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# Calcium Ionophore A23187 Inhibits Human Parainfluenza Virus Type 2 Growth and Monoclonal Antibody against CD98 Heavy Chain Recovers the Inhibition

Sahoko Kihira[1], Jun Uematsu[2], Yuko Ishiyama[1], Misaki Chindoh[1], Aya Baba[1], Rina Kazuta[1], Tomomi Hasegawa[1], Keiko Fujmoto[1], Aya Funauchi[1], Hidetaka Yamamoto[3], Mitsuo Kawano[4], Masato Tsurudome[4], Myles O'Brien[5], Hiroshi Komada[2]

[1]Department of Clinical Nutrition, Faculty of Health Science, Suzuka University of Medical Science, 1001-1 Kishioka Suzuka, Mie, 510-0293, Japan
[2] Microbiology and Immunology Section, Department of Clinical Nutrition, Graduate School of Suzuka University of Medical Science, 1001-1, Kishioka, Suzuka, Mie, 510-0293, Japan
[3]Faculty of Pharmaceutical Science, Suzuka University of Medical Science, 3500-3, Minamitamagaki, Suzuka, Mie, 513-8670, Japan
[4]Department of Microbiology, Mie University Graduate School of Medicine, 2-174, Edobashi, Tsu, Mie, 514-8507, Japan
[5]Graduate School of Mie Prefectural College of Nursing, 1-1-1 Yumegaoka, Tsu, Mie, 514-0116, Japan
*The first 2 authors equally contributed to the work.

**Abstract:** Calcium ionophore A23187 (A23187) inhibited human parainfluenza virus type 2 (hPIV-2) replication in $LLCMK_2$ cells and the inhibition was almost completely recovered by monoclonal antibody (mAb) against CD98 heavy chain (CD98HC). A23187 considerably reduced the number of viruses released from the cells. Virus nucleoprotein (NP), fusion (F) and hemaggulutinin-neuraminidase (HN) gene syntheses were not inhibited. However, a direct immunofluorescence study showed that A23187 largely inhibited virus NP, F and HN protein syntheses. Using a recombinant green fluorescence protein (GFP)-expressing hPIV-2 without matrix (M) protein (rghPIV-2DM), it was found that virus entry into the cells was not inhibited, and that cell-to-cell spreading of virus was not blocked by A23187 either. The effects of A23187 on both actin microfilaments and microtubules were observed, and it was found to cause disruption of both of them. The effect of CD98HC mAb on the inhibition induced by A23187 was analyzed. CD98HC mAb recovered the inhibition by A23187. Virus production was considerably recovered. Virus protein was synthesized by the addition of CD98HC mAb. Almost normal actin microfilaments and microtubules were observed in the presence of CD98HC mAb. These results suggested that the inhibitory effect of A23187 was mainly caused by the inhibition of virus protein synthesis and disruption of cytoskeleton. The precise mechanisms of the inhibitory effect of A23187 and recovery from A23187-induced inhibition by CD98HC mAb are unclear, but the modulation of cytosolic $Ca^{2+}$ concentration may be important for viral protein synthesis and for the preservation of cytoskeleton.

**Keywords**: human parainfluenza virus type 2, calcium ionophore A23187, CD98HC monoclonal antibody, a recombinant green fluorescence protein expressing hPIV-2 without matrix protein

## Introduction

Human parainfluenza virus type 2 (hPIV-2) is one of the major human respiratory tract pathogens of infants and children. hPIV-2 is a member of the genus *Rubulavirus* in the family *Paramyxoviridae*, and it possesses a single-stranded, non-segmented, negative-stranded RNA genome of 15,654 nucleotides [1]. hPIV-2 has 7 structural proteins, nucleoprotein (NP), V, phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large (L) proteins. The gene order of hPIV-2 is 3'-(leader)-NP-V/P-M-F-HN-L-(trailer)-5'. All genes of hPIV-2 were sequenced by our group [2-7]. Monoclonal antibodies (mAbs) were made, and antigenic diversity of clinical isolates was investigated, by Tsurudome [8]. The infectious hPIV-2 from cDNA clone was constructed by Kawano, and

Hiroshi Komada (Correspondence)
komada@suzuka-u.ac.jp
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it was shown that its growth property was the same as that of hPIV-2 [9,10].

