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BOVINE PARVOVIRUS ENTRY AND TRAFFICKING
IN EBTr CELLS

by

Enkhmart Dudleenamjil

A dissertation submitted to the faculty of
Brigham Young University

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Microbiology and Molecular Biology

Brigham Young University

December 2009

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

Of a dissertation submitted by

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This dissertation has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

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As chair of the candidate's graduate committee, I have read the dissertation of Enkhmart Dudleenamjil in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT
BOVINE PARVOVIRUS ENTRY AND TRAFFICKING
IN EBTr CELLS

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Doctor of Philosophy

Bovine Parvovirus (BPV) belongs to the genus *Bocavirus*, family *Parvoviridae*. BPV is the leading etiologic agent among the pathogens that cause primary gastroenteritis of cattle. Many of the intracellular events associated with virus replication are unknown. In this research project, we investigated BPV internalization into the host cell and trafficking in the cytosol. Preliminarily, EBTr cells had abundant clathrin, virus attached to purified clathrin, and EM micrographs revealed virus in endocytic vacuoles. Assays detecting virus infectivity (i.e. viral protein synthesis), virus production (completion of the replication cycle), and quantitative PCR (qPCR) to detect viral transcripts were used to evaluate virus uptake and subsequent trafficking events in the presence of selective inhibitors. Cell toxicity mediated by the drugs was evaluated by the MTT test. Virucidal effects of the drugs were assessed. A control virus was used to verify the inhibitor technology. Immunofluoresceinated virus particles were found in clathrin-rich early endosomes. Clathrin-mediated endocytosis (CME) was examined by clathrin polymerization inhibiting agent (chlorpromazine), lysosomotropic agents (ammonium chloride and chloroquine), a vacuolar ATPase inhibitor (bafilomycin A1), and a blocker of transition between endosomes (brefeldin A). Caveosome pathway inhibitors included phorbol 12-myristate 13-acetate (a suppressor of caveolae formation), nystatin and methyl-beta-cyclodextrin (lipid raft blockers), and genistein (a tyrosine kinase phosphorylation inhibitor). Trafficking of BPV was investigated using specific inhibitors of proteasomal activity, actin-myosin function, and microtubule-dynein function. The proteasomal protease suppressor (lactacystin), and a proteasomal chymotrypsin inhibitor (epoxomicin) were used. The role of actin was probed by cytochalasin D, latrunculin A, and ML-7. The microtubule inhibitors nocodazole, vanadate, and EHNA were used to probe microtubule function. The inhibitors of CME reduced virus production and reduced infectivity, a result confirmed by qPCR. The blockers of caveolin-mediated entry did not interfere with virus production nor virus infectivity. Proteasome activity blockage did not affect the virus replication. But the virus cycle was affected by actin blockage and by microtubule blockage detected by qPCR. Taken together these data indicate that BPV uptake is mediated by clathrin coated pits and is acid-dependent. Further processing of BPV in the cytosol does not require proteasomal enzymes. Actin-associated vesicular transport appears to be essential to virus replication and trafficking to the nucleus appears to be mediated by microtubules.

Key Words: Bovine parvovirus, clathrin mediated endocytosis, virus trafficking, proteasomes, actin, microtubules, qPCR

ACKNOWLEDGEMENTS

People to whom I express my gratitude, who have helped me arrive at this point in my career, are many. I am thankful for your friendship, support, encouragement, generous comfort, and trust in me and without you this work could not have been accomplished. I gratefully appreciate your friendship and full trust in me and you will be precious to me for my entire life-time.

My special thanks to my adviser, Dr. Brent Johnson, for his encouragement, effort, moral support, and confidence in me to come to this point. It has been a great opportunity and experience to be your graduate student, and my graduate experiences will help me to be better and make differences in my life and others'. My committee members, Dr. Richard Robison, Dr. Brent Nielsen, Dr. Byron K. Murray, Dr. Paul Fields and Dr. Scott Grimshaw: I am thankful for your advice, comments, and your time. I am honored to have had you on my committee.

I have collaborated with wonderful graduate students and faculty. It was great to collaborate with Dr. Lin and his students, Thank you very much. With all of you my graduate career has been meaningful, enjoyable (many fewer tough times and trying days), and full of experiences that enrich my professional and personal life. Special thanks to Devin Dredge for technical assistance.

For financial support I express my gratitude to the University for the Brigham Young University Doctoral Fellowship, the Department of Microbiology and Molecular Biology for personal support, and research support provided by the Department, a grant from MicroVir Laboratories, and The Wesley T. Johnson Memorial Virus Research Fund.

I am eternally thankful to God and my Angels who guard me and watch over me.

TABLE OF CONTENTS

Title page	i
Graduate committee approval	ii
Dean's approval	iii
Abstract	iv
Acknowledgement	v
Table of contents	vi
Introduction	1
Endocytosis	3
Inhibitors of endocytosis and cell trafficking	7
Materials and Methods	14
Viruses, cells, and media	14
Detection of clathrin distribution in EBTr cells	15
Virus binding dot-blot assays	15
Transmission electron microscopy	16
Pharmacological treatment of EBTr cells	16
Cell viability assay	17
Assessment of virucidal effects	17
Virus infectivity and inhibitor assays	18
Virus production and inhibitor assays	20
Entry of a positive control virus	21
Fluoresceination of virus	21
Effect of actin inhibitors on cells	22
Real-time PCR	23
Results	24
Clathrin distribution in EBTr cells	24
BPV binding assays	26
Observation by transmission electron microscopy	27
BPV entry is dependent on clathrin	31
Cell viability assay	38
Trafficking through early to late endosomes	38
Test for virucidal activity	39
Assessment of techniques on a control virus	40
Tracking using fluoresceinated virus	42
BPV uptake is independent of caveolin	44
The ubiquitin-proteasome system	50
The role of actin	52
The microtubule network	57
Discussion	60
References	72

INTRODUCTION

The Parvovirus family (*Parvoviridae*) has expanded over recent years as new isolates have been identified as small single stranded DNA viruses that infect either vertebrate or nonvertebrate hosts. Members of the family have been recognized as unique, simple viruses containing two to three open reading frames with one to three promoters. The proteins, as final gene products, are produced by a combination of post-transcriptional splicing and leaky scanning for initiation of protein synthesis. Thus, these viruses present a variety of interesting molecular mechanisms involved in gene expression. Moreover, many of these viruses are important pathogens of humans or animals that invariably target mitotic cells. Further, the adeno-associated viruses in this group have a long history of use as experimental gene vectors for human gene therapy of inherited genetic disorders. (TABLE 1. Classification of the *Parvoviridae* family).

Bocaviruses

Bovine parvovirus (BPV) is a member of the genus *Bocavirus*, family *Parvoviridae* along with canine minute virus (CnMV) and human bocavirus (HBoV). BPV is a small (22 nm), non-enveloped, icosahedral virus. BPV is a pathogen of cattle found worldwide (28) that causes severe gastroenteritis in calves, mild respiratory infection, and may cause reproductive failure (2, 77). Infections often result in a viremic phase. Therefore, this virus is a contamination risk for commercially prepared bovine serum and bovine products. BPV has often been used as a model for studies on viruses that are now classified as bocaviruses. The genome consists of single stranded DNA (ssDNA) that is composed of about 5515 nucleotides (19, 20, 103, 113, 122). The genome contains non-identical palindromic sequences at the two ends (20). The

palindromic sequences have *cis* signals that are important for genome replication (9, 20, 110). The negative ssDNA strand is the predominately encapsidated form although a minor proportion of virions contain plus strands. The genome has three open reading frames (ORFs): the left ORF encodes the nonstructural proteins NS1 and NS2; the central ORF encodes the nonstructural protein NP1; and the right ORF encodes three structural proteins VP1, VP2, and VP3 (19, 68,103). The virus does not encode polymerases. BPV genome replication relies on host cell DNA polymerase and replication factors found in S-phase cells (9, 20), and transcription is carried out by cell RNA polymerase II (9, 20). Intriguingly, the genomic organization of HBoV, the second human-pathogenic parvovirus known (discovered after parvovirus B19), closely resembles the other known bocaviruses BPV and CnMV (2, 10, 63, 75, 122). The mid-ORF product of HBoV is homologous to NP1 of BPV and CnMV, and these proteins have 47% amino acid identity. The proteins of the two major ORFs of HBoV have 42-43% homology with NS1, VP1, and VP2 proteins of BPV and CnMV (2, 10). It has been reported that HBoV is one of the most common viruses in respiratory secretions of ill patients, and the virus has recently been detected in blood and fecal samples.

Concerning BPV replication and virus-cell interactions, Thacker and Johnson (124) identified the BPV hemagglutination receptor on erythrocytes as glycoporphin A (GPA), and Blackburn et al. (11) showed BPV binding to α -2,3-linked sialic acid located on the O-linked oligosaccharides of the GPA molecule. Moreover, BPV attachment occurs on both α -2,3-O-linked and α -2,3-N-linked sialic acids on nucleated bovine host cells (61). However, the processes of viral penetration into the cell and trafficking in the cytoplasm to the nuclear compartment have not been well defined beyond receptor binding.

Viral entry into the host cell includes the transport of the viral genome across the cytoplasmic membrane and subsequent release of the genome into the cytosol and, for DNA viruses such as BPV, subsequent trafficking to the nucleus. Nonenveloped viruses like BPV often exploit receptor-mediated endocytosis to deliver their genomes into the cytoplasm (56, 78, 98, 102). Enveloped viruses that utilize acid-mediated endocytosis generally undergo membrane fusion events that allow for pore formation between the viral envelope and the vacuolar membrane releasing the viral nucleocapsid into the cell cytoplasm where viral replication ensues. Some enveloped viruses fuse with the cytoplasmic membrane directly avoiding the necessity of vacuolar uptake.

TABLE 1. Classification of the *Parvoviridae* family^a

Family	Subfamily	Genus	Type species
<i>Parvoviridae</i>	<i>Parvovirinae</i>	<i>Parvovirus</i>	<i>Minute Virus of Mice</i>
		<i>Erythrovirus</i>	<i>Human parvovirus B19</i>
		<i>Dependovirus</i>	<i>Adeno-associated virus type 2</i>
		<i>Amdovirus</i>	<i>Aleutian mink disease virus</i>
		<i>Bocavirus</i>	<i>Bovine parvovirus</i>
		<i>Densovirus</i>	<i>Junonia coenia densovirus</i>
	<i>Densovirinae</i>	<i>Iteravirus</i>	<i>Bombyx mori densovirus</i>
		<i>Brevidensovirus</i>	<i>Aedes aegypti densovirus</i>
		<i>Pefundensovirus</i>	<i>Periplaneta fuliginosa densovirus</i>

^aModified after C.M. Fauquet et al., Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses. 2005. Elsevier Academic Press, San Diego CA

Endocytosis. Several possible pathways of receptor-mediated endocytosis are recognized: clathrin- and caveolin-mediated endocytosis, macropinocytosis, and novel nonclathrin/noncaveolin pathways (16, 27, 31, 64, 78, 81, 82, 84, 98, 99, 112, 119). To date, for viral infections that have been studied, viruses mostly take advantage of clathrin-associated endocytosis for their internalization (78, 98, 112). During clathrin-dependent endocytosis, transport vesicles are surrounded by a clathrin coat, which is a

three-dimensional array of triskelia. A triskelion is composed of three clathrin heavy chains (CHCs) (approximately 190-kDa) and three light chains (CLCs) (25-29 kDa), and has three-fold rotational symmetry (40, 85). Ligands that are to be transported to the cytosol are concentrated on the cell surface, and the concentrated ligands as a patch trigger recruitment of clathrin-adaptor proteins to the cytoplasmic side of the plasma membrane. Clathrin-adaptor complexes (APs) include the main distinct complexes, AP1 and AP2, and the third protein, AP180. APs bind to membranes by recognizing phosphoinositides and link clathrin to the membrane. Therefore, clathrin coated vesicles are three-layered: the internalized membrane layer with its embedded receptor/ligand complex, a middle layer that is composed of APs, and the fibrous clathrin coat (40). Clathrin coated vesicles (CCVs) are one of the most common and well defined coated or transport vesicles. These vesicles are classified according to the major components of the protein coat that surrounds them during their formation and early life. CCVs are so-called because the major component of the coat is clathrin, and membrane trafficking through CCVs is named CME. Once fission of the pit has occurred through the action of accessory proteins including dynamin and other proteins forming CCVs, the clathrin coat must be rapidly shed to allow fusion of the vesicle with its target membrane. Uncoating of clathrin is resolved by auxilin and the molecular chaperone Hsc70 (heat shock protein 70). Auxilin interacts with assembled clathrin and binds to Hsc70 via its carboxyl-terminal J domain triggering Hsc70's ATPase activity. Hsc 70 then cleaves clathrin-clathrin interactions, causing shedding of the clathrin coat. Disassembled clathrin and accessory proteins are recycled and promote the CCV cycle (15, 29, 30, 35, 40, 52, 55, 69, 73, 79, 102, 121). The cargo-containing vesicle then matures to the early endosome containing a mildly acidic environment (pH 6.5 to 6.0).

Virus engaged receptors are uncoupled from their ligands at this mildly acidic environment of the early endosomes, and receptor molecules are recycled back to the plasma membrane (126). The early endosomes are major sorting stations where endocytosed cargo can be released to the cytoplasm or can progress to more acidic late endosomes (pH 6.0 to 5.5), and the acidification of endosomes is required for release of uptaken virus into the cytoplasm (26, 45, 66, 78, 98, 108, 112, 115,126). The genome or nucleocapsids of viruses are released into the cytosol by fusion of the viral envelope with the endosomal membrane for enveloped viruses or, for non-enveloped viruses, capsid disassembly occurs in the endosome with subsequent genomic escape to the cytosol (14,118). The acidic pH of endosomes plays an essential role to trigger these events. Further, some non-enveloped viruses begin the uncoating process in the late endosome, but complete uncoating is delayed, and it is a nuclear event. For example, in some instances such as adenovirus and canine parvovirus infections, the genome together with modified capsid components translocate to the nuclear membrane (14, 57,129) where final uncoating occurs. Viruses that exploit clathrin-dependent, acid-mediated entry are sensitive to the inhibitors of endosomal acidification. Thus, inhibition of virus replication by endosomal pH inhibitors is taken as evidence for virus tracking through an acid-mediated endocytic pathway.

In contrast to clathrin-mediated pH-dependent endocytosis, caveolin-mediated entry is a triggered event (37, 54, 96, 112). Binding of virus particles to a receptor molecule on the cell surface induces the clustering of lipid rafts with a high content of cholesterol and sphingolipids. The area of the plasma membrane with the clusters invaginate to the cytosol, and the invaginate is surrounded by caveolins, the most characterized proteins of caveolae-mediated entry. These caveolin coated invaginates

formed at the cholesterol-rich microdomains at the plasma membrane are named caveolae. Caveolins stabilize caveolae, and they are remarkably static in caveosomes (78, 97). Accessory proteins involved in caveolae-mediated entry are dynamin and actin, and they are recruited by tyrosine kinase activities (31, 78, 96, 98, 100, 115). The caveolae invaginates containing the virus/receptor complex, closes, pinches off from the cell membrane, and fuses together forming caveosomes. Caveosomes are certainly part of the endocytic organelles with a neutral pH and the absence of markers for early, recycling, and late endosomes (96, 98). Caveosomes connect with the smooth endoplasmic reticulum (ER), early and late endosomes, and the cell membrane. Release of virus uptaken by the caveolar/raft system can occur in caveosomes (Echo 1), the ER (SV40), and endosomes (polyomaviruses and BK virus) (39, 78, 98). The interaction with endosomes may be crucial for some viruses that are uptaken by caveolae-mediated endocytosis but requires a low-pH environment for escape to the cytoplasm. Pharmacological/chemical inhibitors, targeted for a certain part of interconnected organelles of caveolae entry, are extensively exploited to examine a role of caveolae pathways for virus entry with defined cell entry routes.

Cell entry routes of some members of the *Parvoviridae* family have been described. Parvoviruses are known to utilize a variety of cell surface molecules as their receptor including glycoproteins, glycolipids, and glycans (10, 11, 12, 24, 92, 124). It has been reported that parvoviruses with known cell entry routes are up-taken into clathrin-associated endosomes and establish successful infections (4, 24, 51, 91, 93, 106, 128). Adeno-associated virus (AAV)'s entry into the host cell is mediated by clathrin coated pits and then routes to the late endosomes (3). The virus particles then escape to the cytoplasm where they are partially degraded by the proteasome and

delivered to the nucleus for replication (34). AAV's trafficking to the nucleus appears to be independent from the microtubule network (53). The canine parvovirus uses the transferrin receptor for attachment (92), enters through the clathrin coated pits, and localizes in endosomes (56, 93, 129). Minute virus of mice (MVM)'s cell entry and following events in the cytoplasm were investigated analyzing the effects of drugs that interfere with the endosomal acidification and ubiquitin-proteasome activities. Results suggested that MVM's entry is pH-dependent, and the interaction with the ubiquitin-proteasome system is required for MVM replication (106). The relatively rapid endocytic uptake of parvoviruses appears to be followed by slower traffic along the endocytic compartments toward the nucleus. The endosomal pathway undertaken by parvoviruses appears to be complex and depends on the virus, its concentration, and likely the cell type (33, 76, 117, 120, 132). Conformational alterations in capsid structure probably occur in the endosomal compartment facilitating uncoating and transport to the nucleus. However, the place where the virus particles escape remains unclear (128). As the virus ultimately reaches the nucleus, some evidence suggests that parvovirus nuclear entry might occur independently of the nuclear pore complex (23). Once inside the nucleus and uncoated, parvoviruses' replication depends on the cell S-phase DNA replicative machinery and transcriptional enzymes and factors.

Inhibitors of endocytosis and cell trafficking. As noted above, blocking the entrance pathway with chemical inhibitors with known sites of action can provide information on the specific route viruses exploit for cell entry. This strategy has become a commonly used technique to elucidate viral entry pathways. Following are descriptions of the inhibitors used in the current study and their modes of action.

Chlorpromazine (CHPZ). The interaction of amphipathic CHZP with the plasma membrane lipid bilayer increases lipid fluidity within the plasma membrane. This physical state of the plasma membrane lipids disrupts the formation of large membrane invaginations such as clathrin coated pits. This pharmacological effect eventually breaks clathrin recycling between the plasma membrane and cell organelles including endosomes. Overall, interruption of clathrin recycling, and depletion of functional clathrin coated pits at the cell membrane suppress CME (13, 16, 57, 74, 102, 119). CHPZ treatment blocked cell entry of west nile virus (WNV), mouse hepatitis virus type2 (MHV-2), hepatitis C virus (HCV), equine infectious anemia virus (EIAV), severe acute respiratory syndrome coronavirus (SARS-CoV), feline calicivirus (FCV), mouse polyomavirus (PyV), baculovirus, bovine viral diarrhea virus (BVDV), foot-and-mouth disease virus (FMDV), rubella virus (RV), hantaan virus (HTV), and densonucleosisviruses (DNV). The authors conclude these viruses are taken up by clathrin-mediated, pH-dependent endocytosis taking together results they found (13, 15, 16, 21, 43, 57, 59, 60, 64, 67, 71, 74, 90, 102, 119, 128).

Ammonium chloride (NH_4Cl). The acidification of endocytic vesicles triggers a conformational change in the viral capsid protein or envelope glycoproteins that leads to release of viral genome or nucleocapsid into the cytoplasm. NH_4Cl and chloroquine diffuse across the endosomal membrane and become protonated. As a result of this action, the environment of the endosomal compartment becomes less acidic, and this neutral environment arrests release of the viral genome or nucleocapsid and hinders progress of subsequent replication events if the endosomal acidification is required for early events of virus entry (43, 59, 71). Study groups (35, 52) observed that neutralization of the acidic environment of endosomal vesicles causes the arrest of the

pinching off of coated pits from the cell surface. NH_4Cl used as a selective inhibitor of endosomal acidification has been reported in the investigation of PyV, EIAV, BK virus (BKV), BVDV, DNV, SARS-CoV, and human immunodeficiency virus type 1 (HIV-1) (25, 39, 43, 57, 60, 67, 71, 128). It has been reported that NH_4Cl did not affect infections of amphotropic murine leukemia virus (A-MLV) and hepatitis B virus (HBV) (8, 50).

