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Entry of *Bombyx mori* nucleopolyhedrovirus into BmN cells by cholesterol-dependent macropinocytic endocytosis

Jinshan Huang [a,b,*], Bifang Hao [a,b], Chen Cheng [a], Fei Liang [a], Xingjia Shen [a,b], Xiaowen Cheng [c]

[a] College of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu 212018, PR China
[b] Sericultural Research Institute, Chinese Academy of Agricultural Science, Zhenjiang, Jiangsu 212018, PR China
[c] Department of Microbiology, Miami University, Oxford, OH 45056, USA

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ABSTRACT

Bombyx mori nucleopolyhedrovirus (BmNPV) is a serious viral pathogen of silkworm, and no drug or specific protection against BmNPV infection is available at present time. Although functions of most BmNPV genes were depicted in recent years, knowledge on the mechanism of BmNPV entry into insect cells is still limited. Here BmNPV cell entry mechanism is investigated by different endocytic inhibitor application and subcellular analysis. Results indicated that BmNPV enters BmN cells by clathrin-independent macropinocytic endocytosis, which is mediated by cholesterol in a dose-dependent manner, and cholesterol replenishment rescued the BmNPV infection partially.

1. Introduction

BmNPV is an enveloped DNA virus that can produce virions of two phenotypes (BV and ODV) in the life cycle, ODV is responsible for the primary infection of the host insect while BV is responsible for the secondary infection [1]. BmNPV is a serious viral pathogen in sericulture industry, and no effective drug or specific prevention is available to date. Researchers gave major attention to inhibition of the virus replication by over-expression of antiviral exogenous gene [2] or RNA interference [3,4]. However, mechanisms of BmNPV BV entry into the host cell have not been addressed. Virus-cell interactions provide an area that is still incompletely explored and under-exploited for antiviral strategies, virus entry to the cells was a remarkable target for the development of viral protection methods. BmNPV entry mechanism needs to be obtained for future development of methods to block silkworm from BmNPV infection. In addition, BmNPV are increasingly applied as the tools to produce "foreign" protein using silkworm as a bioreactor [5], understanding on entry mechanism will facilitate its application in biomedical medicine research.

In the infection, viruses first bind to cell surface proteins, carbohydrates, and lipids, its interactions lead to the activation of cellular signaling pathways, then cells respond by internalizing the viruses using endocytic mechanisms [6]. Endocytosis is usually used by viruses to enter the host cell [7]. As a close relative of BmNPV, AcMNPV is the best-understood baculovirus, and it serves as a model for basic molecular research and the baculovirus infection cycle. AcMNPV exploits multiple endocytosis pathway according to the host range or cell type, it enters insect cells by clathrin-dependent endocytosis or direct fusion [8,9], whereas entry into mammalian cell is by clathrin-dependent endocytosis, lipid raft, or macropinocytosis pathways [8,10]. AcMNPV GP64 mediates virus absorption and fusion to insect cells [11–13], the CRAC of GP64 is efficiently bound to cholesterol of mammalian cells, which serves as an anchor in the transduction into mammalian cells, whereas this site is unnecessary for AcMNPV entry into Sf9 cells [14]. Although BmNPV genome is over 90% identical to that of AcMNPV [15], the host ranges of these two viruses have essentially no overlapping [16]. Furthermore, AcMNPV GP64 contains three CRAC sites [14], whereas four CRAC sites were found in BmNPV GP64, so we speculated that BmNPV may took a different entry mechanism with that of AcMNPV.

To address this hypothesis, the entry mechanism of BmNPV BV was explored with inhibitors of specific host cell signaling and cytoskeleton rearrangement, the infectivity of virus was checked by fluorescence expression or qPCR, with the depletion and replenishment of membrane cholesterol, we demonstrated that BmNPV BV entered BmN cells by macropinocytosis mechanism, which was mediated by cholesterol on the cell membrane.