Calcium ionophore A23187 (calcimycin) is a kind of antibiotics possessing weak antimicrobial activity against gram positive bacteria and fungi. A23187 acts as a divalent cation ionophore, allowing $Ca^{2+}$ to cross the cell membrane. It is used to increase $Ca^{2+}$ levels in cells. It causes mitogenic stimulation of lymphocytes and increases the uptake of $Ca^{2+}$ in the stimulated lymphocytes [11]. CD98, originally termed 4F2 antigen, is a 125kDa type II transmembrane protein composed of an 80kDa glycosylated heavy chain and a 45kDa nonglycosylated light chain [12,13]. CD98 is a multifunctional protein and its roles are as follows: amino acid transporter [14], integrin regulator [15], cell fusion regulator [16,17], and inducer of osteoclast [18]. CD98 controls calcium concentration: CD98HC mAb 4F2 specifically inhibits the $Na^{+}$-$Ca^{2+}$ exchanger activity [19], and binding of mAb 4F2 to its cell surface antigen on dispersed adenomatous parathyroid cells raises cytosolic calcium [20].

In the present investigation, the effect of A23187 on hPIV-2 replication was analyzed, and it was found that A23187 had an inhibitory effect on hPIV-2 growth. To investigate the effect of A23187 on virus RNA synthesis, Virus RNA was prepared and amplified by polymerase chain reaction (PCR). Virus protein expression was observed by direct immunofluorescence study using Alexa 488 conjugated mAbs against NP, F and HN proteins of hPIV-2. The inhibitory effects of A23187 on hPIV-2 entry into the cells, replication in the cells and cell-to-cell spreading were analyzed using a recombinant green fluorescence protein (GFP)-expressing hPIV-2 without matrix (M) protein (rghPIV-2DM) [9, 10]. The numbers of viruses released from infected cells cultured with A23187, and with A23187 plus CD98HC mAb, were determined. Cytoskeleton was reported to have an important role in paramyxovirus replication. Actin microfilaments are important in the hPIV-3 life cycle, specifically at the level of viral transport and replication [21]. Tubulin also acts as a positive transcription factor for *in vitro* RNA synthesis by Sendai virus [22]. The effects of A23187 on actin microfilaments and microtubules were analyzed using rhodamine phalloidin and anti-tubulin a antibody, respectively. The effect of CD98HC mAb on the inhibition of A23187 was examined: the effects on RNA synthesis and protein expression were analyzed, and virus entry, cell-to-cell spreading, and cytoskeleton were observed.

**Materials and methods**

*Viruses, cell line and cultivation of cells:*

Virus and recombinant virus were approved by the relevant biosafety committees of Suzuka University of Medical Science. hPIV-2 (Toshiba strain) was used. rghPIV-2DM was constructed according to the method described previously [9,10], and it was shown that it did not produce infectious virus particles without addition of M protein gene *in trans* (data not shown). $LLCMK_2$ cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. Minimum essential medium a (MEMa: Wako, Osaka, Japan), supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin, was used. The cells were cultured at $37^{O}C$ in a humidified atmosphere with 5% $CO_2$. After 3 days, when the cells became confluent ($5x10^5$ cells), the medium was changed to MEMa with 0.5% FCS and 0.1 mg/mL kanamycin. A23187 (Acros Organics, Kanto Chemicals, Tokyo, Japan) was added to the cells, and the cells were infected with hPIV-2 ($3x10^2$ $TCID_{50}$). CD98HC mAb (HBJ127: against T24 human urinary bladder cancer cells) [23, 24] was added just after the addition of A23187 and the cells were infected with hPIV-2.

*Cytopathogenic assay:*

Cell fusion and hemadsorption (Had) were observed at 4 days post infection. Had test was carried out using sheep red blood cells (SRBC). The cells were incubated with 0.4% SRBC at room temperature for 30 min, washed 4 times with PBS, and Had was observed under a cell culture light microscope.

