungbuk 712-220, Korea
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The antiviral mechanism of KT5720 is known to inhibit the cAMP-dependent protein kinase (PKA), on the VSV infection in BHK-21cell cultures. The virus inducted CPE (cell rounding) was almost completely suppressed by KT5720 at 5 uM. The inhibitor, however, did not affect the replication of the virus and the synthesis of viral macromolecules. Immunological studies showed the viral matrix (M) protein displayed intimate association with the cytoskeletal components and probably the cell rounding. KT5720, did not block the cytoskeletal disruption, while the cell rounding was suppressed. These observations suggest that the interaction between the viral M protein and the cytoskeletal components may not be enough to cause the morphological change of the cell. And, the KT5720-sensitive function may be involved in developing the VSV-induced CPE, but not essential for the virus replications.

Key Words : Vesicular stomatitis virus (VSV), protein kinase inibitor, KT5720, cytoskeletal, matrix (M) protein

INTRODUCTION

VSV is a prototype of the Rhabdoviridae, which also belong to 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(1). In our previous studies, we investigated the effect of K-252a (a metabolite of Nocardiopsis sp., which is known to inhibit a broad spectrum of protein kinases) and its derivatives of different inhibitory spectra (K-252b, KT5720, KT5823 and KT5926)(2-4) in order to search for inhibitory agents which might affect the replication of rhabdoviruses(5). In the case of productive infection of rhabdoviruses, morphological changes of host cells is usually associated with the virus replication, such as the cell rounding, and finally with cell death and cytolysis. Sequential destruction of cytoskeletal structures seems to be related to these morphological changes of the infected cell(5, 6). At least two events have been suggested to be involved in the VSV-induced CPE(7). The cytoplasmic events which would cause dysfunction of cellular organelles including cytoskeletons and may also cause morphological changes of the cell. In any case the shutoff of the host macromolecule synthesis may result in gradual destruction of the dynamic homeostasis of organized cellular structures and functions. M protein has been suggested to be an involved essentially in the regulation of viral RNA synthesis(8, 9) and virion formation through budding process at the cell membrane in collaboration with the viral glucoprotein (G) and nucleocapsis(10, 11). Its interaction of M protein with both the viral nucleocapsid and G protein have been demonstrated *in vivo* and *in vitro*(10, 12-14). From the studies with temperature-sensitive mutants, however, M protein has been thought to display cytotoxicity in the infected animal cells(15). Recently, the cytotoxicity could be reproduced in the cells by transfecting the M cDNA with a help of expression vector(8). They also suggested that the C-terminal deletion eliminated the harmful activity of the protein. The mechanism of cytotoxic action of M protein is still unclear, but recent report suggests that M protein interacts with cellular tubulins, resulting in depolymerization of microtubules and cell rounding(16).

In my previous studies(17) on the antiviral screening of several protein kinase inhibitors against the VSV replication in cultured animal cells, I found that KT5720 suppressed the VSV-induced cytopathic effect (CPE) without affecting the virus replication and progeny yield. I further investigated the

† Corresponding Author : Department of Oriental Medical Food and Nutrition, Asia University, Kyungbuk 712-220, Korea,
Tel : +82-53-819-8204, Fax : +82-53-819-8135
E-mail : 2004yskim@naver.com

inhibitory mechanism of KT5720 on the VSV induced cytoskeletal components.

MATERIALS AND METHODS

Viruses, cell culture and infectivity assay

A BHK-21-adapted clone of VSV (New Jersey serotype) was used throughout this study(17). Stockes of the virus were prepared by infecting to BHK-21 cells at a low m.o.i.,and assayed by plaque formation on BHK-21 cell monolayers. BHK-21 cell was propagated in Eagle's MEM containing 10% Tryptose phosphate Broth (TPB; Difco Laboratories, Detroit) and 5% calf serum and maintained in MEM containing 1% fetal bovine serum (Kawai, A. laboratory, Kyoto University) after the virus infection.