Chloroquine (CHLO). This organelle acidification blocker has been employed to examine entry pathways of FCV, EIAV, BKV, simian virus 40 (SV40), BVDV, HBV, DNV, MVM, MHV-2, WNV, and HIV-1 (21, 25, 43, 50, 60, 67, 76, 89, 102, 106, 119, 128). SV40 and HBV, internalized by pH-independent pathways, were insensitive to the lysomotropic agent CHLO (50, 89).

Bafilomycin A1 (Baf A1). The vacuolar proton ATPase (V-ATPase) is a multisubunit enzyme complex, and it is responsible for the acidification of membrane-bounded organelles like endosomes. V-ATPase transports H^+ over membranes against an electrochemical potential under ATP hydrolysis, and H^+ ions acidify endosomal environments. Baf A1 blocks the V-ATPase activity causing neutralization of the acidic environment of endosomes. Baf A1 is routinely used as a selective suppressor of V-ATPases, and it is used to test whether specific viral entry is reliant on endosomal acidification (6, 22, 36, 60, 130). Entry pathway studies of PyV, FCV, EIAV, BVDV, AAV, MVM, MHV-2, poliovirus (PV), baculovirus, HCV, influenza virus, HIV-1, and human rhinovirus 14 (HRV14) have analyzed the effects of Baf A1 and examined the role of endosomal acidification in a specific route that leads to replication (5, 13, 14, 33, 43, 49, 59, 67, 74, 76, 102, 106, 111, 119).

Brefeldin A (BFA). BFA interrupts transition of early to late endosomes and disrupts the Golgi apparatus. But this drug has no effect on early endosomes. BFA was

used in our study to test BPV transport from early to late endosomes. The vesicles containing the virus/receptor complex lose the clathrin coat and mature to the early endosomes. Some viruses escape to the cytoplasm under the effects of the acidic environment (6.5 to 6.0) of the early endosome. The early endosome becomes a transport intermediate recruiting Arf1 dependent coatomer proteins (COPI) (COPI, clathrin, and AP-1) and converts to the late endosome if virus does not escape from this station. Arf 1 or small GTPase's activity is catalyzed by Sec7-type GTP-exchange factors (GEFs). GEFs are primary targets of BFA. Therefore, tubulation or maturation of early endosomes are delayed, and virus transition to the late endosome is blocked (14, 88, 119). Studies reported that BFA also affects transport between Golgi and endoplasmic reticulum with the same mechanism through Arf1. BFA was chosen as a selective inhibitor of pH-dependent endocytosis to investigate early events of PyV, SV40, AAV, DNV, MVM, PV, and FCV interaction with the host cell (13, 27, 34, 46, 49, 76, 106, 119, 128).

Phorbol 12-myristate 13-acetate (PMA). Caveolae contain a population of the protein kinase C (PKC) isoenzyme, which have a high affinity and specificity for caveolae membranes. PKC is essential to regulate caveolae formation, and the precise mechanism is under investigation. Studies showed that PKC activators such as PMA interfere with caveolar endocytosis. PKC activators do not affect ligand clustering in cholesterol-rich membrane domains. But, PMA inhibition of caveolae formation markedly suppresses the internalization of lipid raft associated ligands (41, 94, 101, 114). The role of caveolar endocytosis in cell entry of filoviruses (Ebola Zaire virus and Marburg virus), Newcastle disease virus (NDV), and respiratory syncytial virus (RSV) has been assessed using PMA treatment (59, 72, 131).

Nystatin (Nys). This polyene antibiotic inhibits endocytosis associated with lipid rafts. Nystatin interacts with cholesterol of cholesterol-rich membrane domains creating large aggregates in the membrane. The aggregates accumulate cholesterol and alter the structure and function of the cholesterol-rich membrane domain. Nys exposure of cells causes aberrations of the caveolar shape and dispersion of proteins anchored to the cytoplasmic domain of receptor molecules, thereby internalization of lipid raft ligands is inhibited (18, 27, 105, 112). To address the role of cholesterol-rich membrane domains in entry routes of FCV, lymphocytic choriomeningitis virus (LMCV), Py, SV40, DNV, HTV, RV, filoviruses, and FMDV, Nys was used as a cholesterol sequestering agent (27, 41, 43, 46, 59, 64, 90, 105, 128).

Methyl-beta-cyclodextrin (MBCD). This chemical is a cyclic heptasaccharide containing a hydrophobic core that has a high affinity for cholesterol. The interaction of MBCD with cholesterol forms soluble inclusion complexes and depletes cholesterol at the cell membrane. It has been reported that MBCD treatment alters caveolae morphology and mislocates caveolins. These effects interrupt very early events of caveolin endocytosis. MBCD, as a suppressor of cholesterol-dependent pathway, has been employed in cell entry studies of BKV, A-MLV, LMCV, parvovirus, PV, DNV, EIAV, filoviruses, and SARS-CoV (8, 14, 16, 41, 46, 56, 57, 105, 128).

Genistein (Gen). Caveolar vesicles pinch off from the plasma membrane, fuse together, and route to caveosomes. Caveosomes are considered intermediate stations of the ligand/receptor complex that is up-taken by caveolar endocytosis. Gen blocks caveolae-mediated pathway inhibiting the enzyme, tyrosine kinase, which is involved in the formation of caveosomes (15, 16, 39, 74, 96). In investigations of arenaviruses, BKV, SV40, HIV, HTV, EIAV, PV, and baculovirus' entry routes the effects of Gen were

analyzed to assess whether caveolar pathway is a selective route to initiate a successful infection (14, 16, 27, 59, 67, 74, 127).

Lactacystin (Lac). This natural metabolite from *Streptomyces* spp. irreversibly blocks the 26S proteasome linked chymotrypsin-like and trypsin-like activities modifying the β -subunit of the proteasome (34, 123). Lac treatment has been used to examine whether the ubiquitin-proteasome system is required to establish a productive infection of orthopoxvirus, AAV, BKV, MHV, and MVM. Together, data from these studies suggest that proteasome function is linked to replication of orthopoxvirus, AAV, MHV, and MVM rearranging the capsid (34, 58, 123, 133).

Epoxomicin (Epox). Naturally, Epox is an antitumor agent. It interferes with the chymotrypsin-like activity of the ubiquitin-proteasome system covalently binding to the LMP7, X, MECL1, and Z catalytic subunits of the proteasome. In addition, the trypsin-like and peptidyl-glutamyl peptide hydrolyzing activities of proteasomes are inhibited by Epox treatment to 100- and 1000-fold slower rates, respectively. The inhibition of the chymotrypsin-like activity of Epox is more potent than Lac inhibition of the chymotrypsin-like activity (106, 109). Studies on replication events of MVM and vaccinia virus (VACV) demonstrated that the ubiquitin-proteasome activity enhances a productive replication of these two viruses while blocking the proteasome with Epox. MVM and VACV' replication was reduced in the presence of Epox (106, 109).

Cytochalasin D (Cyt D). After uncoating, the viral genome needs to be transported to proper sites in the cytosol for replication and expression. Viruses, objects in a scale of nanometers, are not able freely to diffuse through the crowded cytosol and efficiently find their proper destination by random movement. Thus, viruses hijack cell cytoskeletal structures to reach proper sites of replication in the cytosol or the nucleus.

Virus trafficking associated with actin is commonly assessed using Cyt D and latrunculin A. Cyt D interrupts actin polymerization by occupying the faster-growing “barbed” ends of actin filaments. This pharmacological inhibition of actin polymerization was used to characterize the cell transport system involved in FCV, LMCV, DNV, EIAV, PV, FMDV, and VACV’s trafficking in the cytosol (14, 16, 21, 43, 72, 105, 119, 128).

Latrunculin A (Lat A). This chemical is one of extensively used substances to block intracellular transport by actin filaments. Lat A binds to monomeric actin, and this interaction prevents incorporation of actin monomers to filaments. Intracellular trafficking of Herpes simplex virus 1 (HSV-1), LCMV, BKV, and SV40 has been studied in the presence of Lat A as an inhibitor of actin polymerization (27, 38, 42, 105).

1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro 1,4-diazapine hydrochloride (ML-7). ML-7 is a highly specific inhibitor of myosin light chain kinase (MLCK), and it selectively suppresses active actin that is involved in intracellular transport. ML-7 blocks phosphorylation of the myosin light chain (MLC). This selective compound has been used to elucidate movements of intracellular components and the contraction physiology of smooth muscle (95, 107).

Nocodazole (Noc). Microtubules, one of three principal components of the cytoskeleton, determine cell shape and mediate a variety of cell movements including the intracellular transport of organelles. Like actin filaments, microtubules are dynamic structures that undergo continual assembly and disassembly within the cell. Tubulin, a heterodimer, consists of α - and β -tubulins and polymerizes to form microtubules. The microtubule-active drug, Noc, binds to tubulin and then delays the polymerization of the heterodimers. In this way, Noc interrupts microtubule associated transport in the cytoplasm (15, 64, 98, 119, 125, 128). Noc was used to study functions of microtubules

in FCV, SV40, DNV, HBV, RV, PV, and FMDV's trafficking in the cytosol (14, 21, 27, 62, 64, 128).

Vanadate (Van). Dynein, a motor protein, transports various cellular cargo including virus/receptor complexes by "walking" along microtubules towards the cell center, which is the nucleus. DNA viruses, excluding poxviruses, amplify their genome and transcribe mRNAs in the nucleus. Van disrupts the dynein motor, which leads to the inhibition of transport along microtubules. This microtubule-active drug has been used in studies of the intracellular trafficking of HSV as a selective inhibitor of this microtubule-based motor protein (7, 32, 65).

Erythro-9-3(2-hydroxyonyl) adenine (EHNA). EHNA is an inhibitor of dynein adenosine triphosphatase (ATPase). Analyzing the effects of EHNA allows examination of the efficiency of the microtubule-based dynein motor on intracellular transport. It has been reported that EHNA has been utilized in studies of intracellular trafficking of HTV, BK, and herpes viruses (7, 65, 87, 104).

Studies concerning BPV interactions with its target cell and steps beyond receptor binding are crucial in elucidating pathogenesis of human parvoviruses and molecular and cellular mechanisms of bocavirus replication. In this paper, we report post-binding events of BPV entry into EBTr cells and subsequent trafficking routes. We confirm the functional importance of the pH sensitive CME in BPV penetration into EBTr cells and show trafficking associated with actin filaments and microtubules in the cytosol.

MATERIALS AND METHODS

Virus, cells, and media. The BPV strain used in this study was the original HADEN isolate obtained from F. R. Abinanti (1). Virus stocks were prepared from infected EBTr

(embryonic bovine tracheal) cells, titers were determined as infected focus-forming units, and stocks were stored frozen at -80°C . EBTr cells, a diploid cell strain (ATCC NBL-4), were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) containing 0.11 % sodium bicarbonate, 10 mM HEPES buffer, 50 μg gentamicin per ml, and either 5% cosmic calf serum (Hyclone) or 1% Fetal Clone III serum (Hyclone). In assays where virus infection was measured, Fetal Clone was used in the medium as cosmic calf serum contains anti-BPV antibodies. BEK (bovine embryonic kidney) cells and BT (bovine turbinate) cells were used for immunofluorescent studies and virus stock production, respectively.

Detection of clathrin distribution in EBTr cells. EBTr cells grown on 12 mm glass coverslips in shell vials were incubated until they reached 80% of confluency. The cells were fixed in cold acetone then stained by anti-clathrin antibody (mouse IgM anti-human clathrin MAB, Chemicon, CBL 188) followed by the secondary antibody (goat anti-mouse IgM, rhodamine-conjugated, Chemicon, AP128R), then observed by fluorescence microscopy using techniques previously described (61). Images were collected and stored as digital micrographs.

Virus binding dot-blot assays. Purified glycoporphin A (GPA) and purified clathrin (Sigma-Aldrich) were fixed on nitrocellulose membranes in a dot-blot format. The dot-blot procedures were followed as previously described (11, 61). Briefly, the virus binding substrates GPA and clathrin together with the positive controls, consisting of BPV antigens purified by ultracentrifugation on CsCl isopycnic gradients and crude antigens as infected cell lysates, were dotted on the membranes. Negative control dots consisted of phosphate buffered saline (PBS). Purified virus (20 μl per dot) was added to the GPA and clathrin wells as well as the control wells and incubated for 30 min at

room temperature (RT). The membranes were then removed from the dot-blot template and washed for 30 min in blotto blocker. Following appropriate washes in PBS the membranes were stained by a protein A-immunoperoxidase complex (IP) followed by incubation in a solution of 4-Chloronaphthol chromogen and hydrogen peroxide for visualization of virus attachment to the membrane-fixed molecules as previously described (124).

Transmission electron microscopy (TEM). At 15 min postinfection cells were fixed in 2% glutaraldehyde-0.06 M cacodylate for 1 h at 4°C and then postfixed for 2 h in osmium tetroxide (1:1 dilution with cacodylate) at RT. After dehydration in a grade series of ethanol solutions, cells were infiltrated in Epon resin. Ultrathin sections (0.1 µm) were mounted on copper grids and stained with uranyl acetate and lead citrate. Observations were performed with a FEI T12 electron microscope at 80 kV. The virus particles and infection-associated structures' images were taken by a Gaton Multiscan 794 digital camera and Image J software.

Pharmacological treatment of EBT_r cells during BPV infection. EBT_r cells were treated with pharmacological inhibitors for 30 min prior to the BPV infection at 37°C and 5% CO₂. The inhibitors were present for the duration of the infection. A group of pharmacological agents was used to study whether BPV is up-taken by clathrin-mediated, acid-dependent pathway or the caveolae-mediated, acid-independent pathway. Virus uptake by CME was tested by employing CHPZ, NH₄Cl, CHLO, Baf A1, and BFA. These drugs were purchased from Sigma Aldrich Chemical Company. Caveolae-mediated entry was investigated by treating the cells with PMA (Fluka BioChemica), Nys (Sigma Aldrich), MBCD (Sigma Aldrich), and Gen (Sigma Aldrich). The next group of drugs was used to detect the role of proteosomal enzymes for BPV

processing in the cytosol. It included Epox and Lac, and they were obtained from Sigma Aldrich. The third group of drugs consisted of Noc (Sigma Aldrich), Cyt D (Fluka BioChemica), Lat A (Sigma Aldrich), Van (Sigma Aldrich), EHNA (A. G. Scientific Inc.), and ML-7 (Alexis Biochemicals). These drugs were used to examine the role of cytoskeletal structures for BPV trafficking in the cytosol to the nucleus. The pharmacological agents were stored and dissolved in solvents following manufacturers' recommendation. The working concentrations of the inhibitors were freshly prepared in DMEM containing 1% Fetal clone III serum and stored at 4°C. Each drug was prepared as a set of increasing concentrations based on cell viability/cytotoxicity tests.

Cell viability assay. For all the inhibitors, a dose-response assay was performed to detect the working doses. Cell viability was assessed by measuring mitochondrial dehydrogenase activity (the Cytotox-MTT test, Anisara Corp.). Viable cells are able to reduce MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and reduction is measured spectrophotometrically (34). These tests were performed according to the manufacturer's instructions. Working concentrations of the selective inhibitors prepared for BPV entry assays was evaluated for toxicity on EBTr cells. Concentrations of the pharmacological inhibitors were chosen for further study that did not exhibit significant toxicity for either virus or cells.

Assessment of possible virucidal effects of the inhibitors. To test for possible direct virucidal effects of used chemicals, media containing the highest concentrations of drugs that were tested were inoculated with virus, incubated at RT for 30 min, and then surviving virus was detected by standard virus assay. Controls included in these tests were cells infected with BPV stock not pretreated with the drugs.

Virus infectivity and inhibitor assays. Infectivity assays were designed to test, in the presence of inhibitor, how many cells would be infected or efficiency of viral protein synthesis when exposed to a virus stock of known titer. EBTr cells were grown in 24-well plates, washed twice with serumless medium to remove any anti-BPV antibody that was present in the growth medium, and renewed in media supplemented with the selective inhibitors (see TABLE 2). These cultures were then incubated at 37°C for 30 min to allow uptake of the chemical inhibitor and the initiation of its biological activity. The cultures were then infected with virus at three different multiplicities of infection (MOI) to ensure countable and significantly valid numbers of infected foci. The cultures were incubated at 37°C for 2 days, the cells fixed with formalin-acetic acid-alcohol (FAA fixative), and stained for detection of BPV infected cells by IP (61). Dark purple cells were counted as an indicator of the BPV infected cells. The experiment included mock-infected, drug toxicity controls, cell controls, and virus infectivity controls. The effects of each drug on BPV infectivity (i.e. protein synthesis) were assessed by three separate and independent experiments with triplicates of each concentration of the drug. The replicate experiments showed similar results.

TABLE 2. Chemical inhibitors and their mechanisms of action

Target	Mechanisms of action
Inhibitors of clathrin endocytosis	
Chlorpromazine	prevents receptor/ligand induced clathrin assembly by interfering clathrin recycling.
Chloroquine	raises endosomal pH and blocks clathrin coated pit formation.
Ammonium chloride	increases endosomal pH and inhibits

Bafilomycin A1	pinching of CCVs from the cell membrane. suppresses endosomal acidification by inhibiting V-ATPase.
Inhibitors of caveolin pathway	
Phorbol 12-myristate 13-acetate (PMA)	active against caveolae formation delaying caveolin-1 assembly at microdomains.
Nystatin	dysfunctions caveolae depleting microdomain cholesterol .
Methyl-beta-cyclodextrin (MBCD)	sequesters microdomain cholesterol at the plasma membrane.
Genistein	disrupts caveosome formation by blocking phosphorylation of tyrosine kinase.
Inhibitors of cytosolic trafficking	
<ul style="list-style-type: none"> • Early to late endosome transition Brefeldin A 	suppresses the early-to-late endosomal trafficking by disrupting endosomal maturation.
<ul style="list-style-type: none"> • Proteasome Epoxomicin 	interferes with the chymotrypsin-like activity of the ubiquitin-proteasome system.
Lactacystin	blocks the 26S proteasome associated chymotrypsin-like and trypsin-like activities modifying the β -subunit.
<ul style="list-style-type: none"> • Actin Cytochalasin D 	impairs actin polymerization by occupying the faster-growing “barbed” ends of actin filament.
Latrunculin A ML-7 (1-(5-iodonaphthalene-1-	prevents incorporation of actin monomers to filament by binding to monomeric actin.

sulfonyl)-1H-hexahydro 1,4-diazapine hydrochloride)	specifically blocks intracellular transport via actin by interfering with phosphorylation of MLC.
<ul style="list-style-type: none"> Microtubule Nocodazole 	delays microtubule formation by binding to tubulins.
Vanadate	inhibits transport along microtubules by disrupting the dynein motor.
EHNA (Erythro-9-3(2-hydroxyonyl) adenine)	inactivates dynein ATPase that results in the impairment of microtubule transport.

Virus production and inhibitor assays. Production assays tested for the yield of progeny virions or completion of the replication cycle in experimental infections in the presence of drug. The virus production assays were carried out as follows. EBTr cell cultures in 25 cm² culture flasks at a confluency of 70% to 80% were washed twice with the serumless medium. The cells were pretreated with the test drug (see TABLE 2) for 30 min at 37°C and then infected with virus at a MOI of 2. The infection was allowed to proceed for 3 days at 37°C in the presence of the drug. The cells were then lysed by three freeze-thaw cycles and then centrifuged at 1000 rpm for 30 min in a tabletop centrifuge to remove cell debris. The supernates were tested for virus yields that were produced in the presence of the drug. The virus titrations were performed as described above. The cells were fixed with FAA fixative and stained with IP staining for detection of BPV infected cells. Virus titers were expressed as virus focus-forming units (FFU) per ml. Values are presented as the averages of triplicate samples. The experiment included mock-infected, drug toxicity controls, cell controls, and virus infectivity controls.

The set of the drug concentrations were assessed by separate and independent experiments with similar results.