*RNA preparation, cDNA synthesis and PCR:*

RNA was extracted from the cells ($2x10^6$ cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's method. cDNA was synthesized with 2 mg of RNA using superscript II reverse transcriptase (Invitrogen), with forward primers for NP, F and HN genes of hPIV-2 [25]. PCR was carried out with cDNA using forward and reverse primers for NP, F and HN genes [25] and Ex Taq (Takara, Shiga, Japan).

*Immunofluorescence study:*

To detect virus proteins in the infected cells, the cells were examined by direct immunofluorescence study. The mouse mAbs against NP, HN and F proteins were conjugated with Alexa fluora 488 according the manufacturer's method using Alexa Fluora 488 Monoclonal Antibody Labeling Kit (Molecular Probes, Eugene, OR, USA), and they were purified using MAb Trap Kit (GE Healthcare Japan, Tokyo, Japan).

The cells were fixed with 10% formaldehyde-PBS at room temperature for 15 min, washed with PBS (to detect NP protein, the cells were further incubated with 0.1% TritonX-100-PBS at room temperature for 15 min), and incubated with the Alexa fluora 488

labeled mAbs against NP, F and HN proteins of hPIV-2 at room temperature for 60 min. After washing with PBS, the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan).

Actin was detected using rhodamine phalloidin (Invitrogen). Microtubules were observed using anti-tubulin antibody against 13 day old chicken embryos produced in rabbit (whole antiserum: product number T3526, the antibody cross-reacts human foreskin fibroblasts HS-68) (Sigma-Aldrich, MO, USA) and Alexa 488 labeled second antibody (anti-rabbit) produced in goat (Invitrogen). The cells were fixed with 10% formaldehyde-PBS at room temperature for 15 min, washed with PBS (to detect microtubules, the cells were further incubated with 0.1% TritonX-100-PBS at room temperature for 15 min).

**Results**

*Inhibitory Effect of A23187 and recovery by CD98HC mAb:*
To investigate the effect of A23187 on the growth of hPIV-2, various concentrations of A23187 were added to the cells and they were infected with hPIV-2. Cell fusion and Had were observed at 4 days post infection. It was found that A23187 (0.05 mg/mL) inhibited cell fusion and Had by hPIV-2 (data not shown). To recover the inhibition by A23187, mAb against CD98HC was added to the A23187-treated cells and they were infected with hPIV-2. The inhibitory effect of A23187 on hPIV-2 replication was considerably recovered by CD98HC mAb (200 mL of culture supernatant): cell fusion and Had were observed at 4 days post infection (data not shown). A23187 and CD98HC mAb did not disturb normal cell morphology at the concentrations used in the experiments, as observed under a cell culture light microscope.

*Titration of Virus released from the cells:*
To analyze whether progeny virus is released from the A23187-treated infected cells, the culture supernatants were harvested at 4 days post infection. The titers of virus released from cells were determined. Without A23187, the virus titer was about $3.2x10^5$ $TCID_{50}$/mL, and with A23187 it reduced to 3.2x10 $TCID_{50}$/mL, indicating that A23187 largely prevented the virus release from the cells. In the following experiments, CD98HC mAb was added to the A23187-treated cells and they were infected with hPIV-2. The addition of CD98HC mAb considerably recovered the inhibition by A23187: virus titer was about $6.2x10^4$ $TCID_{50}$/mL.

*Viral RNA Synthesis:*
RNA was prepared from the infected cells at 4 days post infection, and virus-synthesized RNA was analyzed using hPIV-2 specific primers for NP, F and HN genes by PCR. The number of base pairs between forward and reverse primers of NP, F and HN genes was about 800. There are some non-specific bands, some larger and some smaller than 800 in the drug-treated infected cells, and they were also observed faintly in negative control (Fig.1, lanes 1, 2 and 3: NP, F and HN, respectively), and in positive control (Fig.1, lanes, 4, 5 and 6 : NP, F and HN, respectively). A23187 did not inhibit viral RNA synthesis. (Fig.1, lanes 7, 8 and 9: NP, F and HN. respectively). CD98HC mAb had no effect on viral RNA synthesis (Fig.1, lanes 10, 11 and 12: NP, F and HN, respectively).