Abbreviations: BmNPV, *Bombyx mori* nucleopolyhedrovirus; BV, budded virus; ODV, occlusion-derived virus; AcMNPV, *Autographa californica* MNPV; CRAC, cholesterol recognition amino acid consensus; h p.i, hour post infection; MOI, multiplicity of infection; DMA, N,N-dimethylacetamide; TCID50, 50% tissue culture infective dose; MβCD, methyl-β-cyclodextrin; RTKs, receptor tyrosine kinases.

* Corresponding author at: College of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu 212018, PR China.
E-mail address: jshuang@just.edu.cn (J. Huang).

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2. Materials and methods

2.1. Cells and virus

The BmN cell line was cultured at 27 °C in TC-100 insect medium (Applichem) supplemented with 10% fetal bovine serum (Gibco-BRL) using standard techniques.

BmNPV bacmid (BmBac-GFP) were constructed using pFastDual-hsp70-egfp according to the Bac-to-Bac system manual of Invitrogen [17], the recombinant bacmid was transfected into BmN cells for virus preparation, virus titer was determined by end point dilution assay [18].

2.2. Drugs treatments

Pharmacological inhibitors were purchased from Sigma Company and stock solutions were prepared either in water or in DMSO following the manufacturer's recommendations and used at the indicated concentration. BmN cells (10^5 cells)/dish were pre-seeded in tissue culture dishes for overnight, then the cells were incubated with drugs for the specific times as followed, chlorpromazine, nystatin and progesterone for 30 min; genistein, DMA, rottlerin, and Ehop-016 for 90 min; latrunculin A for 2 h. DMSO (no more than 2%) or PBS was added in the control group. After incubation, drugs were removed and the treated BmN cells were infected with BmBac-GFP at an MOI of 5 TCID50 unit per cell for 1 h without drug incubation. The viruses were then removed and the cells were washed twice with TC100 medium without FBS. The cells were cultured in TC100 medium with 10% FBS. Infectivity was recorded at 6 h p.i. by microscopy, and then the cells were applied to calculate the cells viability.

2.3. Cholesterol depletion

BmN cells were treated with different concentrations of MβCD for 30 min, while treatment with PBS was used as the control. After drug treatment, cells were washed with TC100 medium without FBS twice, and then were infected with BmBac-GFP at an MOI of 5 for 1 h. Unattached viruses were removed and the cells were washed with TC100 medium without FBS twice. The cells were incubated in TC100 with 10% FBS at 27 °C. Infectivity and cells viability were recorded at 6 h p.i. by microscopy.

2.4. Cholesterol replenishment

BmN cells were treated with or without 10 mM MβCD for 30 min. Treatment with PBS was used as the control. The treated cells were washed with TC100 medium without FBS twice, and then an MβCD treatment was replenished with cholesterol (water-soluble cholesterol, Sigma product, final concentration 100 μg/ml). The cells were then infected with BmBac-GFP at an MOI of 5. Following infection, the virus was removed and the infected cells were washed with TC100 medium without FBS twice. The cells were incubated in TC100 with 10% FBS at 27 °C. Fluorescence was detected at 6 h p.i. by microscopy. Treated samples were collected for qPCR analysis.

2.5. qPCR

RNA of samples from cholesterol replenishment was extracted with Trizol (Invitrogen) according to the description. A total of 400 ng RNA was used in RT-PCR. Relative expressions of BmNPV IE1 in the different treatments were analyzed by qPCR with primers (IE1F:TGAGCAGTCTGTTGGTGTGA, IE1R:GCACAGCTTTGA ATTGTGCT). Actin expression set as internal control with primers (Actin F: GACCCTCAAATACCCCATCG, Actin R: CTTCCATACCCAA GAACGAGG).

2.6. Statistical analysis

The infectivity of different treatment was estimated as percentage of GFP-expressing cells for each treatment at 6 h p.i. as described in reference [8] and then the cells was applied to calculated viability by the trypan blue exclusion method according to the insect cell culture protocol of Invitrogen. The statistical analyses were performed by two tail *T* test in excel from three independent experiments.