Entry of a positive control virus. Adenovirus is internalized by a clathrin-mediated pH-dependent pathway (81, 82). Adenovirus entry into HeLa cells was tested by the virus infectivity assay in order to verify specific inhibition effects of the drugs on BPV entry. HeLa cells were pretreated with the selective drugs and infected with Adenovirus type 2 for 2 days in the presence of the drugs. The infected cells were fixed with FAA and stained with IP for visualization of Adenovirus-infected cells. The number of Adenovirus-positive cells in treated cultures was compared to the number of virus positive cells in inhibitor-negative control cultures.

Fluoresceination of virus. Purified virus was obtained by CsCl isopycnic gradient ultracentrifugation. Cultures of bovine turbinate (BT) cells (Invitrogen) at 70-80% confluence in 150 cm² culture flasks were inoculated with BPV stock culture at a MOI of <0.01 and incubated at 37°C until 4+ cytopathic effect was obtained. Following three freeze-thaw cycles, the cell debris was removed by low speed centrifugation. The virus particles, together with small particulate cell structures, were pelleted by ultracentrifugation for 4 h at 85,000 x g. The pellets were allowed to soak overnight in pH 8.2 0.01M tris buffer and then suspended by sonication in the tris buffer. Solid CsCl and CsCl dissolved in tris buffer were combined with the virus to form appropriate volumes of virus suspensions at a CsCl concentration of 1.4 g/cc. These suspensions were then banded in isopycnic gradients by centrifuging at 130,000 x g for 48 h. The virus band was separated by drop collection from the bottom of the tubes. The CsCl salt was removed from the purified virus by dialysis against PBS overnight at 4°C. For fluoresceination of the purified virus, a procedure similar to that reported by Freistadt

and Eberle (44) was used. N-Hydroxysuccinimide (NHS)-Fluorescein (Pierce) was used to directly label purified virus. This reagent was prepared and handled according to the manufacturer's directions. Purified BPV was sonicated to break up possible viral aggregates. A quantity of virus in 0.5 ml of PBS, with a BPV hemagglutination titer of 10,000 HA units per 50 μ l, was combined with 2.5 μ l of NHS-Fluorescein reagent then incubated in the dark at 4°C for 2 h. Following this reaction, the labeled virus was dialysed against PBS overnight at 4°C in the dark against multiple changes of PBS until no more color escaped the dialysis bag. The virus was tested by standard BPV infectivity assays to assess for infectious virus both before and after labeling and it was found that infectivity remained after labeling although diminished by nearly 2 logs. The labeled virus was stored at 4°C in the dark until used. Immediately prior to use, the fluoresceinated virus was centrifuged in a microfuge for 1 min to remove possible viral aggregates. All immunofluorescence observations were done in the Nikon Eclipse E600 epifluorescence microscope.

Effect of actin inhibitors on cells. Verification of the activity of drugs thought to inhibit actin polymerization was performed by observing morphologic alterations in the actin contained in cells exposed to Cyt D or Lat A. EBTr cells as well as BEK cells were seeded in shell vials in standard growth medium. They were allowed to attach to the round glass coverslips (12 mm diameter) for 24 h, then the medium was renewed with either standard control growth medium or test medium containing 0.1 μ M Cyt D or 0.05 μ M Lat A. Incubation was allowed to proceed for 24 h, then the cultures were washed in PBS and fixed in -80⁰ C acetone. Staining for actin was performed by covering the coverslips with mouse anti-actin (Chemicon MAB 1501), incubating for 30 min at 37⁰ C, then washing with PBS-Tween and distilled water. This step was followed by a 30 min

incubation with goat anti-mouse-Texas red conjugate (Invitrogen T862), then washes with PBS-Tween, and distilled water. The cultures were then mounted in antifade mounting fluid containing buffered glycerin, p-phenylenediamine and DAPI. DAPI was used to render color contrast to the cell nuclei and make them more visible.

Fluorescence microscopic observation was performed in the Nikon fluorescence microscope using the Texas red-DAPI filter and photomicrographs were taken under the 40X objective lens.

Real-time PCR. Cells were treated with pharmacological agents 30 min prior to virus infection. Infection (MOI=1.5 TCID₅₀/cell) proceeded for 24 h at 37°C in the presence of the pharmacological agents. The cells were detached by trypsinization and pelleted as recommended in RNA extraction kit (RNeasy mini kit, Qiagen). Total RNA was extracted using the RNA extraction kit method following the manufacturer's instructions. cDNA synthesis was performed with the reverse primer (mProm-II™ Reverse Transcriptase, Promega). Primers for BPV DNA amplification were designed using Primer-BLAST (Primer-Basic Local Alignment Search Tool) which uses Primer3 to design PCR primers for user-selected database.). The forward and reverse primers are shown in TABLE 3. The RPLPO (large ribosomal protein) gene was used as a control gene to normalize DNA quantity between samples and experiments. Amplification and real-time detection of PCR products were performed on the cDNA samples using the Lightcycler 400 system (Roche Diagnostics, Rotkreuz, Switzerland) with SYBR Green (Roche). The fluorescent dye SYBR Green binds to double-stranded DNA. At the end of the extension step of every cycle, the fluorescence was measured. The cycle number at which the fluorescence starts to increase is related to the initial number of target copies. Cycling conditions consisted of a step at 95°C for 10 min to activate the polymerase

enzyme followed by 35 cycles with the following thermal profile: 94°C and 15 s, 66°C and 5 s, and 72°C and 30 s. For each quantitative PCR analysis, 4 µl of diluted DNA was deposited in replicates for a 10 µl total reaction volume. The ratio of the amount of amplified gene with the amount of RPLPO gene allowed us to compare accurately expression of the genes of interest between different samples or treated and untreated samples.

TABLE 3. Primer sets for viral RNA quantification by qPCR

Gene	Protein function	Orientation	Primer sequence (5 ¹ -3 ¹) ^a
NS	Nonstructural early protein, genome replication	For	5 ¹ -CCA GTA CCA GGA AAC GGA GA-3 ¹
		Rev	5 ¹ -GTG TTG GCT GGA TGA AAC CT-3 ¹
NP	Early nonstructural phosphoprotein	For	5 ¹ -CGA CTA GCC AGC AAA GGA AC-3 ¹
		Rev	5 ¹ -AAC AAT TCT CCC ACC CCT TC-3 ¹
VP	Capsid structural protein	For	5 ¹ -CCA GTA CCA AAC GGA GA-3 ¹
		Rev	5 ¹ -GCG TTG ATG GTG TAT GC-3 ¹

House keeping gene (large ribosomal protein) primers

For 5¹-CCT TCC CAC TTG CTG AAA AG-3¹
 Rev 5¹-GAC TCC TCC GAC TCC TCC TT-3¹

a primers in 5¹ to 3¹ orientation; (For) forward; (Rev) reverse.

RESULTS

Clathrin distribution in EBTr cells. To investigate the early steps of BPV infection, we examined the distribution of clathrin on EBTr cells ensuring that these cells display a rich array of this endocytosis machinery and to determine whether the anti-human clathrin antibody reagent to be used in our studies would react with bovine clathrin. Clathrin expression and its recycling between the plasma membrane and CCVs are

crucial for membrane trafficking that is mediated by clathrin-associated vesicles. Therefore, the distribution of clathrin on EBTr cells was investigated by immunofluorescence microscopy with an anti-clathrin fluorescent antibody conjugated with rhodamine. The results appeared to show that clathrin is abundantly distributed in the plasma membrane, cytosol, and Golgi of EBTr cells (Fig. 1). Strong staining in the perinuclear area is consistent with Golgi-associated clathrin. Furthermore, there was strong cross reactivity evident between anti-human clathrin and clathrin of bovine origin.

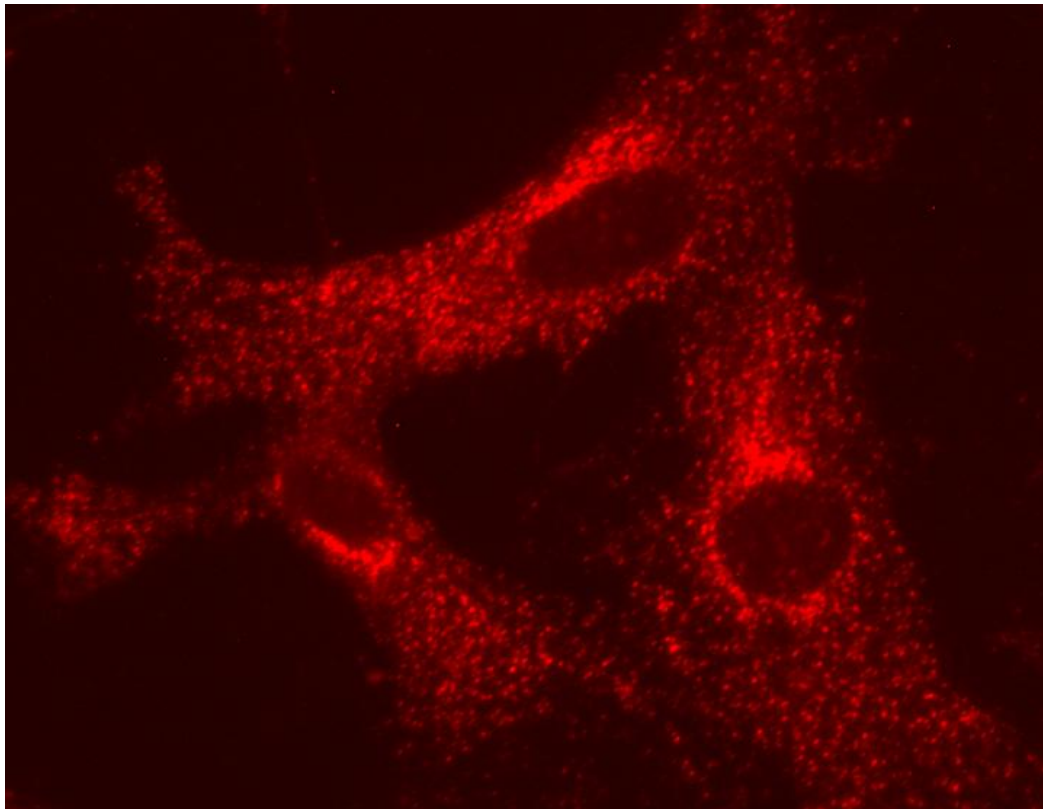


FIG. 1. Distribution of clathrin on EBTr cells seen by immunofluorescence. Mouse anti-human clathrin reacted with bovine clathrin and was visualized using a rhodamine-conjugated secondary antibody.

BPV binding assays. The replication cycle of an animal virus begins with its binding to a receptor expressed on the surface of a susceptible cell. Viruses simply use these cell surface molecules for attachment. Some viruses bind to more than one receptor moiety, and some are transferred to a secondary receptor before or during the penetration process. It is known that BPV binds to the GPA molecule on erythrocytes through α 2,3-linked terminal neuraminic acid located on the O-linked oligosaccharides. GPA on nucleated bovine cells binds to both α 2,3-O-linked and α 2,3-N-linked sialic acids. It is not yet known whether BPV will bind to additional membrane associated molecules or if it goes to a secondary receptor during penetration. Accordingly, dot blot assays were used to examine BPV binding to sialic acids and other cell components fixed on the nitrocellulose membranes. We unexpectedly observed that BPV would bind to clathrin. In this assay purified and crude BPV antigen (positive controls), PBS (negative control), GPA (as an additional positive control), and clathrin were fixed on the nitrocellulose membrane. BPV was added on them. The membranes were then stained by anti-BPV and the immunoenzyme complex for visualization of BPV binding to the membrane fixed molecules. As seen on the dot blot membrane, we observed similar amounts of stain intensity in wells with positive controls, GPA, and clathrin indicating BPV attachment to GPA and, intriguingly, to clathrin as well (Fig. 2). Whatever function BPV binding to clathrin may imply is at present unknown.

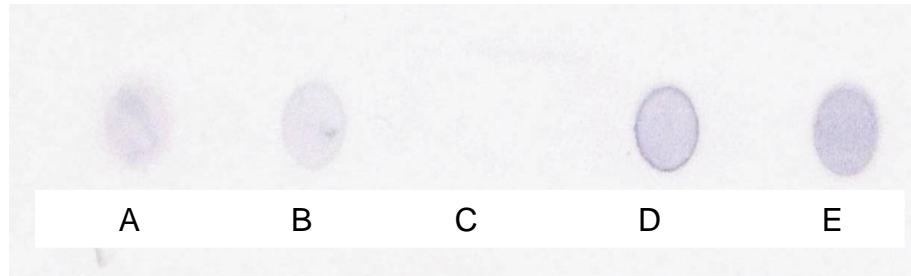


FIG. 2. Binding of BPV to clathrin on a Dot Blot. Dot (A) consisted of the membrane bound clathrin and virus. GPA, known to bind BPV, served as a positive control (B). A negative control, PBS, was dotted (C). A second positive control consisting of crude antigen cell lysate was included (D). Purified virus serving as a positive antibody-enzyme-chromogen control (E) was dotted directly on the nitrocellulose membrane.

Observation of BPV infected cells with electron microscopy. BPV infected EBTr cells were scanned by TEM to observe endocytosis-associated structural components at early points after infection. EBTr cells were suspended in PBS, infected with purified virus stock previously produced on CsCl isopycnic gradients, and incubated at RT for 15 min. EBTr cells not infected with virus served as control. The BPV infected and uninfected EBTr cells were then processed for electron microscopy analysis. Virus particles were found externally at the thickened clathrin-associated regions of the plasma membrane after 15 min of incubation at RT (Fig. 3B). These regions appear to

be thickened as a result of clathrin recruitment at the cytoplasmic side of virus/receptor complexes. The thickened regions of the plasma membrane in control cells were less intense compared to the thickened regions of BPV infected EBTr cells (Fig. 3A, C, E). In thickened clathrin-associated regions of the plasma membrane of BPV infected cells, some areas invaginated to the cytosol forming pits (Fig. 3D), and the two ends of some pits were closed making images of clathrin-coated invaginates ready to pinch off from the plasma membrane (Fig. 4A). Images resembling CCV were also observed in the thickened regions of the plasma membrane (Fig. 4B). Virus particles were found in association with invaginate (Fig. 3D) and in vesicles resembling CCVs (Fig. 3F). Scanning control cells with TEM did not reveal these endocytosis-associated structural components.

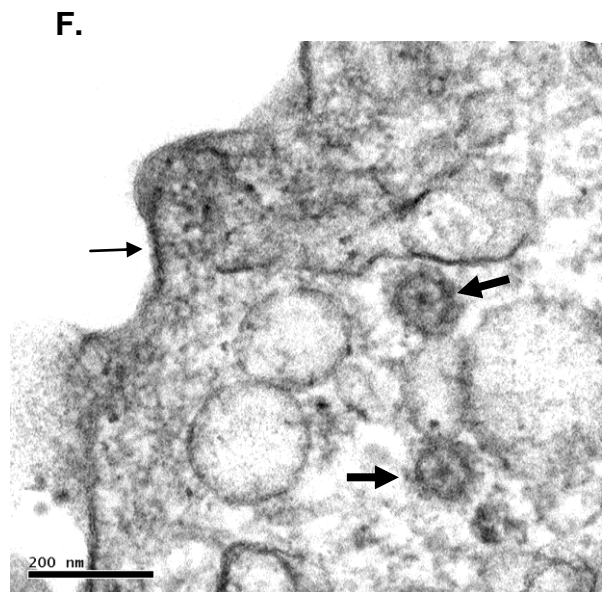
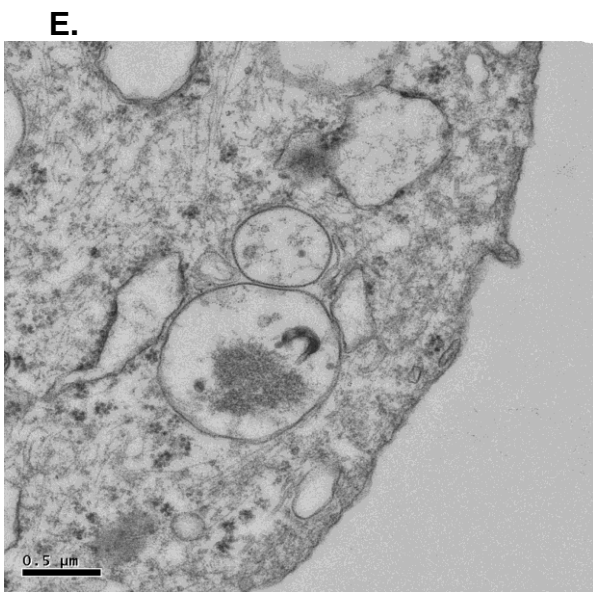
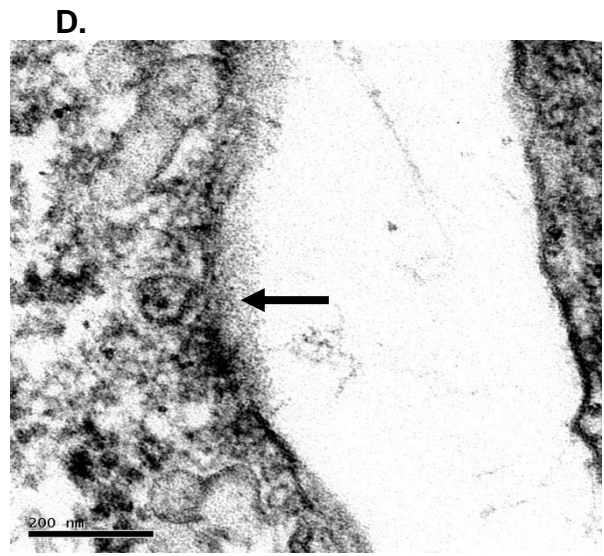
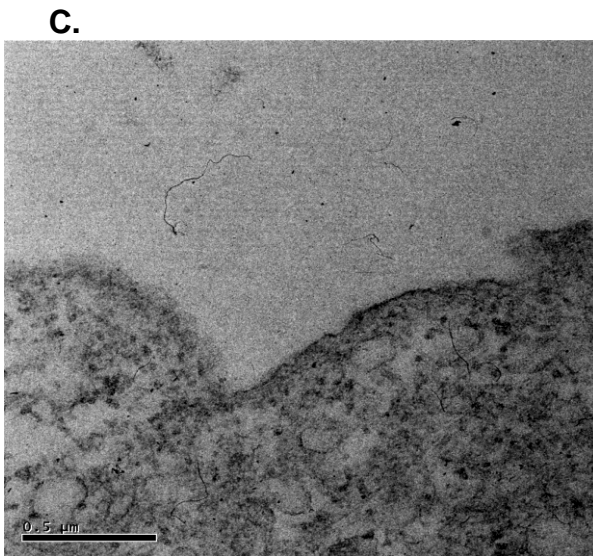
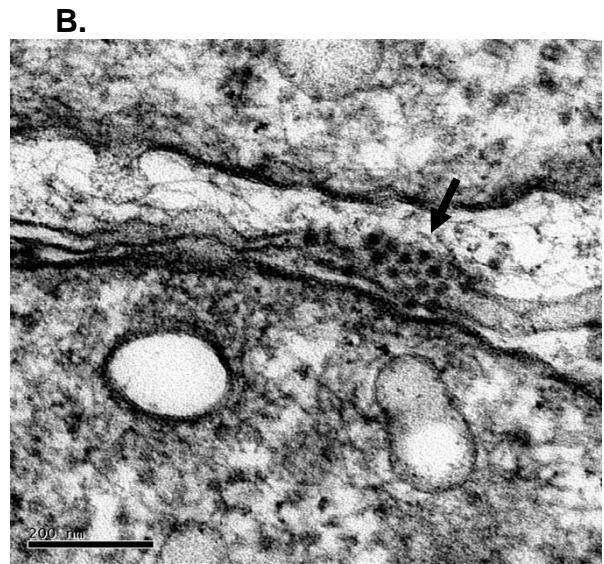
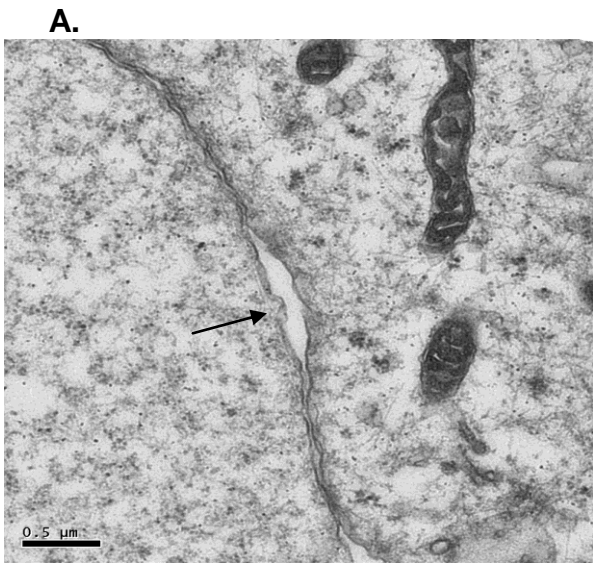


FIG. 3. Observation of control and infected cells using electron microscopy. (A) uninfected cells showing an intercellular space. (B) Cells infected with BPV. Virus particles in intercellular space (arrow). (C) View along the cell membrane of control cells. Cells showing smooth membrane. (D) Virus-containing invaginate with microscopically dense coat. (E) Intracellular compartments of uninfected cells. (F) Vesicles containing virus and dense areas along the cell membrane consistent with clathrin assembly.