Metabolic labeling of viral proteins with radioactive precursor

Radioactive viral proteins were prepared for studies of autoradiography and immunoprecipitation. Infected cultures were prepared as described above, and fed with fresh medium containing 1% serum. At zero time of incubation, protein kinase inhibitors were added at various concentrations as noted on the text. After incubation for 4.5 hours at 37℃, the medium was replaced by the radio-labelling medium which contained a low concentration (1.5 mg/ml) of cold L-methionine and 1% serum. After further incubation for 30 min, ^{35}S-methionine was added to the culture, which were then incubated for metabolic labeling of infected cells for one or two hourses as noted in the text. The cells were lysed with SDS-sample buffer for SDS polyacrylamide gel electrophoresis (PAGE).

Immunoblot analysis

Infected and mock-infected cells were lysed with SDS-lysis buffer. The lysates were applied to 10% SDS-PAGE, and then blotted onto nitrocellulose filter (S & S). After the blocking procedures, the filter was processed for immunological detection voral proteins with rabbit antisera against the VSV G, M and NS proteins and peroxidase-conjugated anti-rabbit antibody. Color was developed as described previouly(4).

Antisera

Anti-VSV M antiserum was prepared by immunizing rabbits with polypeptides which were produced in *E. coli* using the VSV M cDNA inserted into an expression vector (pET3a). Anti-VSV G antiserum was prepared by immunizing rabbits with the G protein sample which was extracted from SDS-PAGE gels after the electrophoresis of purified VS virions. Anti-VSV NS antiserum was prepared by immunizing rabbits with synthetic oligopeptides which were systhesized by mimicking the amino acid sequence of NS protein. Antibodies against the actin, tubulin, and vimentin were purchased from a commercial source (Amersham), FITC and rhodamine-conjugated second antibodies were also purchased from a commercial source (Cappel, MP Biomedicals, Solon, OH 44139 USA).

Immunofluorescence studies

Infected BHK-21 cells were prepared on converslips and fixed at the time indicated in the text with acetone or 3% parafolmaldehyde for 10 to 15 min at room temperature (In the case of the paraformaldehyde-fixed specimens, cell membrabe was permeabilized after the fixation by treatment with 1% Triton x-100 (PBS). The fixed specimens were first stained with the first antibody against the viral proteins or cytoskeletal components for 60 min at 37℃, and then washed for one hour. Then, the specimens were stained with FITC or rhodamine-conjugated second antibody for 30 min at 37℃, and were washed (PBS) for 30 min. The immunostained specimens were observed under a epifluorescence microscope (Nikon Corporation, Tokyo, Japan).

Immunoprecipitation

The ^{35}S-methionine-labeled cells in 35 mm dishes were recovered by lysing with SDS-free IP buffer (200 ul). From each lysate 20 ul was taken and mixed with rabbit anti-M antiserum and incubated overnight at 4. After adding ten volumes of 10% formalin-fixed ghost cell suspension of Staphylococcus aureus (sigma), the mixtures were then incubated on a rotator for one hour at 4℃, then washed three times with IP buffer, and finally dissolved in Laemmli's sample lysis buffer for SDS-PAGE(18). After the electrophoresis, the gel was dried onto whatman 3 mm filter paper and exposed to an imaging plate for autoradiographic analysis as described above.

Chemicals and buffers

PBS is composed of 140 mM NaCl, 2.6 mM KCl, 8 mM sodium phosphate, 0.5 mM $MgSO_4$ and 0.5 mM $CaCl_2$ (pH 7.8). PBS(-) means that magnesium and calcium are omitted from PBS. NTE contained 150 mM NaCl, 10 mM Tris-HCl (pH 7.8) and 1 mM EDTA. TE contained 10 mM Tris-HCl (pH 7.4) and 1 mM EDTA. IP buffer is composed of 0.1% SDS, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate and 10 mM Tris-HCl (pH 7.5). K-252a-derived protein kinase inhibitors, KT5720 and KT5926(13, 19) were purchased from Kyowa Medex Inc. (Tokyo). ^{35}S-methionine or Tran^{35}S-label (specific activity = ~1000 Ci/mmol) and [5-^{3}H]uridine (specific activity = ~30 Ci/mmol were of ICN Radiochenicals (Irvine, CA).