2.7. TEM analysis

BmN cells in the dishes (60 mm) were incubated with/without 10 mM MβCD for 30 min, then the drug was removed and the cells were washed twice. The cells were then infected with BmBac-GFP using an MOI of 30 for 1 h at 4 °C. The virus-containing medium was removed and the cells were washed with PBS twice and fixed by glutaraldehyde for microscope observation and TEM analysis [19].

3. Results

3.1. BmNPV entry into BmN cells by clathrin-independent macropinocytosis

Since the closely related baculovirus AcMNPV take clathrin-mediated endocytosis enter host cell, effect of chlorpromazine, an inhibiter of clathrin-mediated endocytosis, was checked firstly. Result indicated chlorpromazine had no inhibitory function on BmNPV entry into BmN cells (Fig. 1A), even at a high concentration, cells, therefore, were infected efficiently, meanwhile, infectivity and viability of BmN cells incubated with 1 mM chlorpromazine decreased, which indicated chlorpromazine showed toxicity to cells. This result suggested that BmNPV took a clathrin-independent entry mechanism, so is it caveola/lipid raft endocytosis? Next, nystatin and progesterone were applied to verify whether caveola/lipid raft endocytosis was involved in. However, it had no effect on inhibition of BmNPV entry (Fig. 1B), even at a very high concentration (100 μg/ml), and BmN cell was more sensitive to the treatment of these drugs, cell viability decreased rapidly with the increasing drug concentration.

Viruses with large particles can enter cells via macropinocytosis, so BmNPV entry mechanism was supposed to be macropinocytic endocytosis. To validate this hypothesis, inhibitors of RTKs, Rac1 and PKC involving in macropinocytosis were used, including genistein, Ehop-016, and rottlerin. These chemicals blocked BmNPV entry efficiently without cell toxicity (Fig. 1C). Only 1.1% cells preincubated with 100 μg/ml genistein were infected; and about 3% cells showed fluorescence in treatment with 5 μM Ehop-016; while 1 μM rottlerin, inhibitor of PKC, blocked the virus entry activity completely. These results revealed that inhibition of kinase/GTPase resulted the failure of BmNPV into BmN cells efficiently. Other factors, including actin, Na+/K+ exchangers also involved in virus entry into BmN cells, which proved by the application of latrunculin A and DMA, the drugs decreased the infectivity significantly (Fig. 1C).

Taken together, these results indicated that BmNPV entry into BmN cells was clathrin- and caveolar/lipid raft-independent endocytosis, but was PKC-, Rac-1, and RTKs-dependent macropinocytic endocytosis.