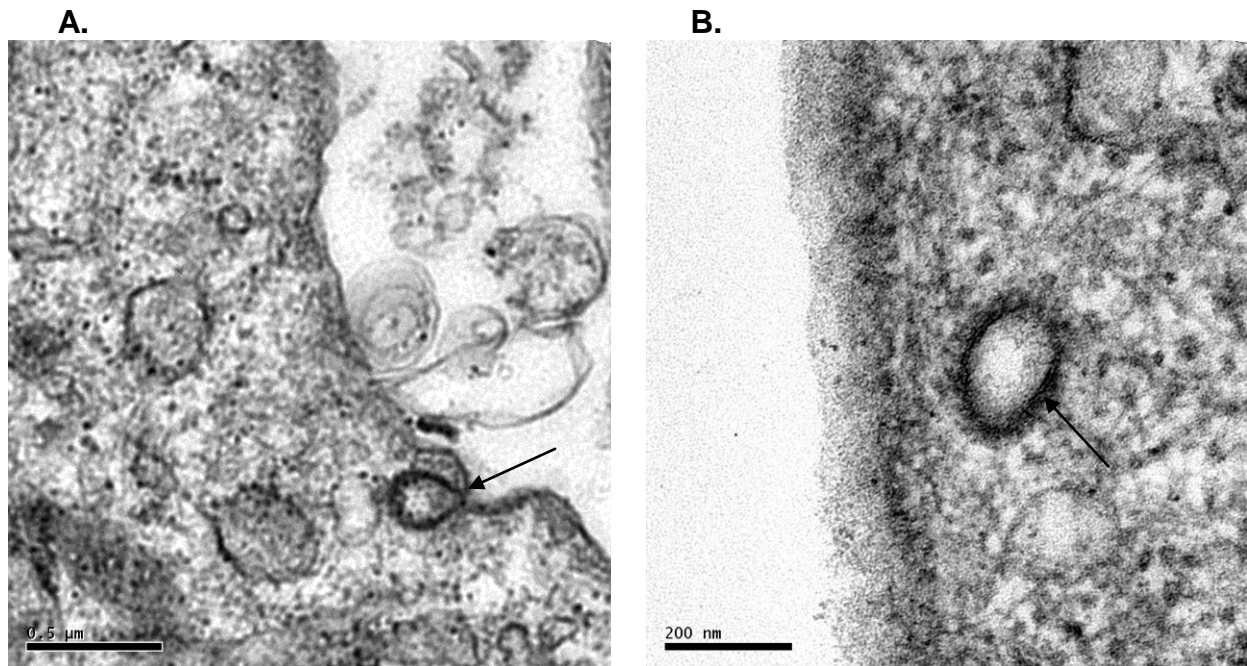


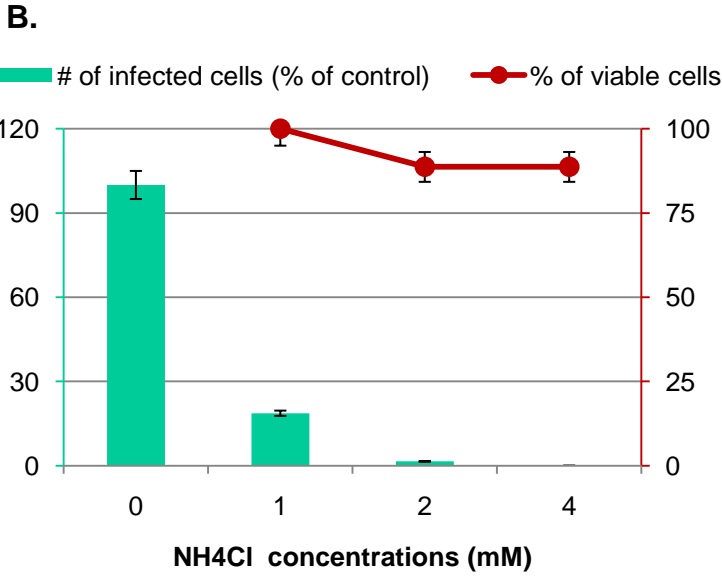
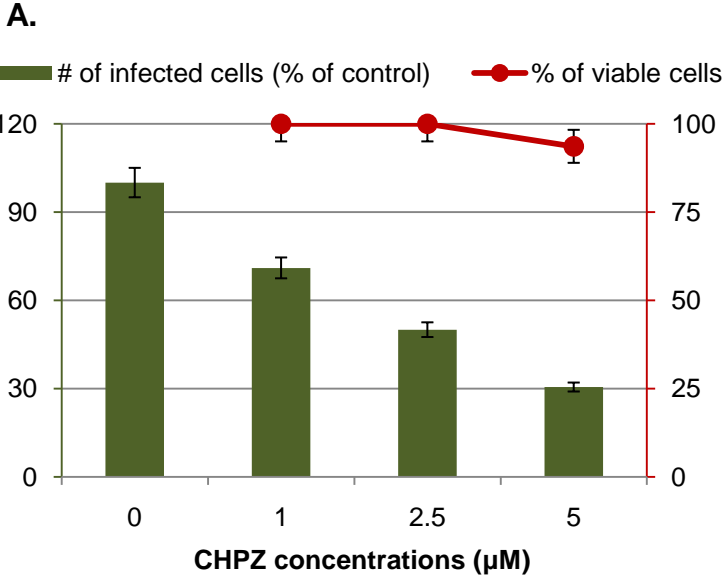
FIG. 4. Apparent clathrin-associated structures in infected cells. (A) an invaginate with a densely coated membrane (arrow). (B) cytosolic vesicle with a dense membrane.

It appears that these endocytosis-associated structural components in EBTr cells were caused by BPV attachment, and BPV entry is mediated by endocytic vesicles. To confirm these results, specific pharmacological drugs were used to test CME for BPV entry into the host cell. At the same time, caveolae-associated receptor-mediated endocytosis was investigated by the same approaches used to examine the clathrin-dependent pathway.

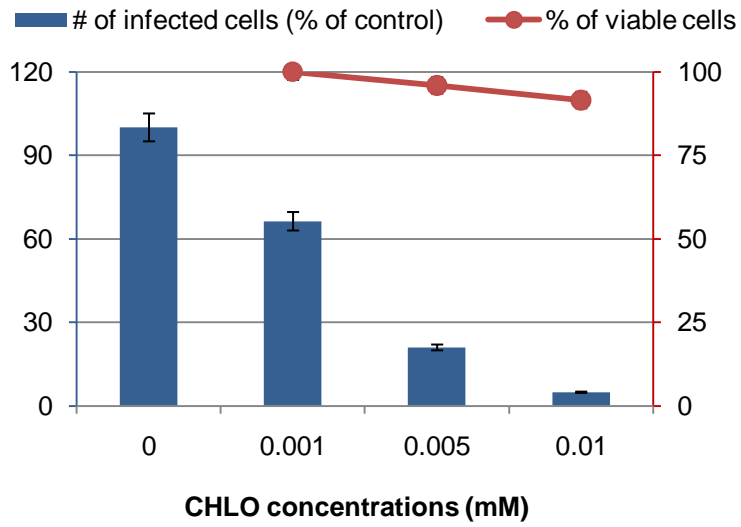
BPV entry is dependent on clathrin. Virus binding to its specific receptor on the cell surface triggers cell signaling pathways involved in virus penetration through the plasma membrane. If virus entry is mediated by clathrin-dependent endocytosis, recruitment of clathrin and its adaptor proteins leads to the formation of clathrin coated pits along the plasma membrane regions where virus/receptor complexes are bound. Thus, we sought to determine whether BPV uptake is affected by CHPZ treatment. Virus infectivity and production assays were carried out with CHPZ. Potential toxic side effects of the drug were determined by cell viability/toxicity tests. The inhibition of formation of clathrin coated pits by CHPZ treatment strongly reduced virus infectivity from 29% to 70% (Fig. 5A) and titers of progeny viruses from 36% to 58% in a dose-dependent manner (Fig. 6A). These data indicate that BPV entry appears to be mediated by clathrin-dependent pathway of endocytosis.

Because CME is a prominent mechanism for introducing cargo into cells, and because it is dependent on endosomal acidification, blocking endosomal acidification was undertaken and the results are shown in Figs. 5, 6, and 7. Inhibitors of endosomal acidification are grouped into three classes based on their modes of action. The first class is composed of the lysosomotropic weak bases, such as NH_4Cl and CHLO. They diffuse across membranes in a dose-dependent manner and rapidly become protonated. This results in neutralization of the acidic environment of endocytic vesicles. The second class of agents alters the endosomal pH by exchanging protons for potassium and sodium. The third class of inhibitors consists of blockers of vacuolar H^+ -ATPases such as Baf A1 that hinders transport H^+ over membranes against an electrochemical potential under ATP hydrolysis. Whether BPV uptake is affected by acidification inhibitors was examined by virus infectivity and production in the presence

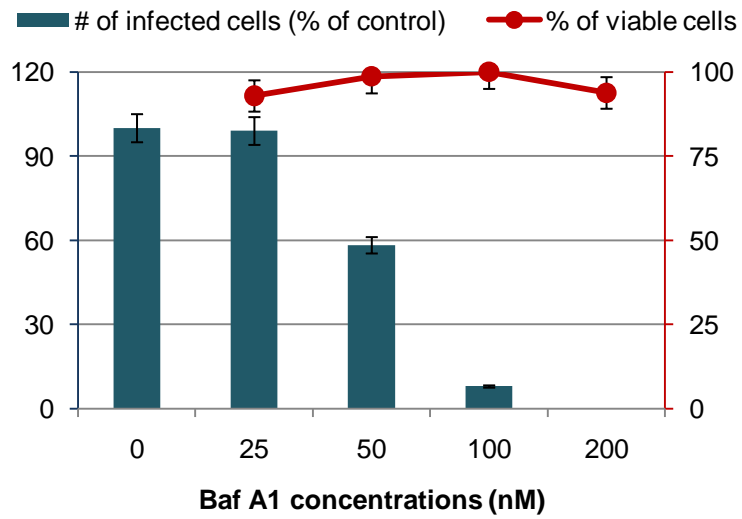
FIG. 5.



C.



D.



E.

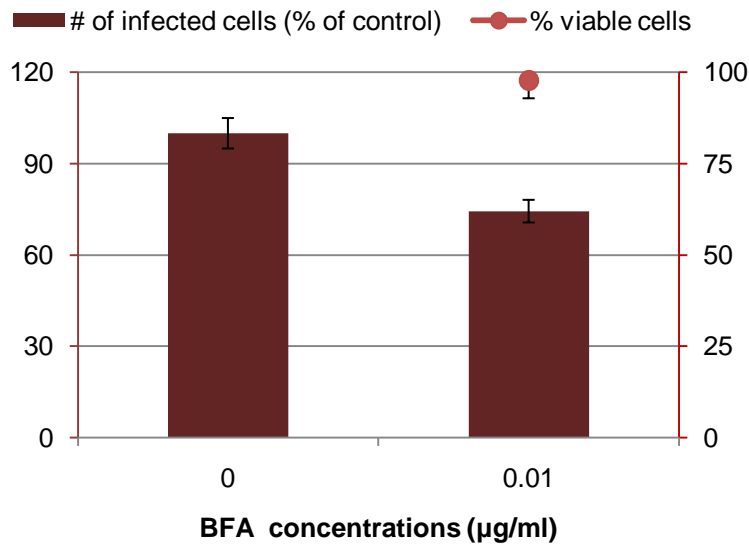


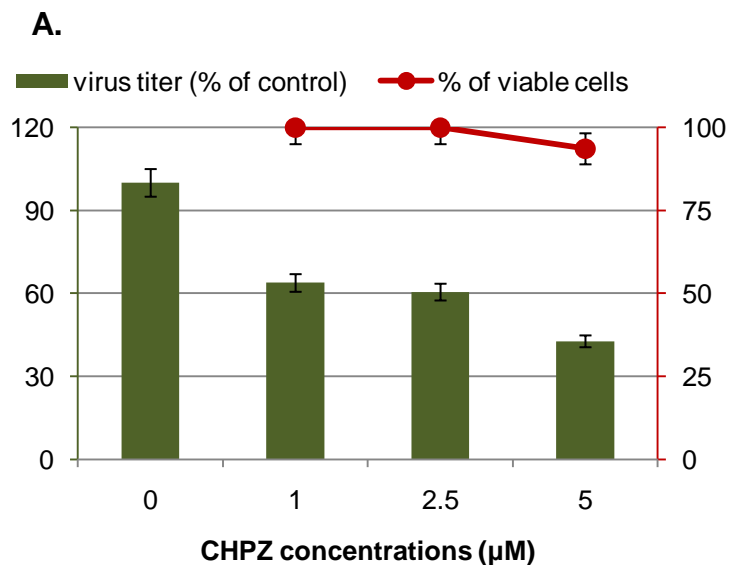
FIG. 5. BPV infectivity in EBTr cells treated with the inhibitors of CME. EBTr cells were treated with CHPZ (A), NH₄Cl (B), CHLO (C), Baf A1 (D), or BFA (E) 30 min prior to the virus infection. The drug concentrations were maintained during 48 hrs of virus infection. Infectious centers were determined by IP staining. The results are expressed as numbers of infectious centers in the treated cultures as a fraction (percent) of infectious centers in untreated cultures (bar plots). Cell viability in drug treated cultures compared to control cells without drug is shown by the data points. The data represent three independent experiments performed with replicate cultures in triplicate per experiment. Error bars represent standard errors of the means.

of NH₄Cl, CHLO, or Baf A1. Virus infectivity depleted from 81% to 100% in NH₄Cl-treated cells (Fig. 5B). NH₄Cl reduced virus production from 42% to 98% (Fig. 6B). CHLO markedly inhibited virus infectivity (34-95%) and production (35-61%) after 48 hours and 72 hours infection, respectively (Fig. 5C and Fig. 6C). Vacuolar H⁺-ATPase inactivation by Baf A1 reduced infectivity to 92% and production to 86% of BPV infectivity and production in control cells (Fig. 5D and Fig. 6D). Collectively, these results suggest that the low pH is required for BPV internalization and supported the

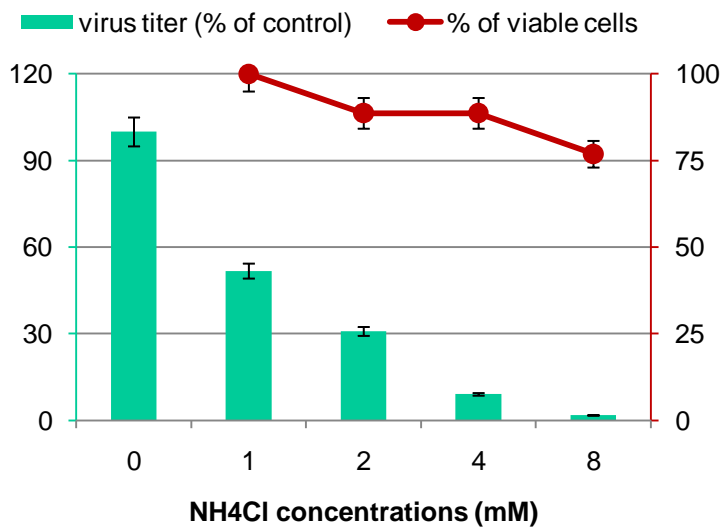
evidence described above that an efficient BPV entry into EBTr cells is mediated by the clathrin-mediated pathway of endocytosis. The inhibitory effect of the drugs was dose-dependent. Working concentrations not toxic to EBTr cells were used to assess an efficient BPV infection reliance on process blocked by the acidification inhibitors.

Confirmatory experiments were conducted by isolating viral RNAs from infected control cells and infected cells in the presence of drugs. cDNA was constructed, then DNA was amplified to determine whether the inhibitors of CME interfere with virus infection in EBTr cells. qPCR results indicated that NH_4Cl (Fig. 7A) and CHPZ treatments (Fig. 7B) inhibited virus replication in the host cell. Taken together, these the the hypothesis data strengthened that the clathrin-mediated, pH-dependent pathway is required for BPV uptake into EBTr cells, and it is essential for the downstream virus replication events to occur.

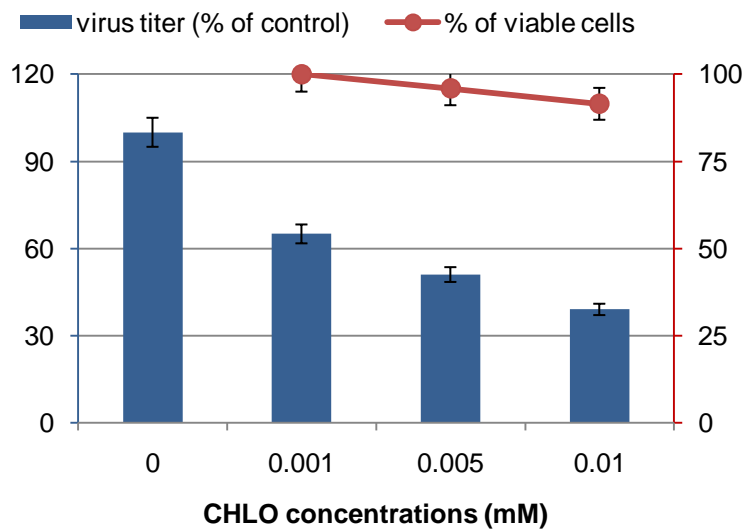
FIG. 6.



B.



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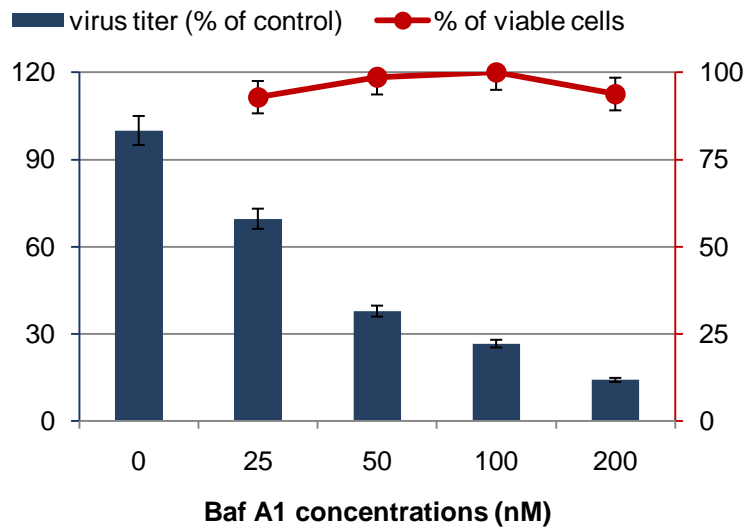


FIG. 6. Production of progeny virus in the presence of inhibitors of CME. Cells were treated with CHPZ (A), NH_4Cl (B), CHLO (C), or Baf A1 (D). The ratio of virus titers in yields is plotted as percent of control (bars). The percent of viable cells was plotted against at working concentrations of drugs (dot plots). Error bars represent standard errors of the means.