RESULTS

Effect of K-252a derivatives on the VSV infection in culture

In hour previous studied on the antiviral activity of K-252a derivatives against the VSV infection in cell cultures, we found that VSV replication was inhibited by a parent compound (k-252a), that is known to inhibit a broad spectrum of protein kinases. Similar results were also obtained with a myosin light chain kinase inhibitor, KT5926(5). Contrasting effect was observed with KT5720, a protein kinase A inhibitor, that suppressed the VSV-induced CPE (cell rounding), but allowed almost a full achievement of VSV replication. The IC50 of KT5720 determined on the basis of CPE suppression was about 0.55 uM, while that of KT5926 was 1.5 uM(7). Difference was observed when the yield of progeny virus was assayed(Fig. 1). In the control cultures, the yield of progeny virus usually reached the maximum level at around 8 to 10 hours of infection. KT5926 decreased the yield in consistent with suppression of the CPE (15 uM KT5926 reduced the yield by 90% or more even at 12 hr), while 5 uM KT5720 only slightly inhibited the virus replication, and the yield reached the maximum level at around 12 hr. Similar effect was observed with these inhibitors in the viral RNA synthesis. Time courses studies performed with these compounds at the higher m.o.i. of VSV infection demonstrate that KT5926 similarly suppressed the viral replication.

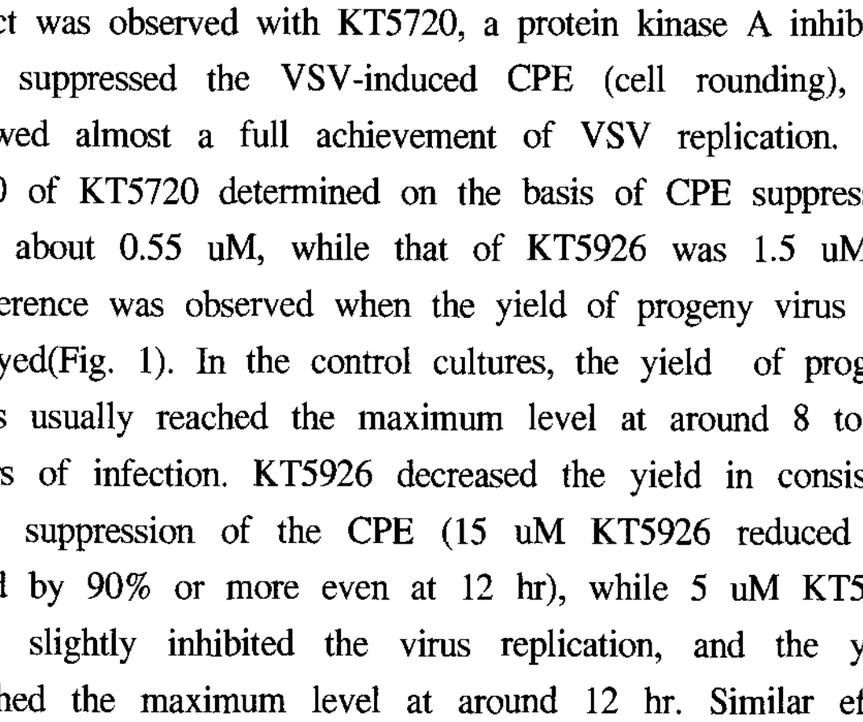
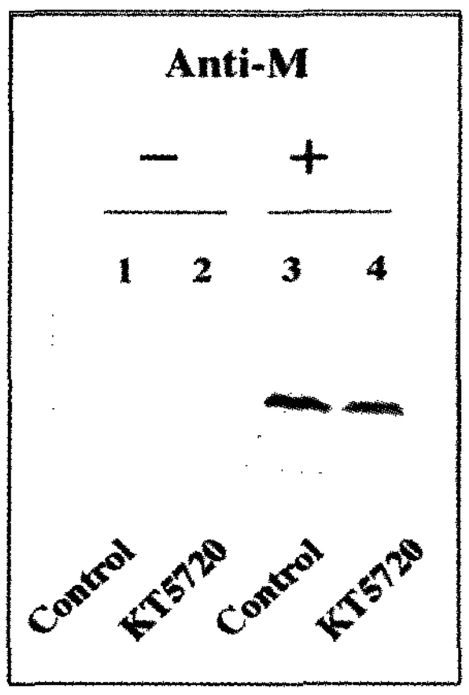