*Viral Protein Synthesis:*
Direct immunofluorescence study was performed to investigate the effect of A23187 on hPIV-2 protein expression. A23187 was added to the cells and they were infected with hPIV-2. At 4 days post infection, the cells were fixed and stained with the Alexa 488 conjugated mAbs against NP, F and HN proteins of hPIV-2. Figs. 2A, 2B and 2C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. In hPIV-2 infected cells, NP, F and HN proteins were observed in almost all the cells: NP protein was observed in many strong fluorescent dots mainly in the cytoplasm; F and HN proteins were in small dots in the cytoplasm and on the cell surface. A23187 largely inhibited the expression of NP (Fig. 2D), F (Fig. 2E), and HN (Fig. 2F) proteins. Interestingly, by the addition of CD98HC mAb, the NP, F and HN proteins (Figs. 2G, 2H and 2I, respectively) were expressed in the A23187-treated infected cells.

*Entry and cell-to-cell spreading of hPIV-2:*
The above results showed that A23187 did not inhibit viral RNA synthesis but largely inhibited protein syntheses. In the following experiment, we determined the effects of A23187 and CD98HC mAb on the entry and the cell-to-cell spreading of hPIV-2 using rghPIV-2DM (Fig. 3). A23187 was added to the cells, or A23187 and CD98HC mAb were added, and immediately after the addition, the cells were infected with rghPIV-2DM ($1x10^5$ $TCID_{50}$), and cultured for 4 days. The cells were fixed and observed under a fluorescence microscope. The uninfected cells had no fluorescence (data not shown). Fig. 3A is a positive control: there are multinucleated giant cells and the cells have strong fluorescence. Fig. 3B shows that A23187 did not inhibit either the entry of rghPIV-2 DM or the multinucleated giant cell formation, but the size was relatively small compared with that of the positive control. Native hPIV-2 (Toshiba strain) did not induce cell fusion when cultured with A23187. However, rghPIV-2DM caused giant cell formation in the presence of A23187. It has been shown that

recombinant Sendai virus without M protein caused extensive cell-to-cell spreading [26]. In the case of hPIV-2, the same result was observed [10], so it may be possible that the low dose of A23187, which inhibited cell fusion caused by native hPIV-2, could not completely inhibit cell-to-cell spreading induced by rghPIV-2DM. CD98HC mAb completely recovered the formation of the multinucleated giant cells (Fig. 3C).

*The Effect of A23187 and CD98HC mAb on Cytoskeleton:*

A23187 was added to the cells and cytoskeleton was observed under an immunofluorescence microscope at 20h of cultivation. Figs. 4A and 4B show normal actin microfilaments and microtubules in $LLCMK_2$ cells, respectively. As shown in Figs. 4C and 4D, A23187 disrupted both actin microfilaments and microtubules. In particular, damage to microtubules was outstanding: microtubules became short, and exhibited a dot like structure. However, when CD98HC mAb was added, almost normal actin microfilaments and microtubules were observed (Figs. 4E and 4F, respectively).

**Discussion**

The present study showed that a low dose of calcium ionophore A23187 had an inhibitory effect on hPIV-2 replication *in vitro*, and that the inhibition was recovered by CD98HC mAb. A23187 inhibited virus protein synthesis and disrupted actin microfilaments and microtubules, but did not inhibit either viral RNA synthesis or virus entry into the cells. CD98HC mAb recovered virus protein synthesis and normal structures of both actin microfilaments and microtubules.

It was attempted to measure the cytosolic calcium ion concentration using fluorescent dye (Calcium Kit-Fluora 3. Dojindo, Kumamoto, Japan) by a fluorescence spectrophotometer. However, no difference was found between the free calcium concentration of normal cells and that of A23187-treated cells. This is because the concentration of A23187 was low and total cytosolic calcium ion concentration might not increase. Cytosolic calcium concentration might increase only in some parts and, as a result, the calcium might be involved in the disruption of both actin microfilaments and microtubules. CD98HC mAb HBJ127 controlled the calcium ion concentration via CD98HC and the cytoskeleton might be recovered. CD98 mAb 4F2 specifically inhibits the $Na^+$-$Ca^{2+}$ exchanger that is an anti-porter membrane protein that removes calcium from cells. Binding of mAb 4F2 to its cell surface antigen on dispersed adenomatous parathyroid cells increases cytosolic calcium [20]. CD98 has been identified as a major surface receptor for a mammalian galactosidase-binding protein galection-3 that binds to CD98 and induces an increase in intracellular calcium in human Jurkat T leukemia cells [27]. CD98HC mAb 44D7 inhibited $Na^+$-$Ca^{2+}$ exchanger activity in cardiac and skeletal muscle sarcolemmal vesicles, but mAb 44H7 that reacts with a distinct epitope does not inhibit the exchanger activity [19]. These results indicate that the inhibition of the exchanger activity is dependent on the cell types and on the epitope that is recognized by CD98HC mAbs.