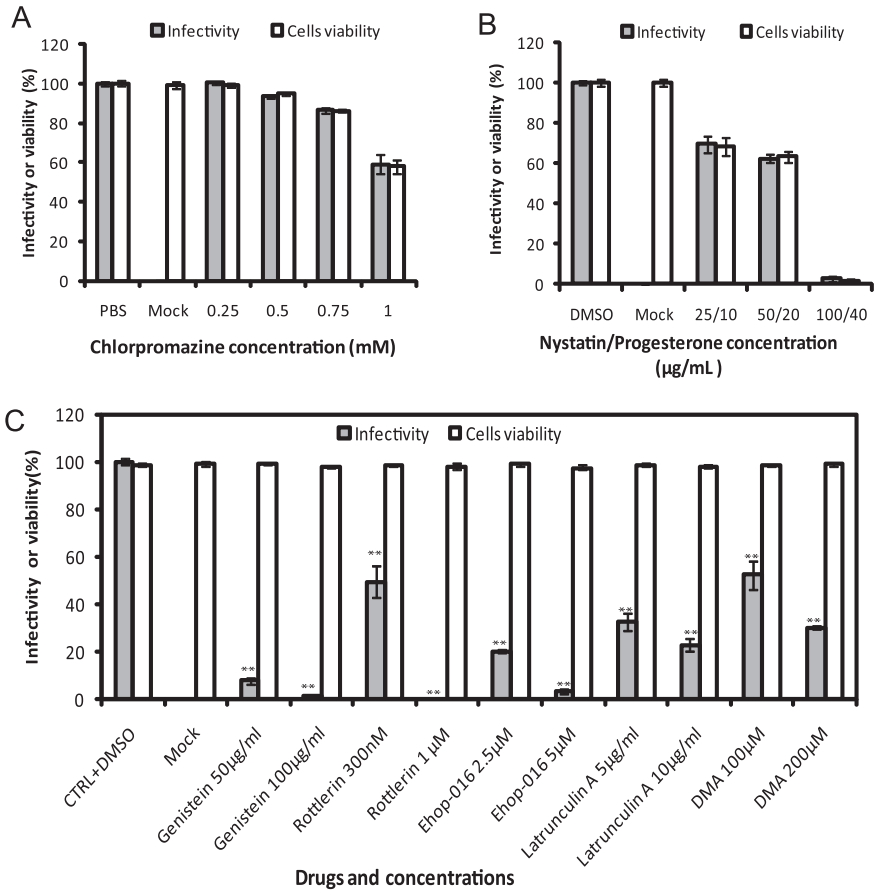

Fig. 1. Drugs effects on BmNPV entry (A–C). (A) BmN cells infectivity and viability treated with chlorpromazine. Cells were pre-incubated with different concentration chlorpromazine for 30 min, then removed the drugs and conducted the infection with BmBac-GFP at an MOI of 5 TCID50, fluorescence was recorded at 6 h p.i., cell viability was calculated by Trypan blue exclusion test. (B) BmN cells pre-incubated with nystatin and progesterone for 30 min and then conduct the infection as previous described. (C) Effects of other drugs on virus entry. Virus infectivity was quantified as the percentage of GFP-expressing cells relative to total cell number. The data shown are the means and standard deviations from three independent experiments, statistical analysis were conduct by two tail *T* test, and significant difference indicated by asterisk (**$P < 0.001$), error bars present the standard deviations.

3.2. BmNPV entry mediated by membrane cholesterol in dose dependent manner

Cholesterol was the necessary factor for membrane ruffling of macropinocytic endocytosis, thus, the role it played in BmNPV infection was determined. Our investigation showed that MβCD, a cholesterol depletion drug, reduced BmNPV infectivity efficiently with the increasing drug concentration. MβCD at 10 mM inhibited BmNPV entry completely whereas fluorescence was observed in the control cells, which indicated that viruses entry were associated with cholesterol on the cell membrane, and high concentration MβCD showed no toxicity to cells (Fig. 2B). In addition, when water soluble cholesterol (final concentration 100 μg/ml) was replenished into 10 mM MβCD treated cell growth media for 30 min and then conduct the infection, fluorescence was observed again in some cells (Fig. 2A and C). The abortive infection resulted from cholesterol depletion was rescued partially by cholesterol replenishment (62% cells were infected). This rescue was further confirmed by qPCR (Fig. 2D), there were almost no relative expression of IE1 in MβCD treated cells, while cholesterol replenishment treatment presented one-third expression of the control group. These results indicated macropinocytosis of BmNPV was mediated by cholesterol.

3.3. Cholesterol depletion results in failure of membrane ruffling

To further confirm that macropinocytosis is an uptake pathway for BmNPV and the function of membrane cholesterol, light microscope and TEM analysis were applied to observe membrane change at the beginning of infection. Results indicated that membrane ruffles formed around cells membrane at a transient pattern when BmN cells challenged by BmNPV (Fig. 3A), while MβCD treated cells presented more smooth membrane (Fig. 3B). TEM images showed membrane ruffling formed around the all cell's membrane (Fig. 3C), viral particle in the micropinosome in the cytoplasm was also observed (Fig. 3D), and the shrunk micropinosomes located around the nucleus were found (Fig. 3E). However, no ruffling structure was observed around the cell membrane treated with MβCD, cells showing smooth cell membranes (Fig. 3F), these results suggested that cholesterol was a necessary factor for membrane ruffling of macropinocytosis.