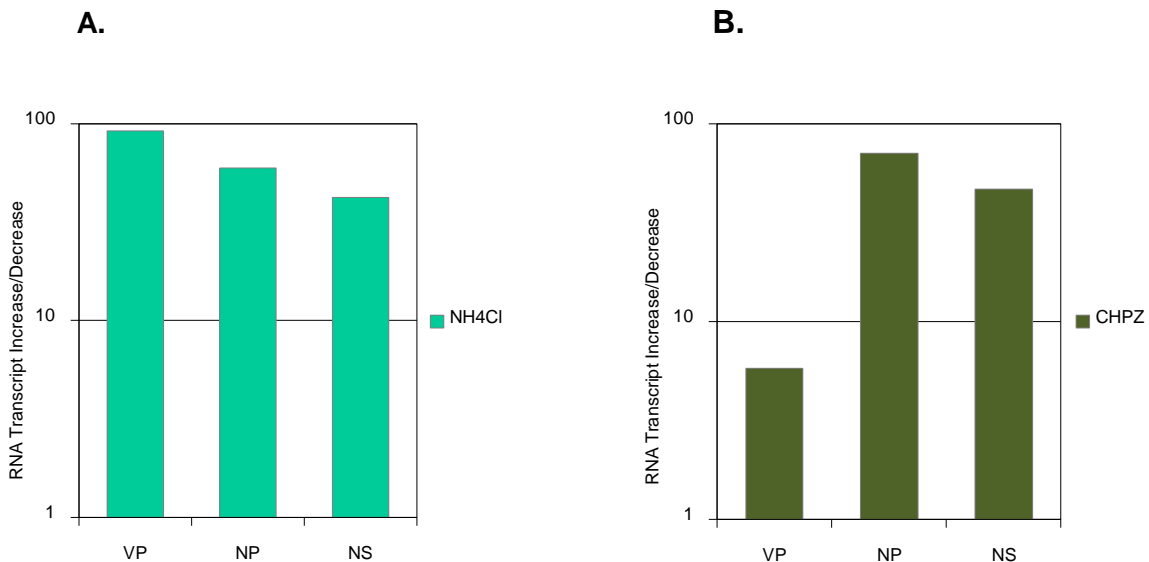


FIG. 7. Viral RNA synthesis quantification in cells treated with CME inhibitors. NH_4Cl (2 mM) or CHPZ (5 μM) pretreated cells infected with virus for 24 h, and RNA was extracted. Viral VP, NP, and NS genes' expressions were measured by qPCR and as described in Materials and Methods. Numbers >1 indicate gene expression is less than

control cultures or cultures infected with virus in the absence of drugs. (A) NH₄Cl or (B) CHPZ compared to untreated infected cultures.

Cell viability assay. It was important to clarify whether the inhibitions seen in the previous experiments were due to inhibition of the intracellular virus-associated machinery or if it was due to death of the cells caused by drug cytotoxicity. Therefore, the effects of the drugs at working concentrations on cell viability were tested by using the MTT test for cytotoxicity. EBTr cells, were grown to confluency in 96-well plates, treated with drugs and were incubated under the identical conditions at which the drugs were used to analyze BPV entry and trafficking. The drug concentrations used in all experimental cultures in this study did not significantly affect EBTr cell viability. This implies that the inhibitory effect of drugs on virus infectivity, production, and transcription was not due to drug toxicity. These results affirmed the selective inhibitory effects of the drugs on virus replication events. Cell viability is plotted on Figs. 5 and 6 as well as subsequent figures as indicated.

Trafficking of BPV through the early to late endocytic compartments. BPV particles routing through the endosomal compartments was investigated. CCVs that carry cargo or virus/receptor complexes lose the clathrin coat and mature to early endosomes. Viruses of some families can escape into the cytoplasm from the early endosomes or some traffic through late endosomes. Several groups of studying parvoviruses have reported that endosomal acidification is crucial for parvovirus infection, inducing capsid rearrangements followed by release of the virus into the cytoplasm. Thus, we hypothesized that trafficking through endosomal compartments might be essential for BPV entry. To identify BPV transit through endosomal compartments, EBTr cells were treated with BFA, a blocker of endocytic traffic from

early to late compartments. The drug inhibited BPV infectivity in a dose-dependent manner. (Fig. 5E) These results indicated BPV transit through the early and late endosomes, and it also supported results of the experiments run with the inhibitors of endosomal acidification. BPV traffic through endosomal compartments and their acidic environment is required for productive replication of BPV. The inhibitory effects on BPV replication are noteworthy, and the concentrations we used did not have any direct inhibitory effect on BPV particles (see next section) or on EBTr cell growth.

Test for virucidal activity of the drugs used in BPV entry studies. Possible direct virucidal activity of the drugs on the virus strain we used in this study was tested by treating virus stock with the highest concentration of drugs. Virus stock was incubated for 30 min at RT with the highest doses of the drugs, and cell cultures were infected with drug treated virus stock to test surviving virus. Infected monolayer cells were incubated at 37°C and 5% CO₂ for 2 days, and cells then processed for the detection of BPV infected cells. The number of infected cells was scored in comparison to mock-treated virus control cultures. For the most part we did not observe differences between the control cultures and the cultures of drug treated virus (Fig. 8), although at these drug concentrations CHLO and CHPZ slightly reduced virus titers. These results confirm that the drugs we used in the study of BPV entry had no or only slight virucidal activities on virus, and the depletion of BPV infectivity and virus production in EBTr cells we noted in this study are not caused by direct inactivation of the BPV strain by the drugs.

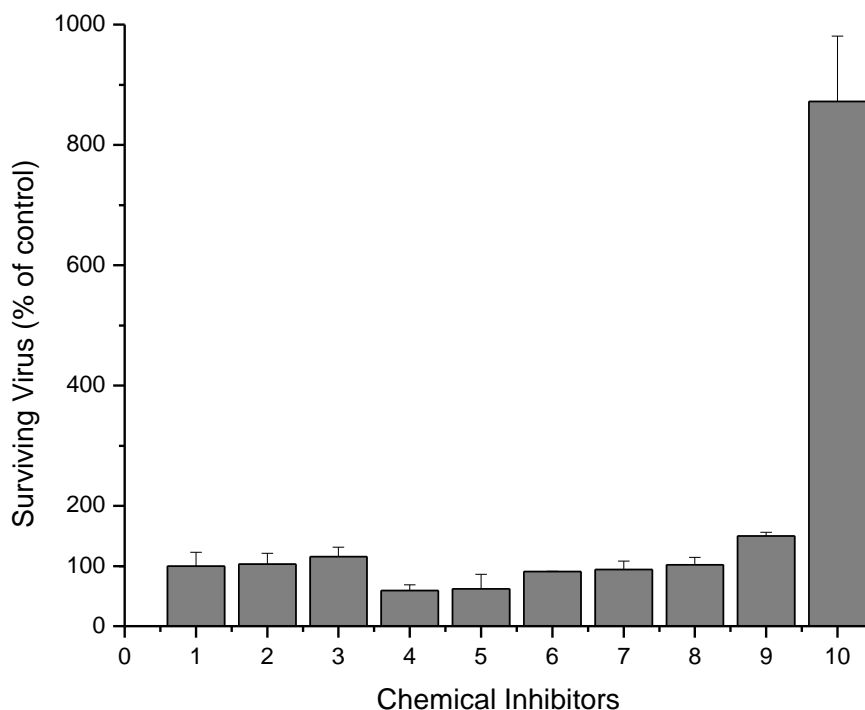


FIG. 8. Assessment of chemical inhibitors for direct virucidal activity. Virus was exposed to the inhibitors at the highest drug concentration used in any of the studies. Exposure was at RT for 30 min, then surviving virus assayed by standard techniques. The control (1), infected with virus stock not treated with drugs, was normalized to 100% survival. The survival rates of the other tests were compared to the control. The inhibitors and concentrations were (2) NH_4Cl , 4mM; (3) Baf A1, 200 nM; (4) CHLO, 0.01 mM; (5) CHPZ, 5 μM ; (6) BFA, 0.05 $\mu\text{g/ml}$; (7) Nys, 1 $\mu\text{g/ml}$; (8) MBCD, 2.5 mM; (9) Gen, 200 μM ; and (10) PMA, 1000 nM.

Assessment of our techniques on a nonparvovirus control virus. To determine if the techniques we used in this study could replicate results published for another virus, we used an adenovirus model. Ad virus is a nonenveloped, DNA virus, which exploits the clathrin-mediated pathway for cell penetration. Based on reports in the science literature, we performed Ad virus infectivity assays in HeLa cells with inhibitors of clathrin- (NH_4Cl and CHPZ) and caveolae-mediated (Nys and PMA) pathways. The

effect of these drugs on BPV replication was analyzed by infecting cells in the presence of the drugs. HeLa cells growing in 24-well plates were treated with the drugs for 30 min followed by Ad virus infection at 37°C and 5% CO₂ in the presence of the drugs for 2 days. Infected cells were fixed with the FAA fixative and processed for the detection of positive cells in relation to the number of virus antigen-positive cells in mock-treated cells, and it is expressed as the percentage virus antigen-positive cells. Clathrin-dependent pathway inhibitors depleted Ad virus internalization 55% and 38% (NH₄Cl and CHPZ, respectively) (Fig. 9). Neither Nys nor PMA suppressed Ad virus entry into HeLa cells. Ad virus infectivity was increased from 134% to 250% in cells treated with Nys and PMA, an enhancement effect consistent with our observations on BPV replication in Nys and PMA treated-EBTr cells.

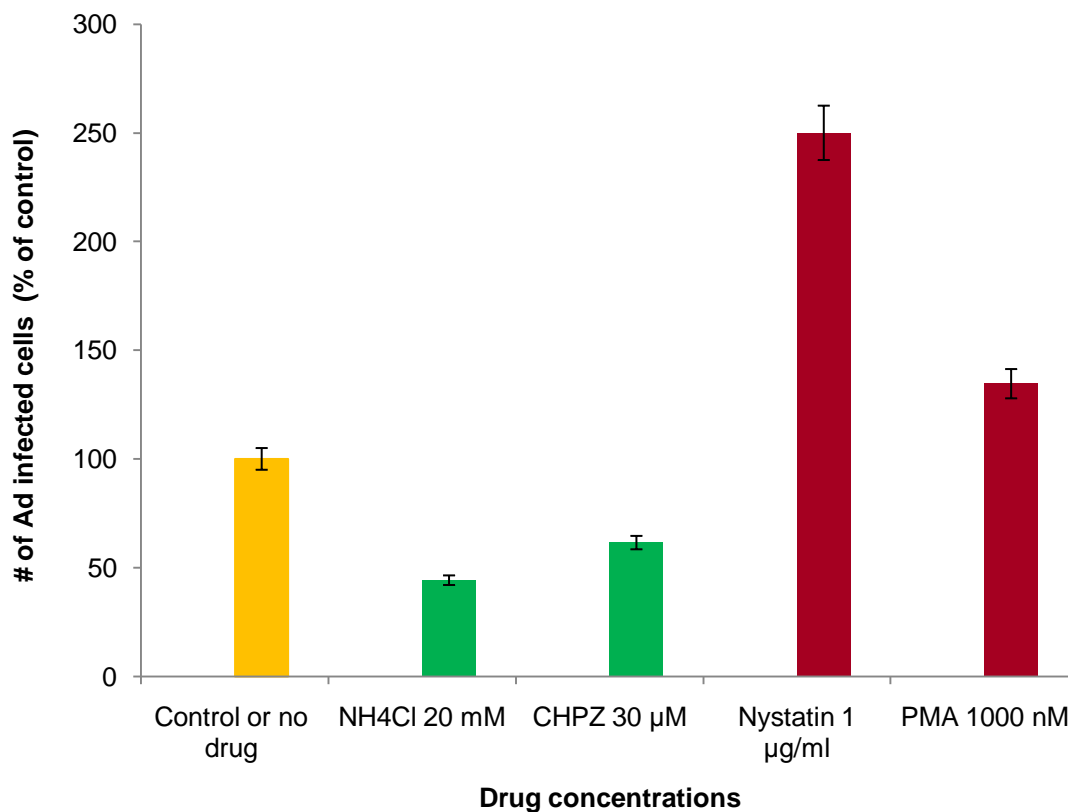


FIG. 9. Ad virus infectivity in HeLa cells in the presence of drugs. Ad virus is internalized by CME. The effect of inhibitors of clathrin- (NH_4Cl and CHPZ) and caveolin- associated (Nys and PMA) pathways on Ad virus infectivity in HeLa cells was examined. HeLa cells were pretreated with drugs and then infected with Ad virus. After 48 h infection, Ad virus positive cells were scored in cultures treated with drugs and compared to Ad virus positive cells in control cultures or untreated cultures.

Tracking virus entry using fluoresceinated virus. EBTr cells sheeted on 12mm round coverslips in shell vials were infected with 10 μl of fluoresceinated virus per vial. Immediately prior to infection, the virus was clarified by centrifugation for 1 min in a microfuge to remove possible virus aggregates. At the end of the appropriate incubation period, the cultures were washed in PBS, fixed in cold acetone (-80°C), air dried, washed in a stream of PBS, and then distilled water. After co-staining for clathrin the cultures were observed for fluorescence. Cultures fixed at 5 min post-infection, and 60 min and 90 min were observed. The virus (fluorescein label) could be distinguished from clathrin (rhodamine label) (Fig. 10). At 5 min post-infection (A) the virus appeared to be evenly distributed on the surface of the cells without aggregation and appeared to be attached to viral receptors because it did not wash away during processing. The clathrin, at this magnification, also appeared distributed about the cell and not collected into endosomes. In contrast, by 60 min post-infection, the virus was clearly aggregated (C) as was the clathrin (D). These co-localized structures appeared to result from endosomal formation and contained large numbers of virus particles.

At 90 min post-infection (Fig. 11) cells observed at a higher magnification clearly show large aggregates of virus associated with clathrin and appeared to result from endosomal formation. The structures were commonly distributed throughout the cytoplasm, even in long cytoplasmic processes. In some cells virus was observed to be

somewhat concentrated in the perinuclear area of the cell. In merged photomicrographs (not shown) the clathrin- and virus-associated structures exhibited co-localization.

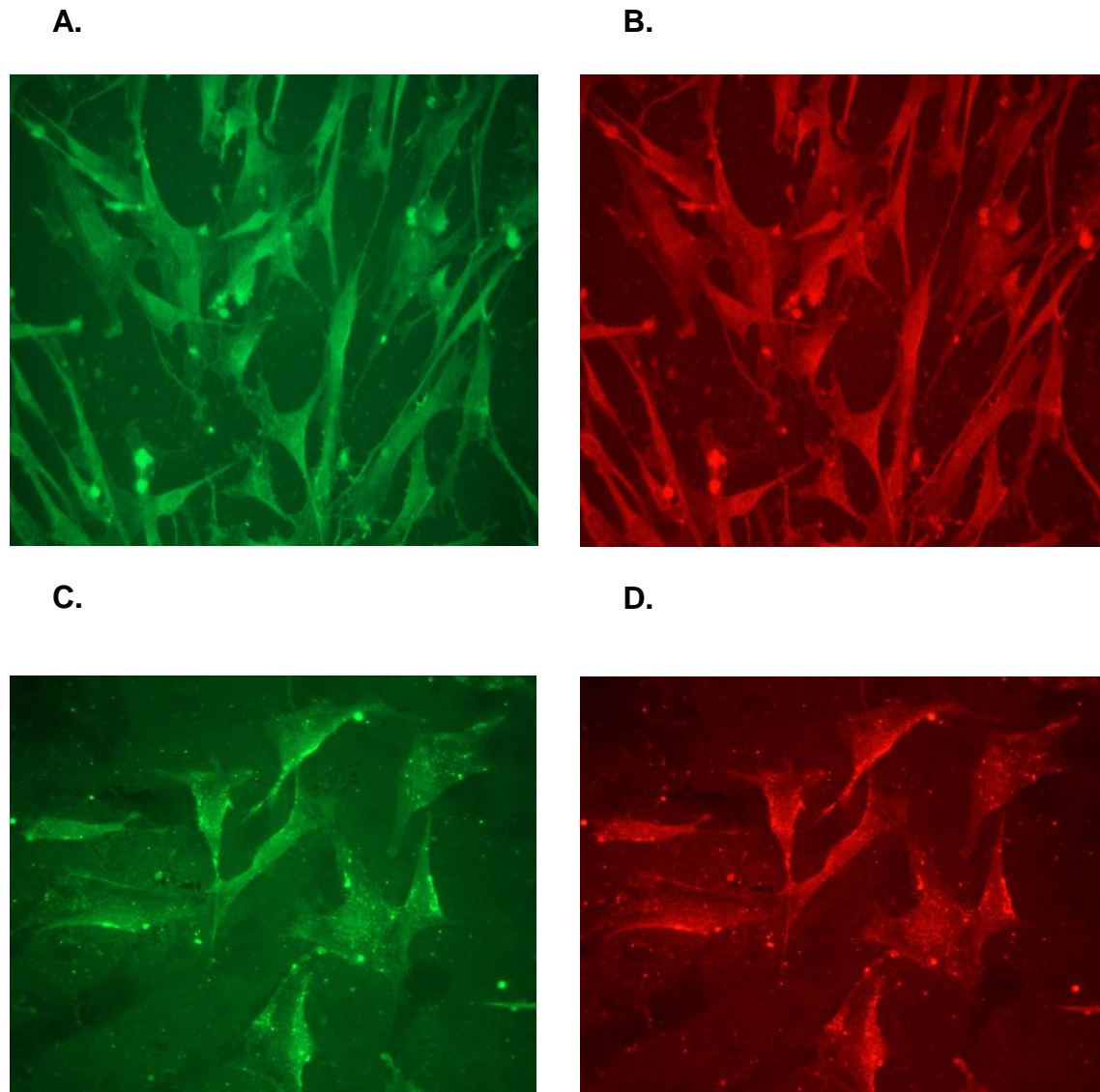


FIG.10. Interaction of fluoresceinated BPV particles with EBTr cells. The green images show the virus particles and the red images show rhodamine-stained clathrin. At 5 min post-infection (panels A and B) the virus particles were associated with the cytoplasmic membrane primarily in a smooth, even pattern (A) showing the viral material started out as non-aggregated virus particles. The clathrin was found distributed comparatively evenly over the cells (B). By 60 min post-infection (panels C and D), the virus particles were markedly aggregated (C) as shown by the granular distribution of virus. The

cellular clathrin distribution (D) corresponded in a granular pattern with the virus aggregates with co-localization of virus and clathrin. Magnification, 20X.