Figure 1. Time course study of the effect of KT5727 and KT5926 on the VSV replication. Monolayer cultures of BHK-21 cell were prepared in 35 mm dishes and, on the following day, infected with VSV at on m.o.i. of 10 p.f.u. per cell. After the viral adsorption, maintenance medium was added to the cultures, to which 5□□M KT5720 or 15□□M KT5926 was added. The cultures were incubated at 37℃ for 12 hours, from which culture fluids were recovered at 3 hr interval and assayed for the progeny yield by plaque formation (symbols: □, control; ●, KT5720; △, KT5926).

Studies on the effect of KT5720 on the viral protein synthesis

Immunoblot analysis anti-M antisera did not show any difference between the viral proteins produced in the KT5720-treated and mock-treated infected cells(Fig. 2). Semi-Quantitative immunoblot assay indicated that the rate of viral protein synthesis did not seem to be altered by KT5720. These results suggest that viral protein synthesis was not affected by KT5720, in other words, the KT5720-sensitive functions are not involved in the viral protein synthesis.



– ; mock-infection
+ ; VSV-infestion

Figure 2. Immunoblot analysis of viral protein synthesis in the KT5720 and KT5926-treated cultures. VSV infected mock infected cells were prepared as described for (9) and incubated in the presence or absence of either 5 μM KT5720 or 15 μM KT5926 for 5 hr at 37℃. Then, the cells were recovered with rubber scrapers, and lysed with a sample lysis buffer. The lysates ware subjected SDS-PAGE and immunoblot analysis with rabbit antibodies against the viral M protein as described under materials and methods. Immunoblot stained with anti-M antibody: lane 1, uninfected untreated control; lane 2, uninfected KT5720-treated control; lane 3, VSV infected control; lane 4, VSV infected and KT5720-treated.

Studied on the distribution of viral antigens in the cell

We next performed immunofluorescence studies on the viral protein synthesis. Immunoblot analysis with anti-M, anti-G and anti-NS antisera did not show any difference between the viral proteins produced in the KT5720-treated and mock-treated infected cells(7). Semi-Quantitative immunoblot assay indicated that the rate of viral protein synthesis did not seem to be altered by KT5720. These results suggest that viral protein systhesis was not affected by KT5720, in other words, the KT5720-sensitive functions are not involved in the viral protein synthesis. Since the viral matrix protein (M) is thought to be responsible for the virus-induced cell rounding(16), we next checked whether the inhibitor affected the intracellular behaviours of viral M protein. Fig. 3 (VSV-infected) compares the distribution of M antigen mostly display fine dot-like and sometimes granular distribution in the cytoplasm(Fig. 3. mock-infected), and the granular antigens gradually become abundant, but most of such cells seemed to eventually take

rounded cell morphology. In the KT5720-treated infected cultures, however, most cells also displayed similar dot-like or granular distrubution of viral M antigen in the cytoplasm(Fig. 3. VSV-infected). KT5720 did not cause any changes in the uninfected cultures for at least 12hr of incubation. These observations indicate that KT 5720 suppressed the VSV-induced cell rounding without affecting the distribution of M protein(data not shown).