Taken together with these findings, it was suggested that the inhibition of hPIV-2 replication by A23187 was mainly caused by the protein synthesis inhibition and by the disruption of actin microfilaments and microtubules. Also, CD98HC mAb HBJ127 recovered protein synthesis and structures of actin microfilaments and microtubules. As a result, the production of progeny virus, which was inhibited by A23187, was also considerably recovered by CD98HC mAb. The precise mechanisms of inhibition by A23187 and recovery by CD98HC mAb were unclear, but calcium ion may be involved in the inhibition and the recovery.

**Acknowledgement**

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**Figure Legends**

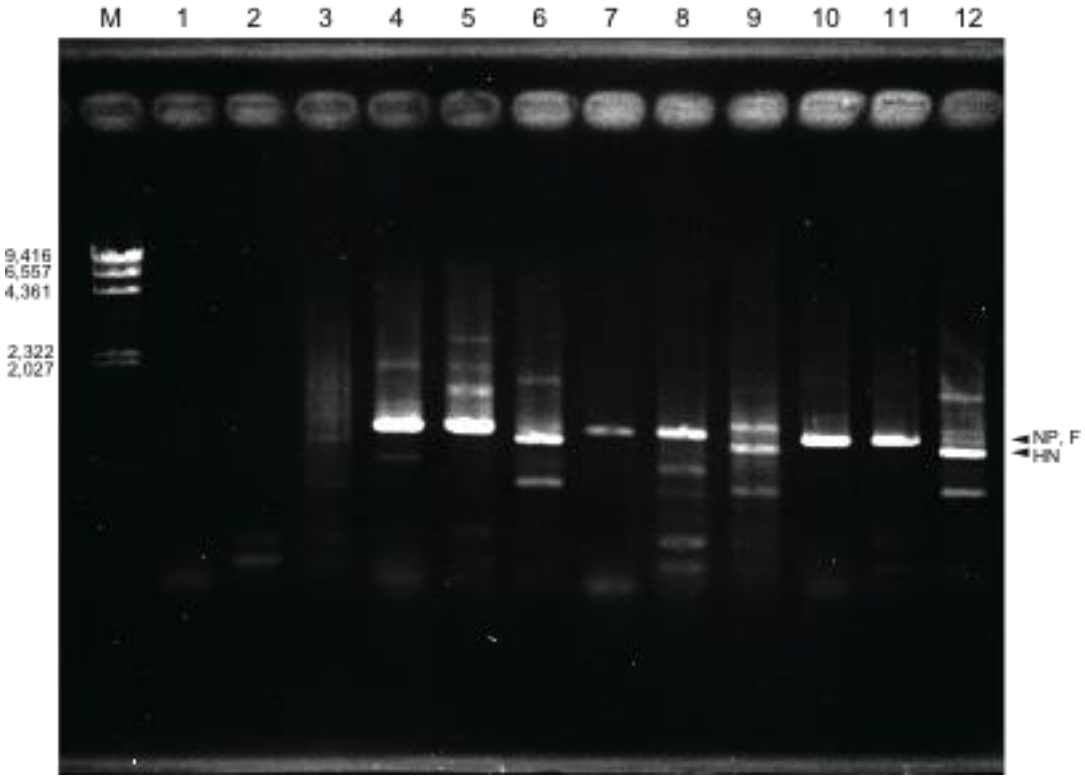
Figure 1. A23187 did not inhibit viral RNA synthesis. Lane M: marker (base pair), lanes 1, 2, 3: non-infected cells, lanes 4, 5, 6: hPIV-2 infected cells, lanes 7, 8, 9: hPIV-2 infected cells cultured with A23187, lanes 10, 11, 12: hPIV-2 infected cells cultured with both A23187 and CD98HC mAb. Lanes 1,4,7,10:NP gene, lanes 2,5,8,11:F gene, and lanes 3,6,9,12:HN gene.