4. Discussion

Viruses developed a number of strategies to enter target cells. These include endocytosis via clathrin-coated pits, caveolae/lipid-raft, phagocytosis, and macropinocytosis [6,20]. The classical clathrin- or caveolin-1 mediated endocytotic vesicles are commonly smaller than 200 nm and 100 nm, respectively [21,22]. Thus, it seems reasonable to assume that BmNPV, a rod-shaped virus particles, use macropinocytosis mechanisms for cellular entry. Macropinocytosis refers to formation of macropinosomes, which are large endocytotic vesicles formed at specific locations of the plasma membrane. This process is suited for uptake of extracellular material such as fluid, antigens, or even large apoptotic

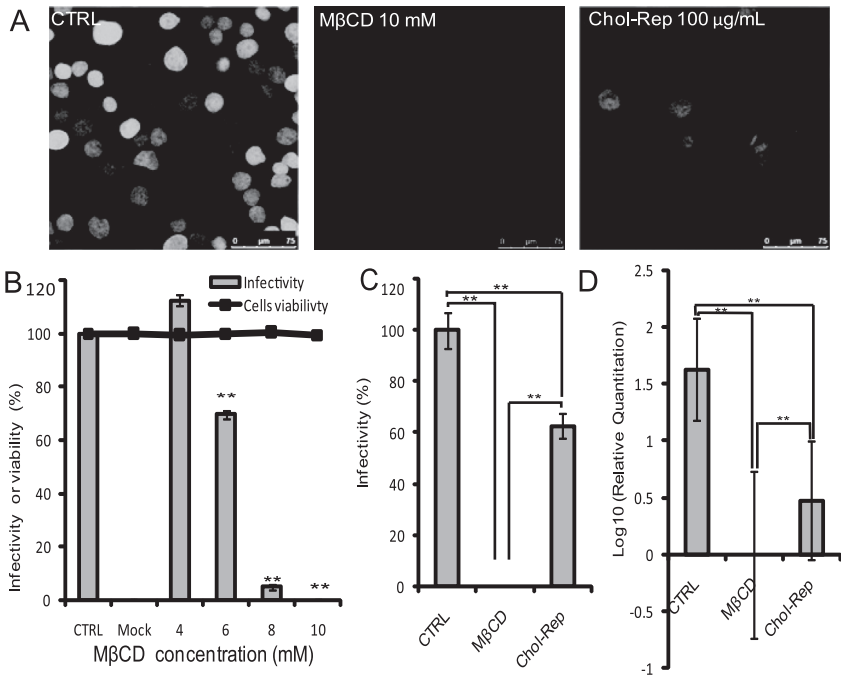

Fig. 2. BmNPV entry into BmN cells mediated by cholesterol in dose dependent. BmN cells were treated with or without 10 mM MβCD for 30 min. Treatment with PBS was used as the control. The treated cells were washed with TC100 medium without FBS twice, and then an MβCD treatment was replenished with cholesterol (final concentration 100 μg/mL). The cells were then infected with BmBac-GFP at an MOI of 5 TCID50. The cells were washed for twice and incubated in TC100 with 10% FBS at 27 °C. (A) Fluorescence expression in cholesterol depletion cells and cholesterol replenishment cells (Chol-Rep) infected by BmBac-GFP; scale bar, 75 μm. (B) BmNPV infectivity of different groups treated with increasing concentration of MβCD. (C) Cholesterol replenishment partially rescued the viral infectivity. (D) Cholesterol replenishment partially rescued the viral infectivity by qPCR. Statistical analyses were performed by *t* test. Significant differences are indicated by asterisks (**$P < 0.001$); error bars represent the standard deviations.

cells [23]. Macropinocytosis was employed to invade host cells by several animal viruses including human viruses, such as African swine fever virus, Ebola virus, Nipah virus, vaccinia virus [6].