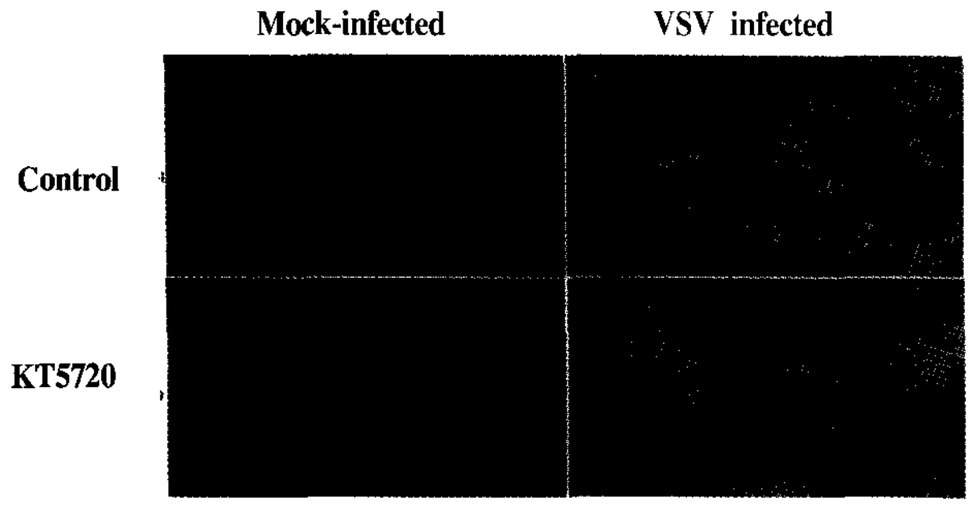


Figure 3. Immunofluorescence study on the distribution of viral M antigen. BHK-21 cells sown on coverslips were mock-infected with VSV (m.o.i. =5 p.f.u./cell), and were incubated in the presence and absence of 5 μM KT5720 for 6 hours. Then, the cells were fixed with acetone and were stained with rabbit anti-M antibody (first antibody) and then with rhodamine-conjugated second antibody (A: VSV infected control; B: VSV infected KT5720-treated; C: mock-infected untreated control; D: mock-infected and KT5720-treated).

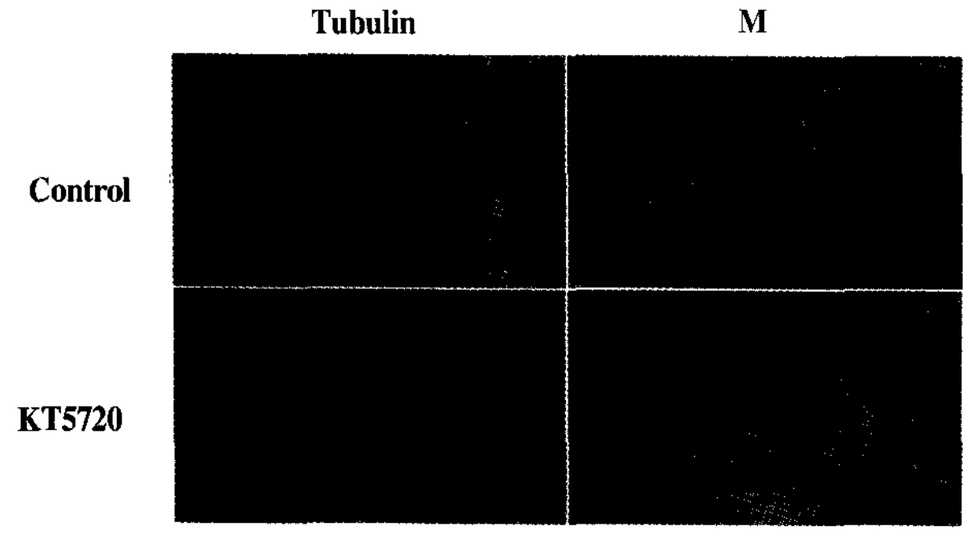


Figure 4. Immunofluorescence study of the cytoskeletons in the VSV infected and KT5720-treated cells. VSV infected BHK-21 cells were prepared as described for Fig. 3, and fixed with acetone (or with 3% paraformaldehyde and membrane permeabilization with 1% Triton X-100 for immunostaining with anti-action antibody) for 15 min at room temperature. Specimens were doubly stained with rabbit anti-VSV M antibody and mouse antibody against the β-tubulin, vimentin or actin, which was followed by the second staining with goat rhodamine-conjugated and FIFC-conjugated antibodies against the rabbit Ig and mouse Ig, respectively. Top row (A, B, C, and D): anti-M antibody; middle row (E, F, G, and H): anti tubulin antibody; bottom row (I, J, K, and L): anti-vimentin antibody. Left half columns (A, B, E, F, I and J): VSV infected: right half columns (C, D, G, H, K and L): mock-infected. The first and third columns (A, C, E, G, I and K): mock-treated control; the second and fourth columns (B, D, F, H, J and L): KT5720-treated.