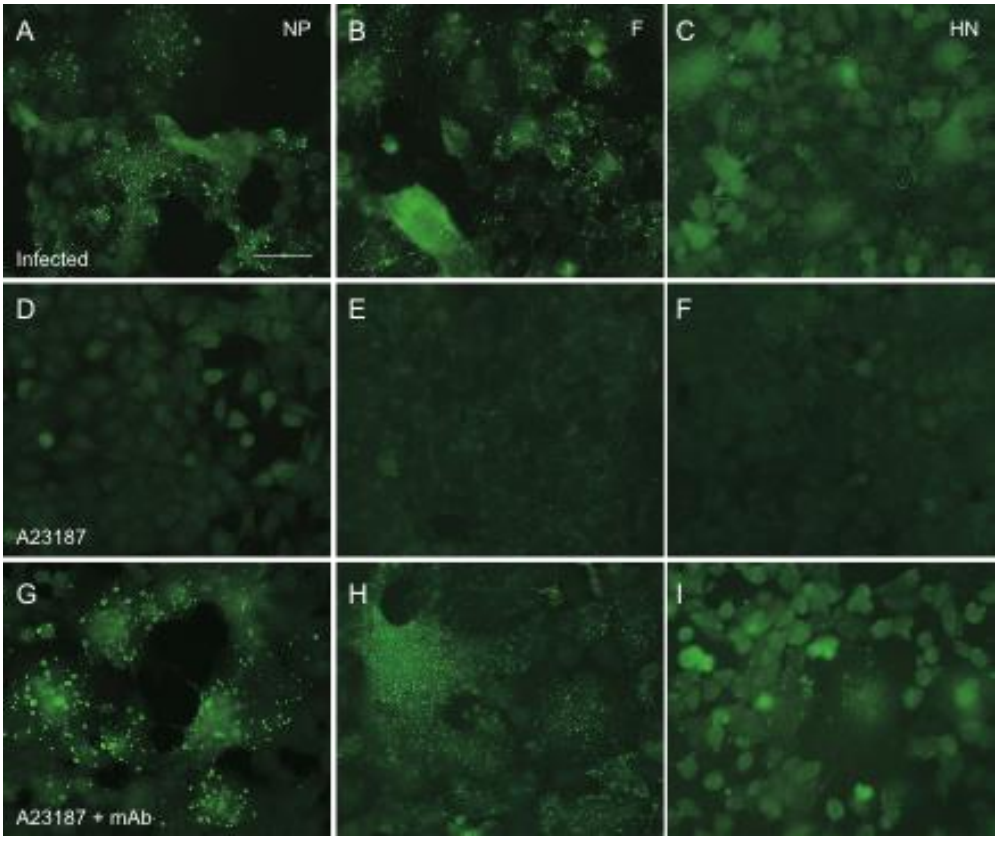
Figure 2. A23187 inhibited the expression of NP, F and HN proteins of hPIV-2. The expression of NP (A), F (B) and HN (C) proteins of hPIV-2 infected cells. A23187 inhibited the expression of NP (D), F (E) and HN (F) proteins. CD98HC mAb recovered the expression of virus proteins that were inhibited by A23187: NP (G), F (H) and HN (I) proteins. (bar: 50 mm)