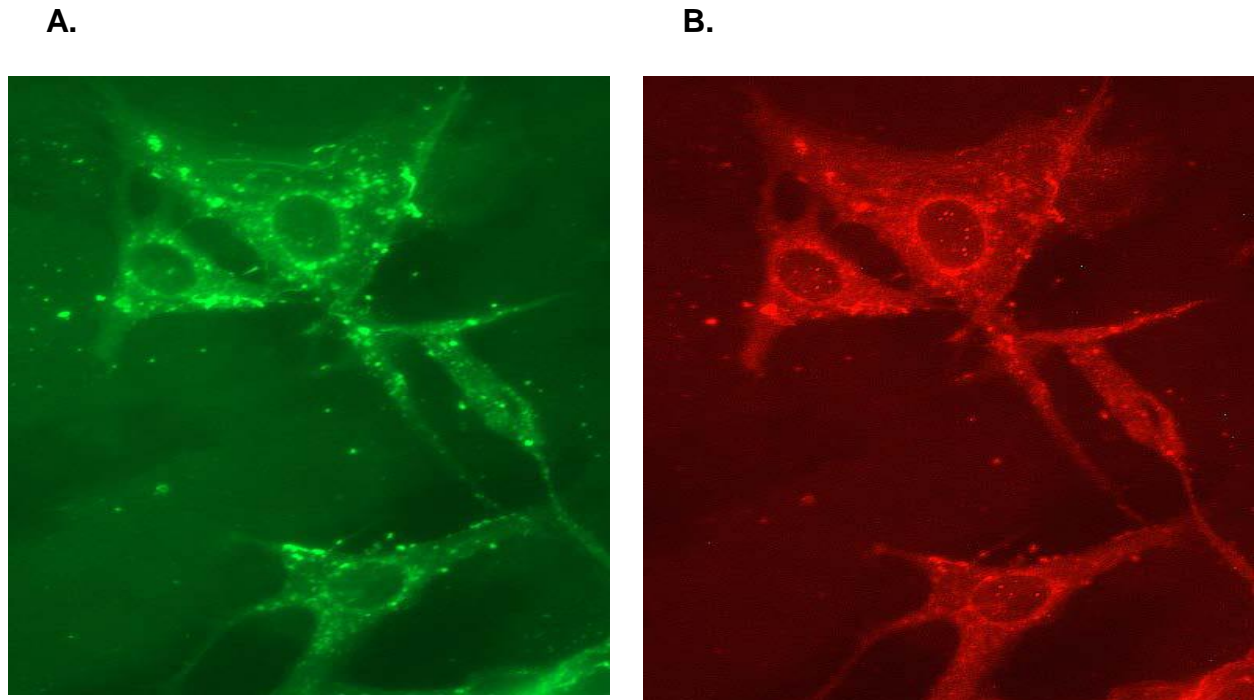
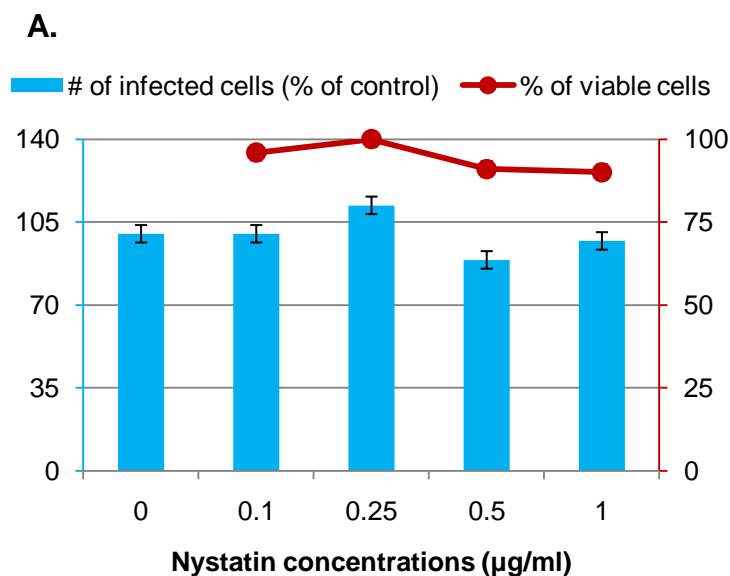


FIG. 11. Uptake of fluoresceinated BPV particles in EBTr cells at 90 min post-infection. At this time point, the virus (panel A, green) appeared as large aggregates distributed throughout the cytoplasm in association with endosomal clathrin (panel B). In some cells, viral material collected around the perinuclear area. In merged images (not shown), endosomal clathrin and viral protein aggregates co-localized in apparent endosomes. Magnification, 40X.

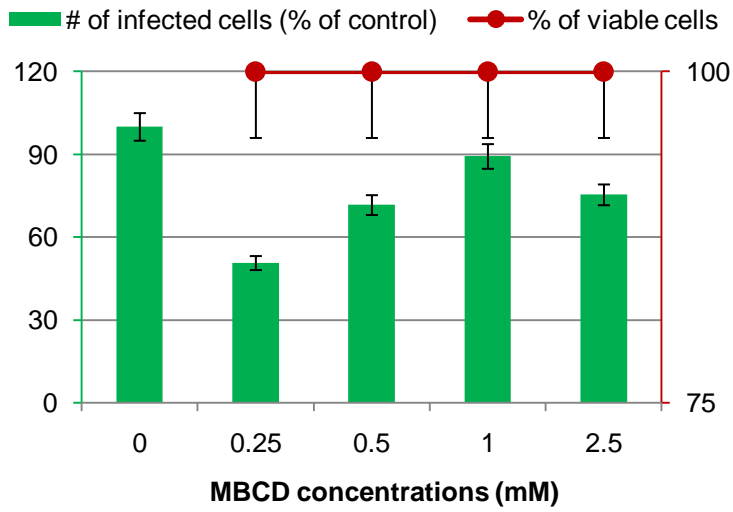
BPV uptake by EBTr cells is independent of the caveolin pathway. Inhibitors of the caveolin pathway also were used to test a role of caveolae-associated endocytosis for BPV entry. To determine a role of caveolae pathway for BPV internalization into EBTr cells, we analyzed the effects of a caveolin depletion agent (PMA), lipid raft blockers (Nys and MBCD), and a tyrosine kinase blocker (Gen) with virus infectivity (Fig. 12) and production assays (Fig. 13).

The selective drugs allowed us to target interconnected organelles of caveolae entry. None of these drugs affected BPV infectivity or production in EBTr cells (Fig. 12 and 13). We observed increases of BPV infectivity and virus production relative to control with some drugs. Further investigations will be required to determine the precise mechanisms of how some of these selective inhibitors enhance BPV replication. However, enhancement is not unusual as seen in the adenovirus control experiments (Fig. 9). All together, these results strongly indicated that BPV uptake by EBTr cells is not by caveolin-associated, pH-independent pathway of endocytosis. Working concentrations of the drugs used in the caveolae experiments were tested for toxicity by the MTT test and for virucidal action. Viral RNA in Nys and PMA treated-EBTr cells was measured by qPCR (Fig. 14). As expected, Nys did not inhibit virus transcription, but interestingly, PMA decreased transcription of all three markers (NS, NP, and VP). What role PMA plays in this blockage, whether relating to caveolin or another sensitive point will require further analysis.

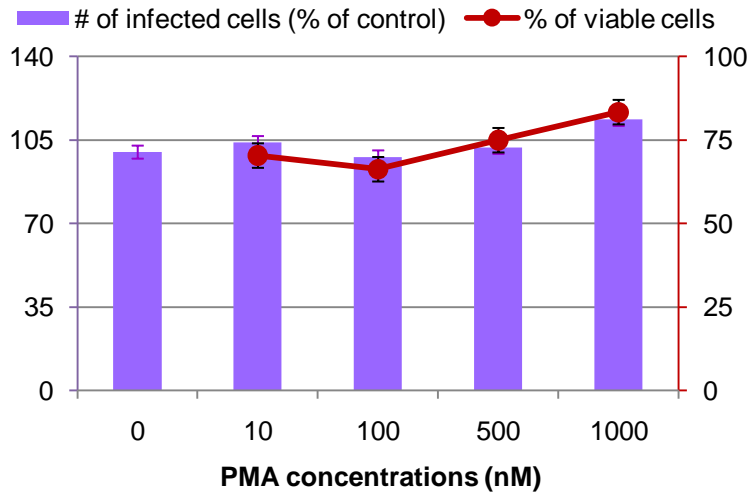
FIG. 12.



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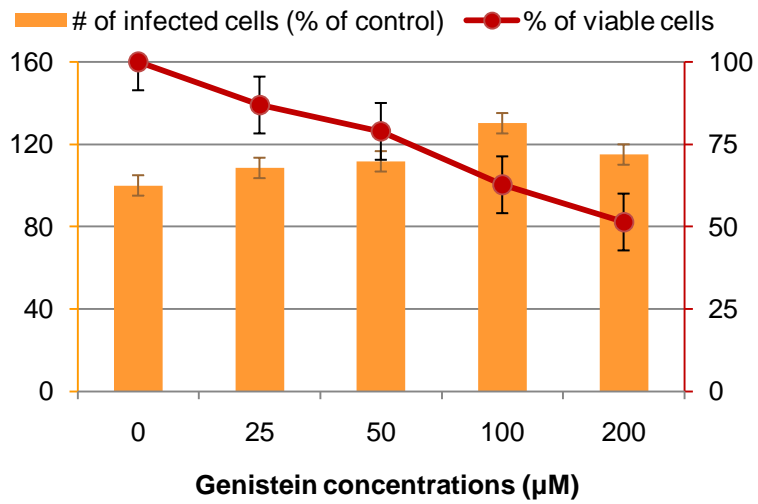
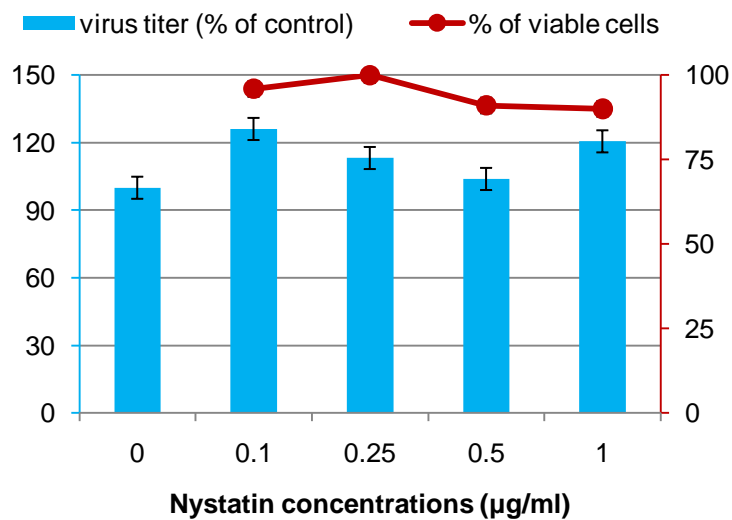


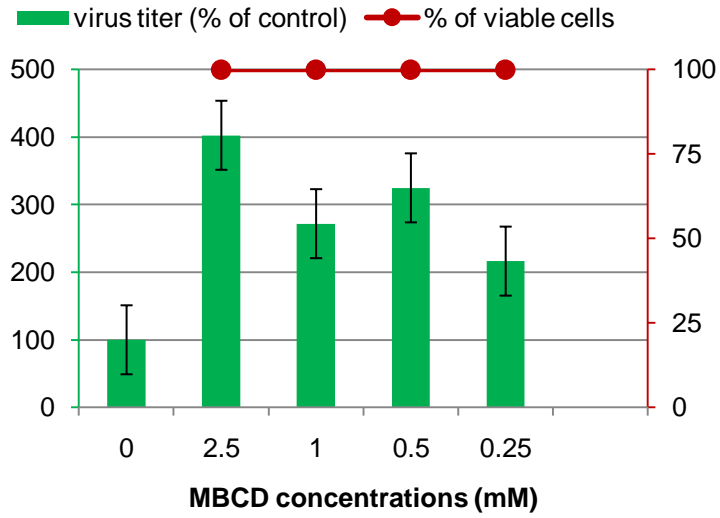
FIG.12. BPV infectivity in EBTr cells treated with the inhibitors of caveolae-mediated endocytosis. For details please refer to FIG. 5 legend. Cells were treated with Nys (A), MBCD (B), PMA (C), or Gen (D). Error bars represent standard errors of the means.

FIG. 13.

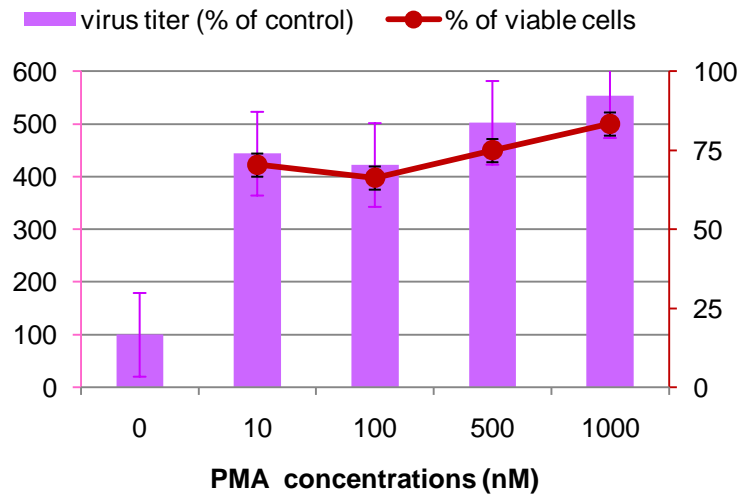
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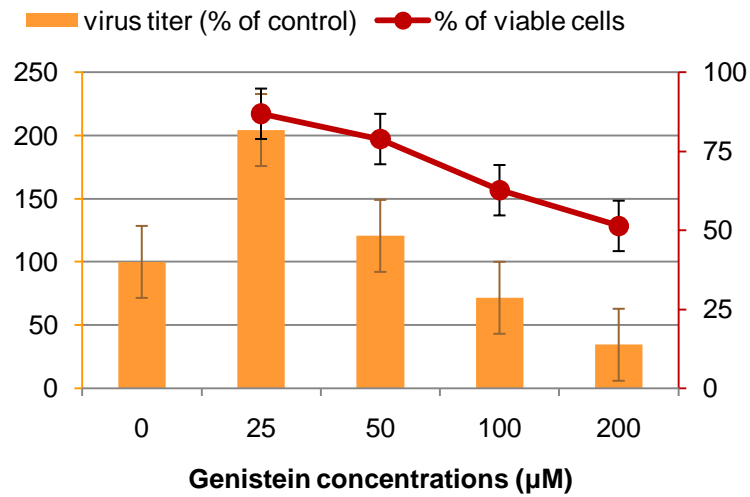


FIG.13. Production of progeny virus in the presence of inhibitors of caveolae-mediated endocytosis. For details please refer to FIG. 6 legend. Cells were treated with Nys (A), MBCD (B), PMA (C), or Gen (D). Error bars represent standard errors of the means.

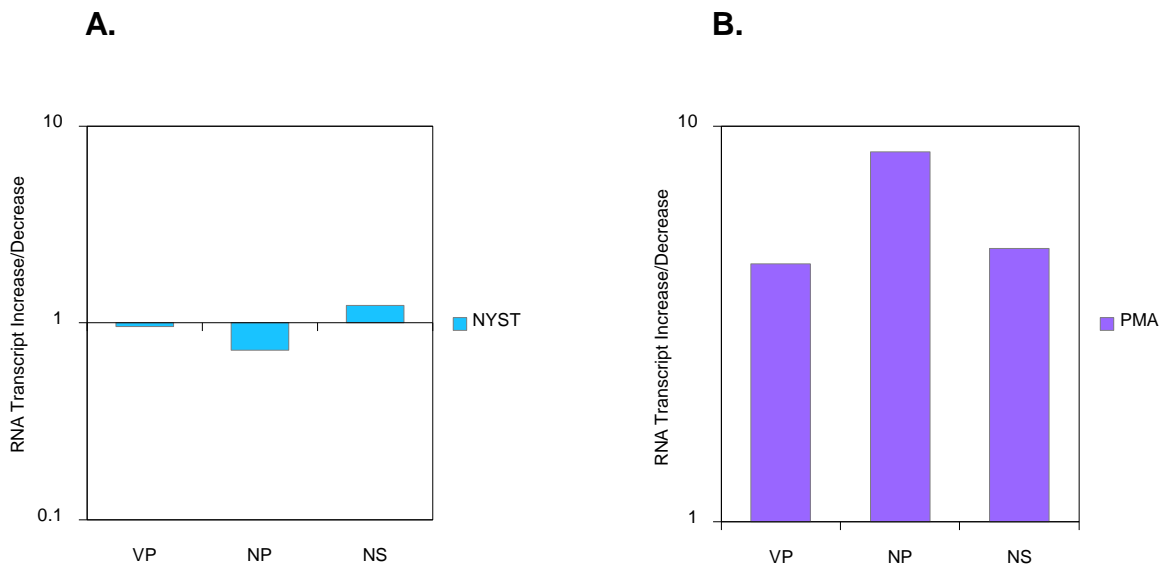
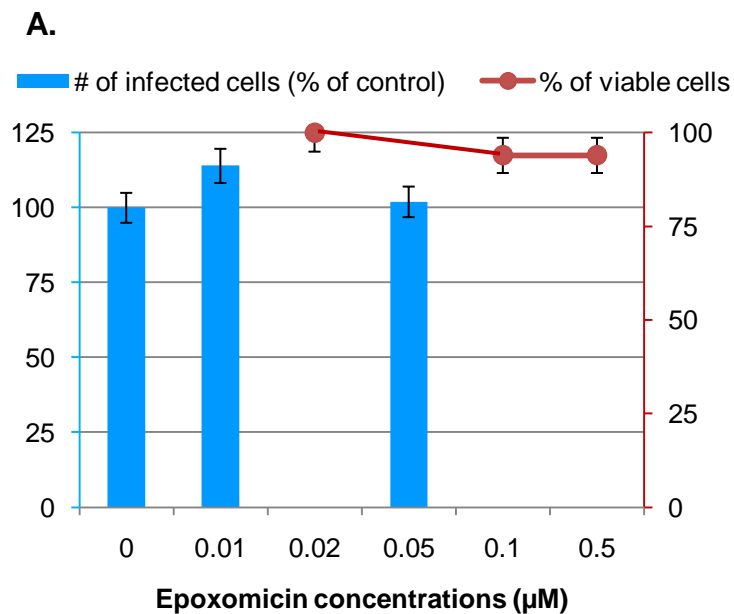


FIG.14. Quantification of viral RNA in cells treated with inhibitors of the caveolae pathway. For details please refer to FIG. 7 legend. (A) Nys (0.25 $\mu\text{g/ml}$) and (B) PMA (1000 nM).

The ubiquitin-proteasome system and BPV replication. It has been reported that a variety of virus species is dramatically dependent on the ubiquitin-proteasome system to establish productive infection through a diversity of distinct mechanisms. To determine whether a relationship exists between BPV and the ubiquitin-proteasome system, EBTr cells were treated with the antiproteasome drugs Lac and Epox prior to BPV infection. The drugs were maintained in the cultures for the entire time of infection. Lac suppresses the 26S proteasome related chymotrypsin- and trypsin-like activities. Epox inactivates the chymotrypsin-like activity of the proteasome. The results demonstrated that suppression of proteasome activity by selective inhibitors did not affect BPV infectivity in the host cell (Fig. 15). Quantitative PCR results did show a significant difference of viral transcripts in untreated and drug treated-EBTr cells (Fig. 16). Taken together, these results suggest that BPV processing following clathrin-mediated pH-dependent endocytosis does not require the proteasome-ubiquitin system. The drug doses used in infectivity assays were not toxic to EBTr cells.



B.

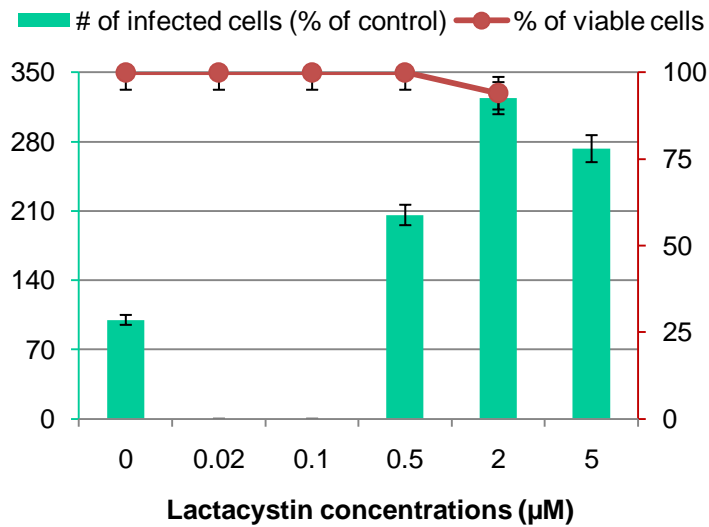


FIG.15. BPV infectivity in proteasome enzyme inhibitors treated-EBTr cells. For details refer to FIG. 5 legend. Cells were pretreated with Epox (A) and Lac (B), and the drugs were maintained during the virus infection. Error bars represent standard errors of the means.