Effects of KT5720 on the cytoskelatal structures

Since morphological changes of virus infected cells may reflect the alteration of cytoskeletal structures, we next examined whether the distribution of cytoskeletal components was affected by VSV replication. Fig. 4 shows that microtubular networks were disrupted in the viral antigen-positive cells, and the tubulin antigen became to be detectedin the dot-like and/or granular structures in the cytoplasm, which seemed to occur at around 6 hr of infection in accordance with the onset of cell rounding. Intermediate filament networks were also displayed granular appearance as seen in the specimens stained with anti-tubulin antibody. In addition, both the tubulin and vimentin antigens displayed a colocalized distribution with viral M antigen in the cytoplasmic granular structures. Structures of actin filament were very faint in the fluorescent antibody-stained specimens, accordingly we could not examine so far whether the actin filament is disrupted in the VSV infected cells. When 5 uM KT5720 was added to the infected cultures, the morphological change (cell rounding) was abolished(Fig. 3 and 4), however, the disorganized structures of the cytoskeletal components were still observed, and the colocalized distribution of the viral M antigen and cytoskeletal antigens was not changed at all(Fig. 4). In orther words, the inhibitor did not block the VSV-induced disruption of microtubules and intermediate filaments.

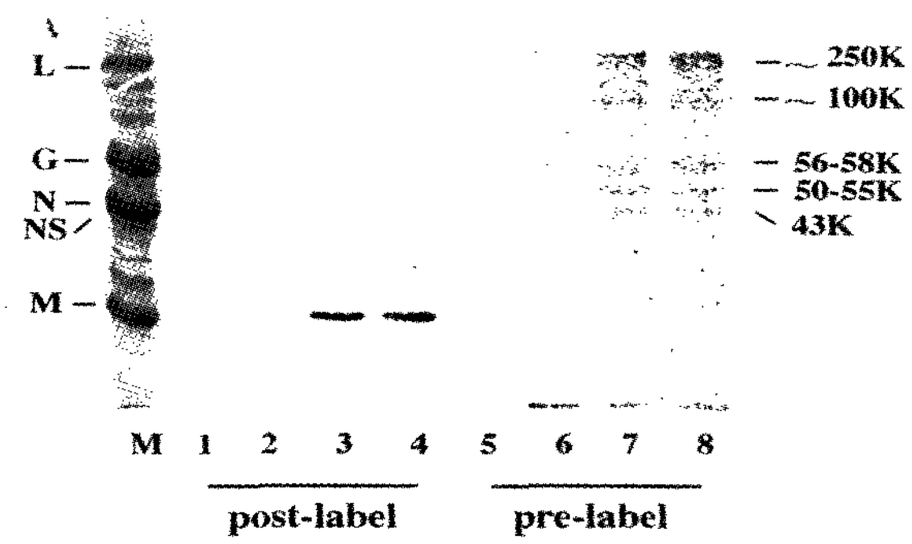


Figure 5. Immunoprecipitation studies on the interaction of viral M prorein with cellular components. BHK-21 cells sown in 35 mm dishes were preincubated with ^{35}s-methionine for 6 hours, and then infected with VSV at an m.o.i. of 5 p.f.u./cell, and incubated in the presence or absence of 5μM KT5720 for 6 hours at 37℃. Post-labeled cells were also prepared in parallel; BHK-21 cells were infected with VSV and incubated for 6 hours in the presence or absence of KT5720, and labeled with ^{35}s-methionine for one hour from 5 hr to 6 hr of infection. Then, the cells were recovered by lysing in 200 μl SDS-free IP buffer and subjected to the immunoprecipitation with anti-M antibody, and the precipitates obtained were applied to SDS-PAGE and autoradiographic analysis as described under materials and methods (Lane M: VS virion proteins as the molecular weight marker. Lanes 1 to 4: post -labeling; lanes 5 to 8 prelabeling. Lanes 1 and 5: uninfected mock-treated control; lanes 2 and 6: uninfected KT5720-treated control; lanes 3 and 7: VSV infected and mock-treated; lanes 4 and 8: VSV infected and KT5720-treated).