In our study, we demonstrate that BmNPV BV entered the BmN cells by clathrin-, caveola/lipid raft independent endocytosis through drugs application (Table 1). Preliminary experiments showed that the presence or absence inhibitors in the infection process had no significant difference on virus entry (data not shown), however, chlorpromazine and nystatin/progesterone showed toxicity to BmN cells (Fig. 1), these results were consistent with that chlorpromazine caused bioelectric toxicity in mammalian cells [24] and high concentration nystatin resulted in transmembrane aqueous channel formation [25]. Though nystatin and MβCD could interacted with cholesterol, the mechanism was different, lower concentration nystatin binding with membrane cholesterol [25], but MβCD can remove membrane cholesterol efficiently [26]. Total cholesterol concentration of the cell treated with 10 mM MβCD were decreased to 0.07 μM compare to 0.143 μM of the control in our test (unpublished data), and cholesterol replenishment rescued the virus infectivity partially (Fig. 2), these results revealed cholesterol was a key factor for BmNPV infection.

Here we have identified a macropinocytosis process as the critical mechanism for BV of BmNPV particle uptake into BmN cells. This discovery is consistent with findings in recent reports that AcMNPV entry into mammalian [14], but not entry into insect cell. It is well-known that AcMNPV entered insect cell by clathrin-mediated endocytosis [8,9], so why BmNPV is particular for this mechanism since all baculoviruses are rod-shaped? It might be due to several reasons, firstly, the endocytosis pathway that virus taken dependents on cell type or origin [7], BmN cells derived from ovary, which was not the most susceptible cells, as we know that hemolymph and fat body of *Bombyx mori* larvae were the most susceptible tissues to BmNPV [27]. Secondly, inherent characters of BmNPV resulted in this entry mechanism, there were some reports on Adenovirus 2 and 5 entry into cells by clathrin-mediated endocytosis [28–30], as well as Adenovirus 3 take macropinocytosis mechanism [31,32], hence, it is reasonable that BmNPV and AcMNPV take difference entry mechanism though both are member of Group I baculovirus. Lastly, difference on GP64, a membrane fusion protein mediated virus infection. Compare to three CRAC sites of AcMNPV GP64 [14], four CRAC sites were found in BmNPV, the additional one CRAC site located in eighteen amino acids at N-terminus of GP64, predicted as the signal peptide (SignalP 4.1), which was absence in AcMNPV. Unlike AcMNPV, the BmNPV GP64 signal sequence alone may not be sufficient for protein secretion [33], other research also revealed that insufficient BmNPV GP64 function result in the failure of virions transport into nuclear [34]. GP64 was acquired by an ancestral Group I virus in the baculovirus evolution [35]. AcMNPV may acquire other mechanisms through the evolution of GP64 to invade more cells by clathrin endocytosis.

Cholesterol is a necessary factor of macropinocytic endocytosis [6]. Cholesterol depletion is probably caused by the redistribution of phosphoinositides in the plasma membrane affecting the localization of Rac1, Arf6 and other signaling factors [36,37]. Here, we showed that it was necessary for BmNPV entry, which was similar to other virus infection [38,39] and AcMNPV entry into mammalian cells [14]. Cholesterol depletion did not affect BmNPV attachment to the cell surface, which was similar to AcMNPV [14]. However, cholesterol depletion inhibited membrane ruffling of BmN cells (Fig. 3F), therefore, cholesterol may serve as the signal transducer in BmNPV entry. It was reported that actin modulation involves in membrane ruffling while many upstream signals transduction involves in actin reorganization and macropinocytosis