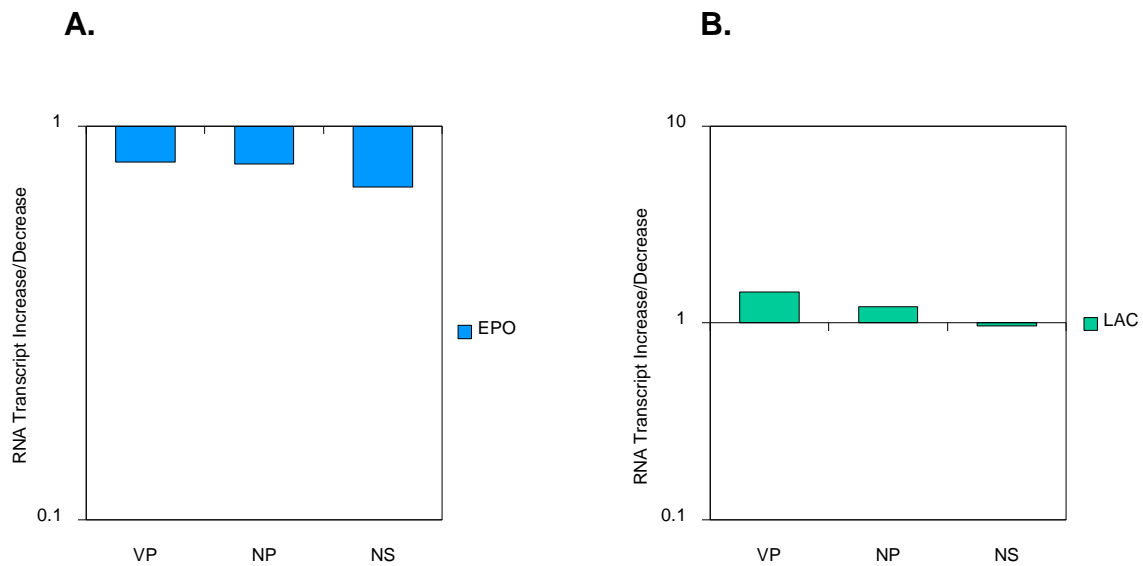
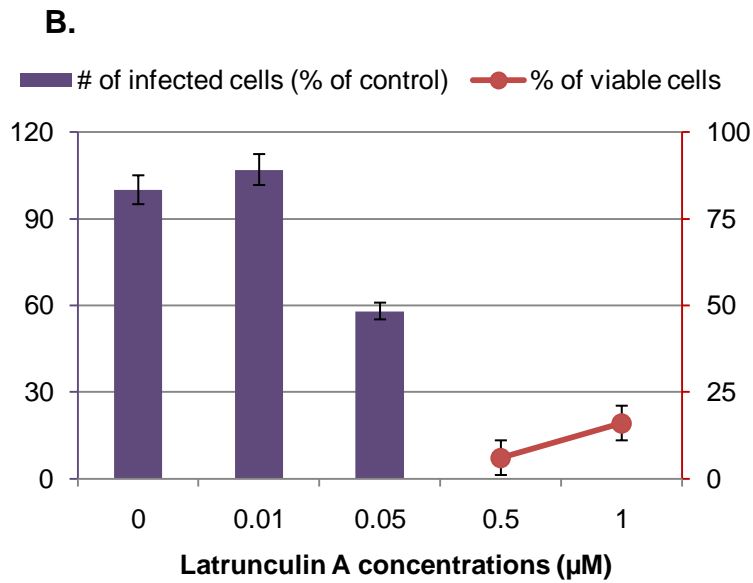
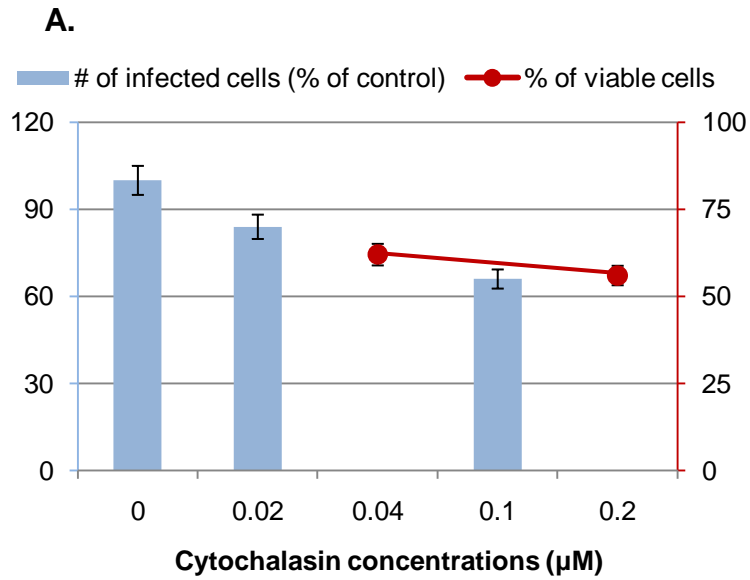


FIG.16. Quantification of viral RNA synthesis in the presence of proteasome activity blockers. For details refer to FIG. 7 legend. Viral VP, NP and NS transcripts were measured by qPCR in (A) Epox (0.05 μM) and (B) Lac (2 μM) treated-EBTr cells.

The role of actin in intracellular trafficking of BPV. Virus attachment to its receptor molecule induces cell signaling on behalf of virus replication. Virus uptaken by the host cell interacts with cell cytoskeletal structures and regulate them to reach to appropriate sites of replication. Virus associated trafficking routes may involve microtubules, dynein and kinesin motors, or actin filaments, and myosin motors. To characterize intracellular trafficking of BPV, cells were treated with the specific blockers of cell cytoskeleton synthesis or function and infected with virus. Viral protein expression and viral RNA synthesis were detected in the drug treated and mock treated cells to identify which component of the cell cytoskeleton may contribute to intracellular trafficking of BPV particles. Growing evidence has suggested a tight interaction between the actin network and CME. To analyze the role of actin filaments for BPV trafficking in the cytosol, EBTr cells were treated with increasing noncytotoxic concentrations of actin inhibitors, Cyt D, which inhibits actin polymerization, and Lat A, an inhibitor of actin function. Following drug treatment cell cultures were infected with virus stock. An efficient entry was scored as viral protein-expressing cells. Cyt D treatment resulted in a reduction of BPV infectivity at 48 h post-infection in a dose-dependent manner (Fig. 17A). Lat A similarly depleted virus infectivity (Fig. 17B). The actin-myosin inhibitor, ML-7, also inhibited infectivity (Fig. 17C).

The inhibitory effects of CME blockers strongly affirmed that CME is a functional route of BPV entry into the host cell. Results shown in Fig. 17 indicate that actin filaments participation for BPV trafficking and additionally confirm that CME is a functional route of BPV uptake by EBTr cells. Further evidence was obtained by testing all three of these inhibitors for inhibition of transcription. Viral RNA isolated from untreated and drug treated cultures was detected by qPCR. Drugs active against actin

markedly affected viral gene transcription (Fig.18). Overall, results suggest actin transport is essential for BPV infectious cycle.



C.

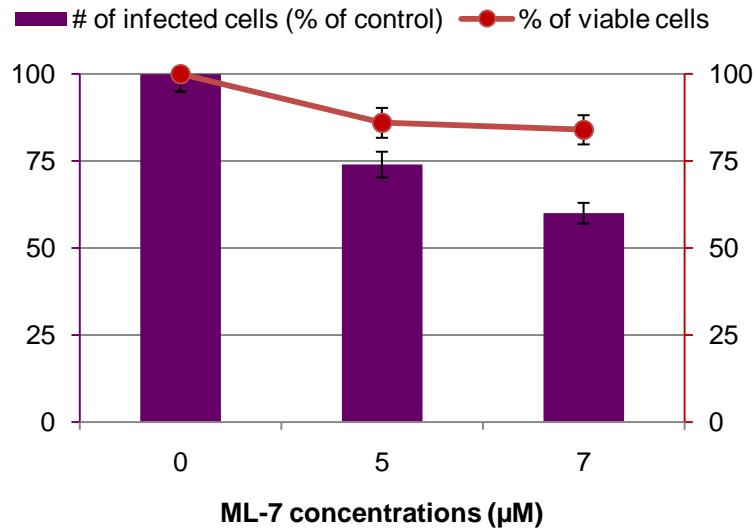


FIG. 17. BPV infectivity in EBT cells treated with actin blocking agents. For details refer to FIG. 5 legend. Cells were treated with Cyt D (A), Lat A (B), and ML-7 (C).

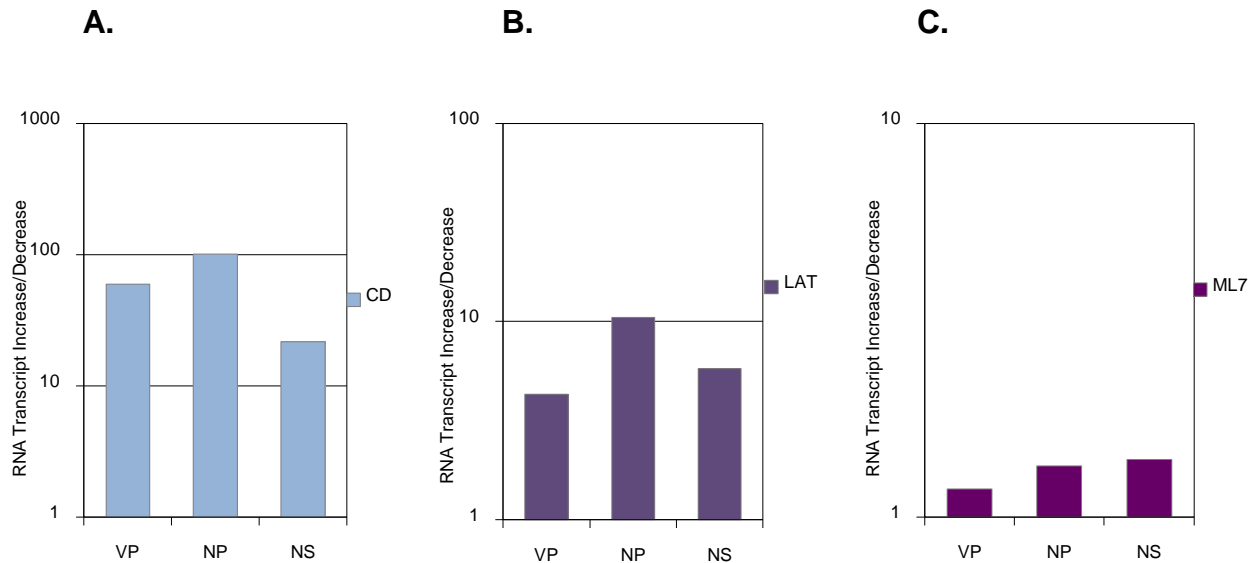


FIG. 18. Quantification of viral RNA synthesis in cells treated with drugs active against actin transport. (A) Cyt D ($0.1 \mu\text{M}$), (B) Lat A ($0.05 \mu\text{M}$), and (C) ML-7 ($5 \mu\text{M}$). Details given in FIG. 7 legend.

We speculated that because actin plays an important role in virus infection, it might be possible to observe morphological changes in cells exposed to actin inhibitors.

Accordingly, EBTr cells together with BEK cells (a cell strain derived from an embryonic bovine kidney primary cell culture established in our laboratory) were exposed to media containing Cyt D or Lat A. Fig. 19 shows the resulting morphological changes induced upon exposure to these two drugs. Actin was stained with monoclonal anti-actin and the fluorophore was Texas Red. The nuclei were stained by DAPI.

The EBTr cell morphology in control medium was typical with long, fibroblastic cells (Fig. 19A). Actin filaments were clear, morphologically distinct, and appeared highly organized. In the cells exposed to Cyt D (Fig. 19B) some actin filaments remained, but most cells showed less clearly polymerized actin arrays and much more amorphous actin. In the cells exposed to Lat A (Fig. 19C), there were very few actin filaments to be seen in any of the cells. Most of the residual actin appeared in amorphous masses. The cells exhibited extensive shrinkage that was consistent with contraction of cell size and flattening due to loss of cytoskeletal structure.

BEK cells are large, cuboidal cells with an extensive actin cytoskeleton. In Fig. 19D BEK control cells can be compared to cells exposed to either Cyt D (Fig. 19E) or Lat A (Fig. 19F). Cells with observably less actin found in filamentous forms and more amorphous actin were seen in the drug treated cultures. Consistent with the Lat A-treated EBTr cells, the BEK cells exhibited cell shrinkage pulling away from each other (Fig. 19F). Interestingly, the cell membranes appeared ruffled along the edges. These results verify the effects of these two drugs on actin and are consistent with their activity as inhibitors of actin polymerization and movement.

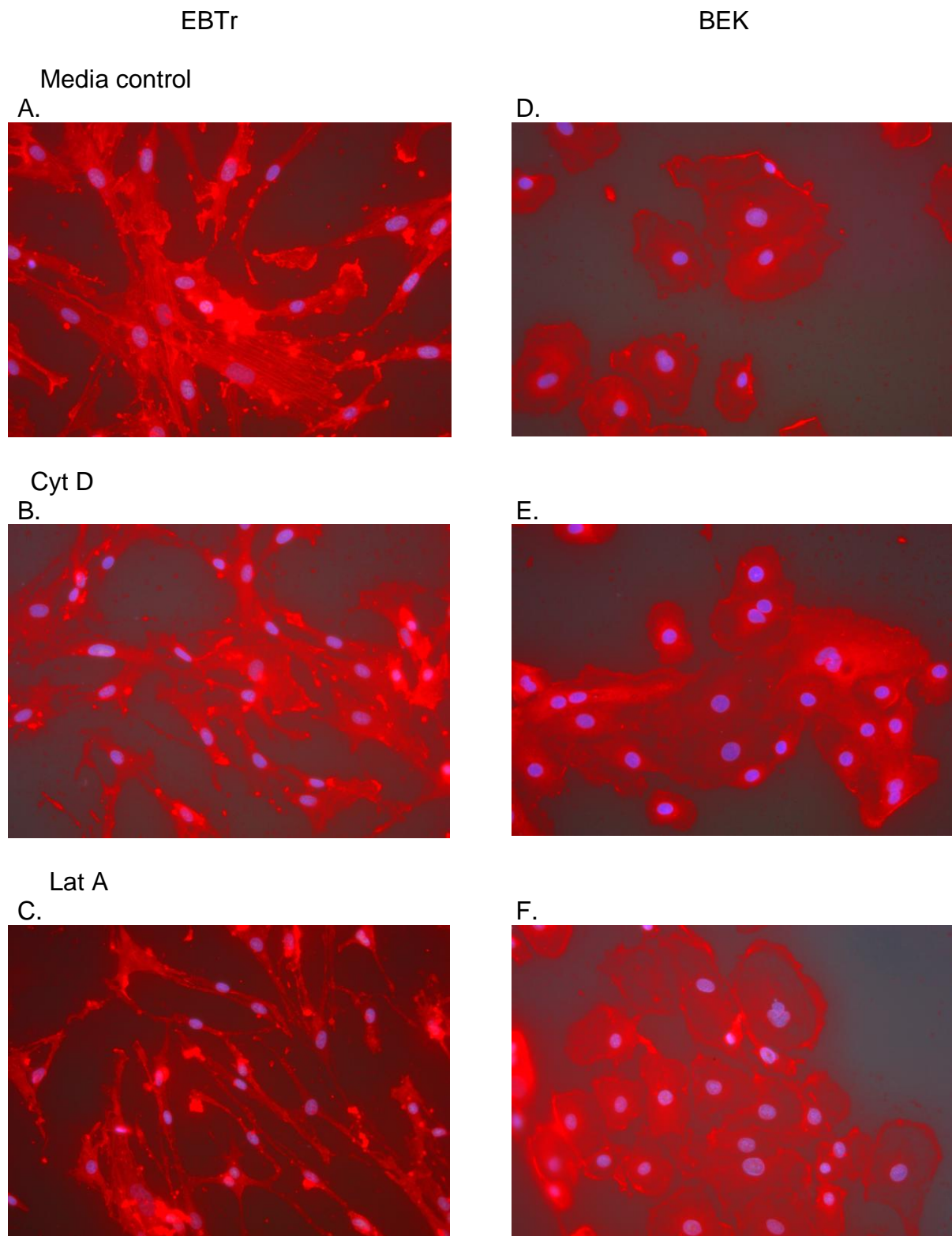


FIG. 19. Immunofluorescence view of cells treated with actin inhibitors. A, B, and C show the results of tests on EBTr cells and the BEK cells are shown in D, E, and F. (A)

and (D) cells in the cell culture medium. (B) and (E) cells treated with Cyt D. (C) and (F) cells treated with Lat A.

Myosin motors move cargo on actin filaments. Myosin function can be blocked by standard drugs. In this study, we employed ML-7, a highly specific inhibitor of MLCK, to examine the involvement of the myosin motor in BPV trafficking. Treatments by ML-7 strongly reduced BPV infectivity in EBTr cells (Fig. 17C). Moreover, the same drug was active in reducing viral transcription (Fig. 18C). These results suggested the myosin motor plays a functional role in BPV transport in the cytosol and affirmed results that demonstrated the actin network interaction with intracellular BPV.

The microtubule network may be required for BPV infection. We also examined a potential role of the microtubule network in the intracellular trafficking of BPV. Trafficking through microtubules can be inhibited by specific drugs such as Noc. EBTr cells were pretreated with Noc at increasing doses and infected with BPV for 48 h. Similarly EHNA and Van were also tested. Noc did not exert any blocking effect on BPV infectivity when the number of the drug treated viral protein expressing cells were compared to the number of mock treated viral protein expressing cells (Fig. 20A). These results suggested that the microtubule network may not be associated with the BPV entry route into EBTr cells.

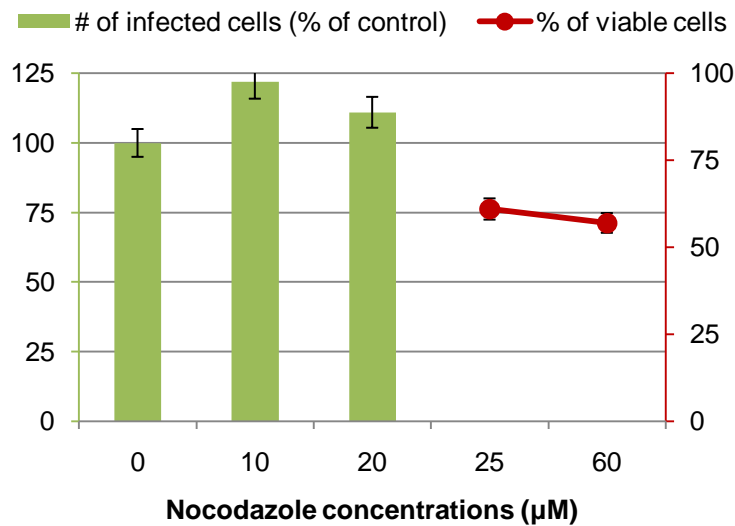
The microtubule-based dynein motor transports cargo, including virus or virus containing vesicles, along microtubules towards the nucleus. A functional role of the dynein motor in BPV infection was tested by using the inhibitors Van (a non-specific dynein inhibitor) and EHNA (a dynein inhibitor). These drugs interfere with different aspects of dynein motor function. BPV infectivity was not affected by treatments with Van or EHNA as both drugs did not show any inhibitory effect on BPV protein synthesis.

These results seemed to confirm that the microtubule network is not involved in BPV trafficking.

Conflicting evidence was obtained when inhibition by these three drugs was determined by blocking transcription as tested by qPCR. Noc, an inhibitor of microtubule formation, showed strong interference with viral RNA synthesis (Fig. 21A). Van did not block RNA synthesis (Fig. 21B), and EHNA produced mixed results with blockage of NS and NP transcripts, but not VP (Fig. 21C). These discrepant results need further study, but in the meantime our hypothesis is that microtubules and the dynein motor traffick this virus to the nuclear membrane.

FIG. 20.

A.