To confirm these observations, we performed immuno-precipitation experiments with anti-M antibody, which showed that the antibody coprecipitated at least five species of cellular

components; their apparent molecular weight was 43 k, 50-55 k, 56-58 k, ~100 k, and ~250 k (Fig. 5, lane 5). Immunoprecipitation with antibodies against the cytoskeletal components indicated that the 43 k, 50-55 k and 56-58 k-Da polypeptides correspond to actin, tubulin and vimentin, respectively (data not shown). Other two of higher molecular weight were not yet identified. Treatment with KT5720 did not abolish the coprecippitation(Fig. 5, lane 6), suggesting that the inhibitor did not affect the interaction between the viral M protein and cytoskeletal components.

DISCUSSION

Our present study showed that KT5720 did not affect the interaction between the viral M protein and cytoskeletal components(Fig. 4 and 5), however, the agent suppressed the cell rounding. Immunological studies suggested that VSV replication caused disorganization of cytoskeletal structures, and the viral M protein displayed colocalized distribution with the tubulin and vimentin, which might result in rounding of the host cell. KT5720 did not block the VSV-induced cytoskeletal disorganization, however, it blocked the cell rounding. These results suggest that the interaction of M protein with cytokeletal components may not be enough for causing the morphological alteration of the cell, but may require some additional KT5720-sensitive function(s) or may affect other cellular structure or function. These points will also be discussed. This apparently contradictory observation may be accounted for by either of two possibilities. One is that the interaction between viral M protein and cytoskeletal components (tubulin and/or vimentin) is not enough to cause the cell rounding, and some KT5720-sensitive function(s) may be required to affect the cell morphology. Another possibility may be that, apart from the interaction with tubulin, M protein also interact with some other cellular component which is/are involved in keeping the flat cell shape, such as some transmembrane protein(s) contributing to cellular adhesion to the substratum, whose function would be regulated by the KT5720-sensitive function(s). If the latter assumption is true, such protein(s) may be coprecipitated with anti-M antiserum. Along this consideration, we can remind two additional unidentified polypeptides (about 100 k-Da and 250 k-Da) of host cell origin in the precipitates obtained with anti-M antibody(Fig. 5). Concerning this problem, I would like reconsider the recent studies performed in the Kawai's laboratory (Kyoto) on the functional interaction of rabies virus proteins with cellular or cytoskeletal components which may be involved in the viral replicative process. They have assumed recently that some of such components may also be incorporated into mature virions as have been reported for the action in the virion(21). And, some virion -associated host cell proteins have been identified: for instance, hsc73 (a constitutive type of that shock protein 70; 22), ezrin-rdixin-moesin family protein (actin-binding proteins(22)) and 130 k-Da polypeptide (a membrane-anchoring transmembrane protein for actin protein filament, which is identified as one of CD44 proteins(19)). These host-derived components were also detected in the highly purified VS virions(21, 22). Furthermore, most of these virion-associated cellular component were also shown to be associated with the viral products, especially with the envelope protein, in the cell(22). In addition to these, they have detected two unidentified polypeptides of 100 k and 20 k-Da in the rabies virion, they have performed immunofluorescent studies by using monoclonal antibodies we prepared. And, we have found so far that the 100 k-Da polypeptide seems to be a membrane component. Accordingly, it is of interest to know whether the 100 k-Da polypeptide in the virion is the same one which was coprecipitated with anti-VSV M antibody. Another polypeptide (250 k-Da) of the two the precipitates may also be a candidate.

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