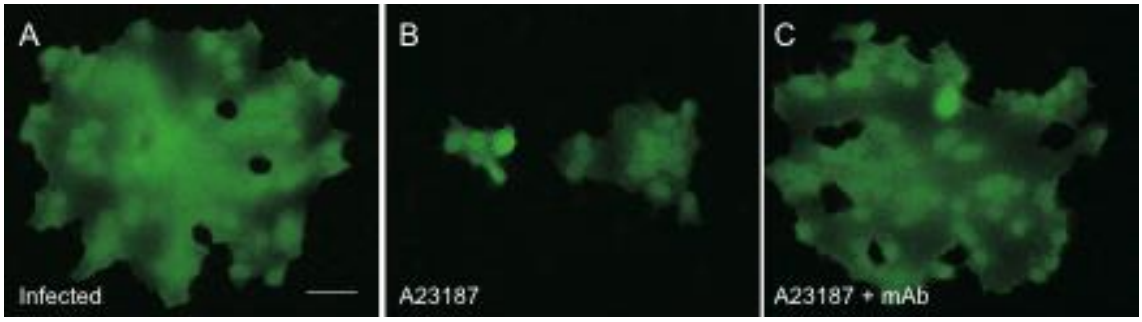
Figure 3. Effects of A23187 and CD98HC mAb on hPIV-2 entry and cell-to- cell spreading. The cells infected with rghPIV-2DM (A), rghPIV-2DM infected cells cultured with A23187 (B), and infected cells cultured with both A23187 and CD98HC mAb (C). In Fig. 3B, relatively small multinucleated giant cells were observed, indicating that A23187 did not inhibit either the virus entry or cell-to-cell spreading of rghPIV-2DM. In Fig. 3C, multinucleated giant cells were observed by the addition of CD98HC mAb. (bar: 50 mm)

Figure 4. A23187 disrupted both actin microfilaments and microtubules, and CD98HC mAb recovered them. The cells were cultured with A23187 or A23187 plus CD98HC mAb for 20 h, and actin microfilaments and microtubules were stained with rhodamine phalloidin and anti-tubulin antibody,

respectively. Fig. 4 shows actin microfilaments of the control cells (A), the cells cultured with A23187 (C), A23187 plus CD98HC mAb (E), and also exhibits microtubules of the control cells (B), the cells cultured with A23187 (D), and A23187 plus CD98HC mAb (F). (bar: 50 mm)




A
Infected
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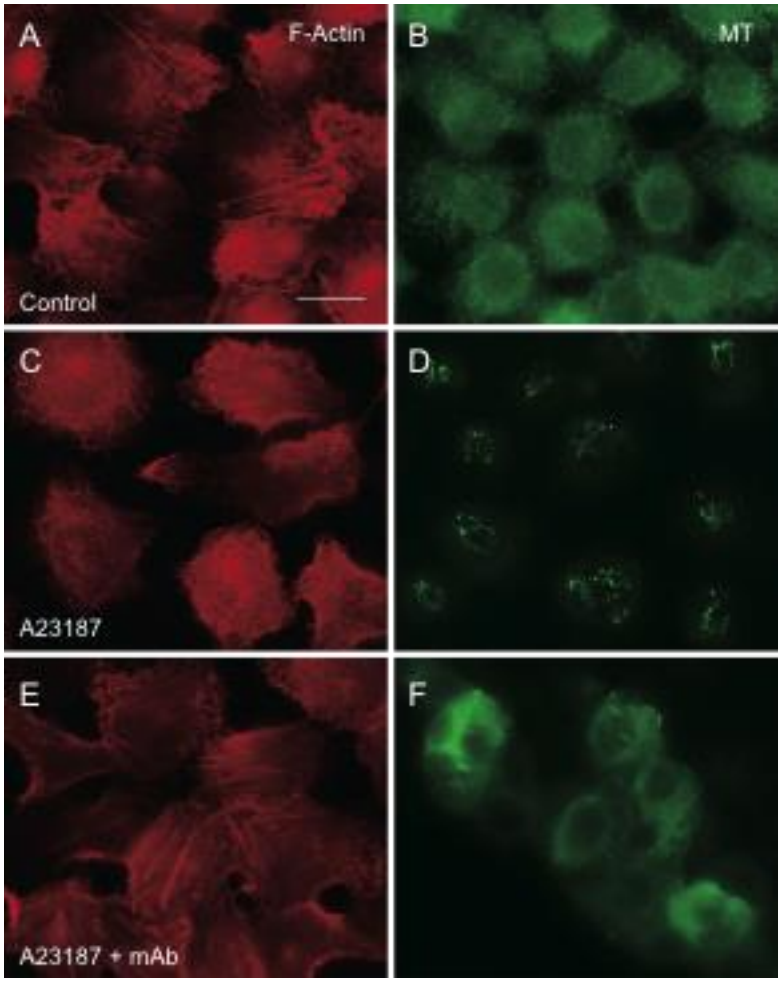
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