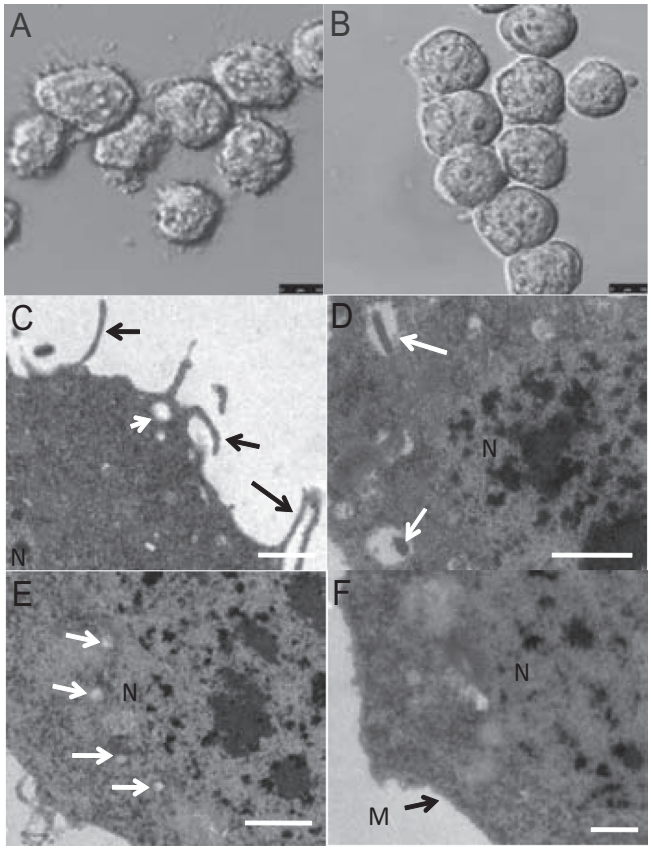

Fig. 3. Microscope observation and TEM analysis of BmN cells infected by with BmBac-GFP. BmN cells were treated with or without 10 mM MβCD, and infected by BmBac-GFP with an MOI of 30 TCID50 for 1 h at 4 °C, cells were then fixed for observation. (A) Membrane ruffles of cells induced by BmBac-GFP infection under microscope; scale bar, 10 μm. (B) No ruffling structure observed around the cell membrane treated with 10 mM MβCD; scale bar, 10 μm. (C) Membrane ruffling around the untreated cells infected with BmBac-GFP under microscope. Arrows showed the membrane ruffling, open arrow showed macropinosome. (D) Macropinosomes and virus particles in the cytoplasm. Open arrows showed the viral particle in the micropinosome. (E) Open arrows showed the shrinking micropinosome around the nucleus. (F) BmN cells treated with 10 mM MβCD for 30 min, no ruffling structure observed around the cell membrane, arrow showed the smooth membrane. Scale bar (C–F), 1 μm; N, nucleus; M, membrane.

Table 1
Cellular factors and processes involved in BmNPV entry into BmN cells.

Cellular factor	Inhibitor	Required for BmNPV entry into BmN cells
Clathrin	Chlorpromazin	No
Cholesterol	MβCD	Yes
Lipid raft	Nystatin	No
Tyrosine Kinases	Genistein	Yes
Rac1	Ehop-016	Yes
PKC	Rottlerin	Yes
Actin	Latrunculin A	Yes
Na+/H + exchangers	DMA	Yes

[36,40]. Here, we revealed that latrunculin A had a significant inhibitory effect, however, virus entry was not inhibited completely at a lower drug concentration, for high concentration drugs application in the test for depolymerization of actin filaments resulted in cell rounding and cell detachment. In particular, inhibition on other signal factors showed similar effects with that of cholesterol. Several hallmarks of macropinocytosis were confirmed, such as activation of RTKs, Rac1, and PKC. Macropinocytosis is usually initiated by external stimulation. These, in turn, activated signalling cascade that induces changes in the dynamics of actin filaments and trigger plasma membrane ruffling [6], once activated, Rac1, a Rho GTPase, is responsible for triggering of membrane ruffles [41], and PKC is activated by RTKs, it promotes ruffling and macropinosome formation [42], so inhibition on any factors in signalling cascade blocked virus entry. Furthermore, Na+/H+-exchanger activity were critical for BmNPV, DMA had a significant inhibitory effect on BmNPV entry.

In summary, it appeared that BmNPV entered host cells by macropinocytic endocytosis and depended among other factors on the host cell, cholesterol on the host cell membrane played a key role in BmNPV infection. Therefore, this research provided a new clue to understand BmNPV infection.

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Conflict of interest

The authors declare no conflict of interest.

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