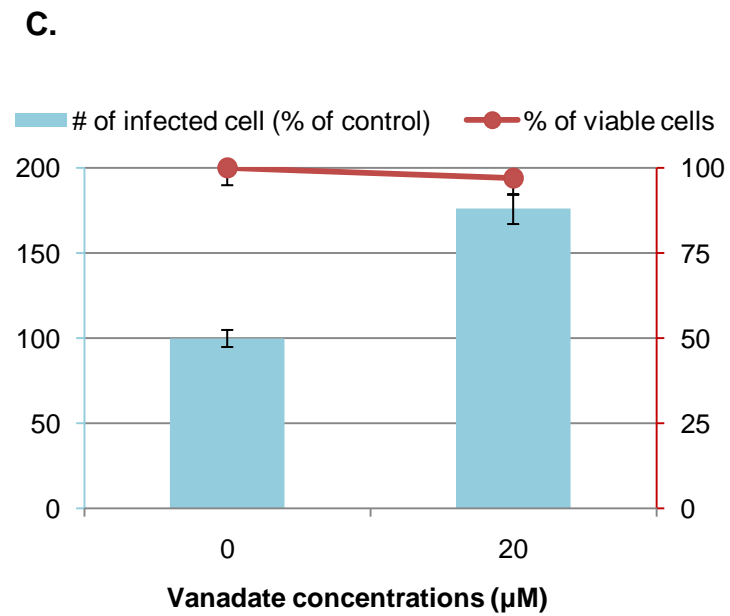
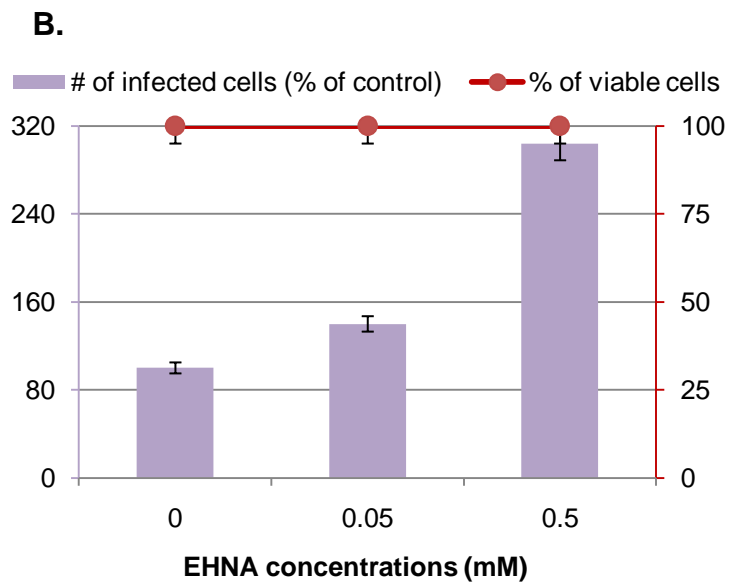


FIG. 20. BPV infectivity in EBTr treated agents active against microtubules. Refer to the FIG. 5 legend for details. Cells were treated with Noc (A), EHNA (B), and Van (C).

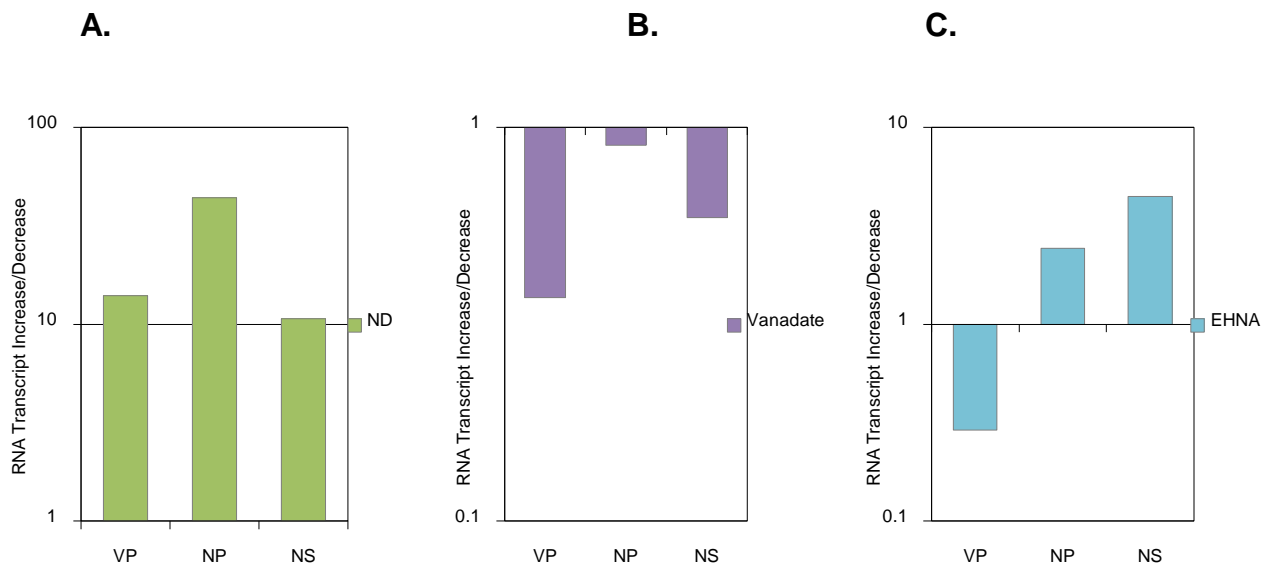


FIG. 21. Quantification of viral RNA synthesis in cells treated with microtubule inhibitors. (A) Noc (20µM), Van (20 µM) and (C) EHNA (0.5 µM) effects were tested against VP, NP, and NS expressions. For details refer to FIG. 7 legend.

DISCUSSION

BPV is a common causative agent of cattle gastroenteritis. Serological surveys indicate a high rate of infection in both dairy cattle and range cattle. Virus infection usually involves multiple organ systems including the respiratory tract and internal organs. It is presumed to transmit through the placental barrier infecting developing fetuses. As with many other autonomous parvoviruses, BPV targets rapidly dividing cells. Many studies on the replication cycles of these viruses have focused mainly on the middle events of the replication cycle including genome replication and transcription. Fewer studies have appeared on the early events of the replication cycle. Therefore, understanding the early events of virus infection, i.e., exploring host factors involved in entry and trafficking processes, will create a clearer global understanding of parvovirus biology. Renewed attention is being given to the Bocaviruses since the identification of the HBoV and BPV is the primary model for the study of Bocavirus infection. The aim of

this work was to investigate the early cellular events that lead to successful BPV infection of the host cell.

We first assessed cellular and viral aspects involved in the most common entry route of viruses, clathrin-dependent endocytosis. Our observations of an abundant clathrin distribution on EBTr cells by immunofluorescence showed strong molecular homology with human clathrin, and that EBTr cells would be a good model to examine CME. Interestingly, we showed that the virus would bind to clathrin in a dot blot format, suggesting a possible transition of bound virus from sialic acid receptors to clathrin (or co-binding) in the transition from surface binding to coated pit, or from the coated pit to the early endosome. However, if direct attachment of virus to clathrin has any role at all in the replication cycle remains to be seen. Electron microscopy images of virus infected cells were consistent with virus internalization mediated by a clathrin-associated pathway.

To confirm these observations we designed assays to block essential cellular functions with specific pharmacological agents and analyzed the resulting effect on virus replication. For a summary of the results, see TABLE 4. In this portion of the study, we applied a variety of drugs to interrupt viral entry and trafficking at appropriate replication compartments and at different intracellular steps. Moreover, blocking the proteasome activity or various cytoskeletal structures and functions revealed previously unknown information about mechanisms of intracellular transport to move the virus to the nucleus for further processing.

Many studies have demonstrated that the clathrin-dependent endocytic pathway is mainly required for uptake of members of the Parvoviridae family into cells (106, 128). To verify BPV entry dependence on clathrin, we used CHPZ, a cationic amphiphilic drug

that inhibits the formation of clathrin-coated pits at the plasma membrane. We found that CHPZ strongly suppressed BPV infection in EBTr cells. Adenoviruses are reported to enter cells also through the clathrin pathway (70). If, in our hands, CHPZ would block Ad entry, such results would lend credence to the methods used in analyzing BPV entry. Ad infection in HeLa cells was also depleted by CHPZ treatment supporting the activity of CHPZ interference with BPV entry. The inhibition effect of CHPZ on Ad is consistent with previous studies using CHPZ for investigation of baculovirus, FMDV, HTV, HCV, MHV-2, PV, RV, SARS-CoV, and WNV entry via CME (13, 14, 21, 57, 59, 64, 74, 80, 90, 102). Since BPV entry into cells was strongly dependent on clathrin assembly at the plasma membrane; we then definitively sought to determine the requirement of endosomal acidification for BPV internalization.

Acidification of endosomes is known to be essential for viruses, which internalize within CCVs. Studies of parvovirus entry reported that MVM, AAV, and CPV internalization require endosomal acidification, and the endosomal acidic environment may induce capsid conformational changes vital for viral release from endosomes to the cytoplasm (4, 34, 76, 106). To define the role of pH in the BPV infection process, the effects of NH_4Cl , CHLO, and Baf A1, selective inhibitors of endosomal acidification, were analyzed by functional assays. The weak bases, NH_4Cl and CHLO, diffuse across the endosomal membrane and become protonated, causing the endosomes to become less acidic. BPV infection was strongly sensitive to these weak bases in a dose-dependent manner verifying acid pH dependency. A selective inhibitor of vacuolar H^+ -ATPase, Baf A1 neutralizes the pH of intracellular vesicles via a different mechanism. The results obtained with Baf A1 were similar to that obtained with weak bases. Thus, altering the endosomal microenvironmental pH toward alkalinity demonstrated that a

low-pH environment is necessary for BPV infection to progress to the next sequential events in the cytoplasm.

Sensitivity to endosomal acidification raised a question regarding duration of the virus within the endosome. That is, whether the virus particles are directly released from the early endosomal compartment to the cytosol or are routed farther into the late endocytic compartment. In an attempt to examine the virus endosomal traffic, we analyzed the effect of a drug that disrupts the endosomal network, BFA. The results indicated that BFA was active against BPV infection, suggesting that the virus particles are not released from the early endosomes but instead traffic toward the late endosomal compartment. Further studies on the location of capsid conformational changes associated with endosomal acidification will help to clarify escape mechanisms and the uncoating process.

The findings obtained by these virus replication studies were supported by tracking the route of fluorescent-labeled BPV in cells. EBTr cells infected with fluorescent labeled BPV (NHS-fluorescein) and stained with anti-clathrin antibodies allowed visualization of virus at the cell surface and tracked it in the cytosol following penetration of the plasma membrane. Photomicrographs showed that BPV co-localized with clathrin concentrated areas at the cell membrane and endosomes in the cytosol. The fluorescence micrographs by themselves are unconvincing, but taken together with the biology of infection studies and the RNA transcript studies are consistent with the trafficking pattern that has emerged.

One of the well known endocytic pathways is caveolae-dependent endocytosis. The possible role of the caveolae-dependent pathway for BPV uptake was examined by using inhibitor chemicals including Nys, MBCD, PMA, and Gen. These chemicals

interrupt the caveolae-mediated pathway through different mechanisms. Functional assays using these selective inhibitors did not affect BPV infection in EBTr cells: Nys, PMA, MBCD, and the two lowest doses of Gen (25 μ M and 50 μ M) were not active against virus production; Nys, PMA, and Gen did not impair virus infectivity; and MBCD slightly depleted virus infectivity. This observation is in agreement with reports that MBCD partially inhibited CME of transferrin receptor, EGFR, and SARS-CoV (57, 111). The results strongly suggested that the caveolae-dependent endocytosis is not responsible for BPV entry into EBTr cells. We observed much higher progeny virus titers produced in cultures treated with Nys, PMA, MBCD, and the lowest dose of Gen; MBCD gave slight suppression of virus infectivity.

Although there is a rich and extensive literature of reports using these same chemical inhibitors for the same purposes that are reported in the current study. It is essential to recognize the pleiotropic nature of these drugs and continually remain cognizant of possible unexpected secondary effects. Our studies revealed marked enhancement of virus infectivity and production in the presence of PMA. PMA was used as a disrupter of caveolin-1 assembly at the plasma membrane to assess the caveolin pathway as a possible entry route for BPV. PMA is a diester of phorbol and is a strong tumor promoter. It is an activator of the signal transduction enzyme PKC. Thus, it mimics the action of the natural diester diacylglycerol (DAG), which is also an activator of PKC. PKC is actually a family of about 10 isozymes with the catalytic region being highly conserved. PKCs, after activation, are translocated to the plasma membrane and exhibit a multiplicity of functions such as modulating membrane structure events, regulating transcription, and regulating cell growth. To date, we do not know the precise

mechanism of PMA virus enhancement, but it may well link to cell growth regulation stimulated by DAG-like activation of the PKC complex.

Virus escape from the endocytic organelles releases it into the cytosol. Once the virus is released, the virus targets the nucleus by cellular factors that facilitate capsid disassembly, exposure of the genome, and movement to an appropriate replication compartment. To identify cellular factors interacting with virus particles immediately after endosomal release, the cellular cytoskeleton and proteasome activity were investigated. Again, tests detecting viral protein production were employed to determine whether the proteasome or the cell cytoskeletal structures were involved in essential events in movement of the virus to the nucleus. Further, inhibition of transcription was analyzed as a measure of blocking movement of the viral genome to the nucleus in the presence of specific inhibitors. Quantitative real-time PCR detecting NS, NP, and VP transcripts provided valuable information. Our observations did not indicate that BPV interacts with the proteasome. In an attempt to define the role of the multiple proteolytic functions of the proteasome in BPV infection, we infected cells in the absence or presence of either Epox or Lac. Epox, a chemical that selectively inhibits the chymotryptic activity of the proteasome, did not affect virus infectivity except a slight enhancement with the lowest dose of the drug. Moreover, Lac, which blocks chymotrypsin-like and trypsin-like activities of the proteasome increased virus infectivity. These results indicate that the ubiquitin-proteasome system may not provide an essential degradation process for uncoating of this virus. However this point should be investigated further.

Finally, we tested viral intracellular trafficking dependence on the cytoskeleton network. It has been reported that viruses that enter via receptor-mediated endocytosis commonly require the cytoskeletal network to take the virus to a replication site (14, 15,

17, 47, 48, 116, 118). Actin blocking agents, Cyt D, Lat A, and ML-7, reduced virus infectivity. Further, to confirm the activity of these drugs on cell structures we tested the effect of Cyt D and Lat A on EBTr cells by immunofluorescent microscopy. Photomicrograph images showed evident changes in cell morphology, which presumably was caused by actin disruption. The virus was also sensitive to microfilament disrupting agents, Noc, EHNA, and Van as observed by RNA transcript reduction but was not detected by reduction in infectivity. These differences were unresolved but will be the subject of future research. The results of this aspect of our studies suggested that BPV intracellular trafficking is dependent on actin and microtubules.

In summary, our study defined early BPV entry events and its intracellular trafficking. The results we obtained allowed us to map the main entry route of BPV and trafficking in the cytosol. BPV, a Bocavirus and a member of the *Parvoviridae* family, is up-taken by clathrin-coated pits, trafficks through the early- and late endosomal compartments, and is dependent on the pH of intracellular vesicles. It is not yet known whether capsid conformational change, which may allow endosomal escape, may occur in the early or late endosome or whether the genome or an intact virion is released to the cytosol. BPV infectivity or transcription failed to be mitigated by blocking proteasomal function. Intracellular trafficking is mediated by the actin and microtubule networks. Future studies will refine our knowledge of the uncoating process and the process this virus uses to translocate across the nuclear membrane to gain access to the nuclear microenvironment.

The model displayed in FIG. 22, illustrates some of the known and proposed events in the BPV replication cycle. Sialic acids are known receptors for BPV

attachment to cells. The genomic structure is well documented with the entire nucleotide sequence known and the locations of the three major open reading frames, NS1, NP1 and VP (capsid), together with the putative NS2 ORF. The single transcription promoter is located at map position P4. The mRNAs are separated from large poly transcripts by a complex set of splices and are polyadenylated at internal and terminal poly A signals. DNA synthesis is carried out by cell DNA polymerase along with cell-specific auxiliary factors with genomic inverted terminal repeats functioning as primers. Multimeric genome forms are generated with final encapsidation of primarily negative sense monomeric genome copies. Egress has been reported to occur by cell necrosis.

Our hypothesis for this project was that BPV is taken into the cell by an endocytosis mechanism rather than direct penetration of the plasma membrane or by a fusion mechanism. Moreover, we proposed that cytosolic trafficking events take the virus to the nuclear membrane. Taken together, data accumulated in this study show virus entry through the clathrin system. That blocking actin function inhibits virus transcription, protein synthesis, and full virus replication, indicates the involvement of actin in vesicular transport through the cytosolic milieu. Actin, together with myosin motors, is known to perform essential functions for the cell. Myosin type I is involved in membrane binding and endocytic vesicles. Myosin type V operates in vesicle transport, and myosin type VI is involved in endocytosis. Thus, actin, together with one or all of these myosin motor types, when inhibited could explain the observations we have seen in this study. Inhibition of virus replication with blocking of microtubules may imply movement of viral particles to the nuclear membrane in association with such structures. That dynein may be the motor that moves the particles along microtubular

filaments is a hypothesis at this time based on the observation that dynein is involved in some models of retrograde movement to nuclei.

There are other hypothetical suggestions in the replication model. The function of the PLA (phospholipase domain of VP) is unknown. The virus has to have some way of escaping the late endosome with enough structural integrity to remain functional and avoiding complete digestion as might be the case after lysosomal fusion. Some viruses do this using the fusion domains of "F" proteins that fuse viral envelopes with endosomal membranes. Because BPV is a nonenveloped virus it cannot use this mechanism. Alternatively, it may provide a frontal attack on the endosomal membrane by activation of phospholipase activity in a proton-rich environment. The freed, now capsid-altered, particle is faced with finding the nuclear membrane. Binding to the microtubule-dynein complex may solve this problem. These aspects of the replication cycle, together with the mechanism of nuclear membrane penetration, are yet to be elucidated.

TABLE 4. Summary of results

Target	Mechanism	Inhibition of virus		
		Inf	Prod	RNA
1. Inhibitors of clathrin endocytosis				
1.1 Chlorpromazine	prevents clathrin assembly at the cells surface.	+	+	+
1.2 Chloroquine	raises endosomal pH and blocks clathrin coated pit formation.	+	+	
1.3 Ammonium chloride	increases endosomal pH and delays pinching of clathrin coated pits from the cell membrane.	+	+	+
1.4 Bafilomycin A1	neutralizes endosomal acidification by inhibiting vacuolar ATP-ase.	+	+	
2. Inhibitors of caveolin pathway				
2.1 PMA	block caveolae formation at the cell surface.	-	-	+
2.2 Nystatin	depletes cholesterol in microdomains at the cell membrane.	-	-	-
2.3 MBCD	sequesters cholesterol in microdomains at the plasma membrane.	+	-	
2.4 Genistein	interferes with caveosome formation.	-	-	
3. Inhibition of cytosolic trafficking				
3.1 the early-to-late endosome		+		
3.1.1 Brefeldin A	interferes with transition from the-early-to-late endosome			
3.2 Proteasome				
3.2.1 Epoxomicin	inactivates the chymotrypsin-like activity of the proteasome.	-		-
3.2.2 Lactacystin	active against the chymotrypsin- and trypsin-like activities of proteasome.	-		-
3.3 Actin				
3.3.1 Cytochalasin D	blocks actin polymerization.	+		+
3.3.2 Latrunculin A	binds to actin monomers delaying transport via actin.	+		+
3.3.3 ML-7	interferes with actin transport blocking phosphorylation of MLC.	+		+
3.4 Microtubule				
3.4.1 Nocodazole	inhibits microtubule formation	-		+
3.4.2 Vanadate	dimishes microtubule transport disrupting the dynein motor.	-		-
3.4.3 EHNA	inactivates the microtubule-based dynein motor through ATPase.	-		+

+ inhibition; – no inhibition; nystatin* NS gene was enhanced; MBCD** (see 57, 111); EHNA*** VP gene enhancement; NP and NS genes inhibition; Inf (infectivity); Prod (productivity); RNA (qPCR)

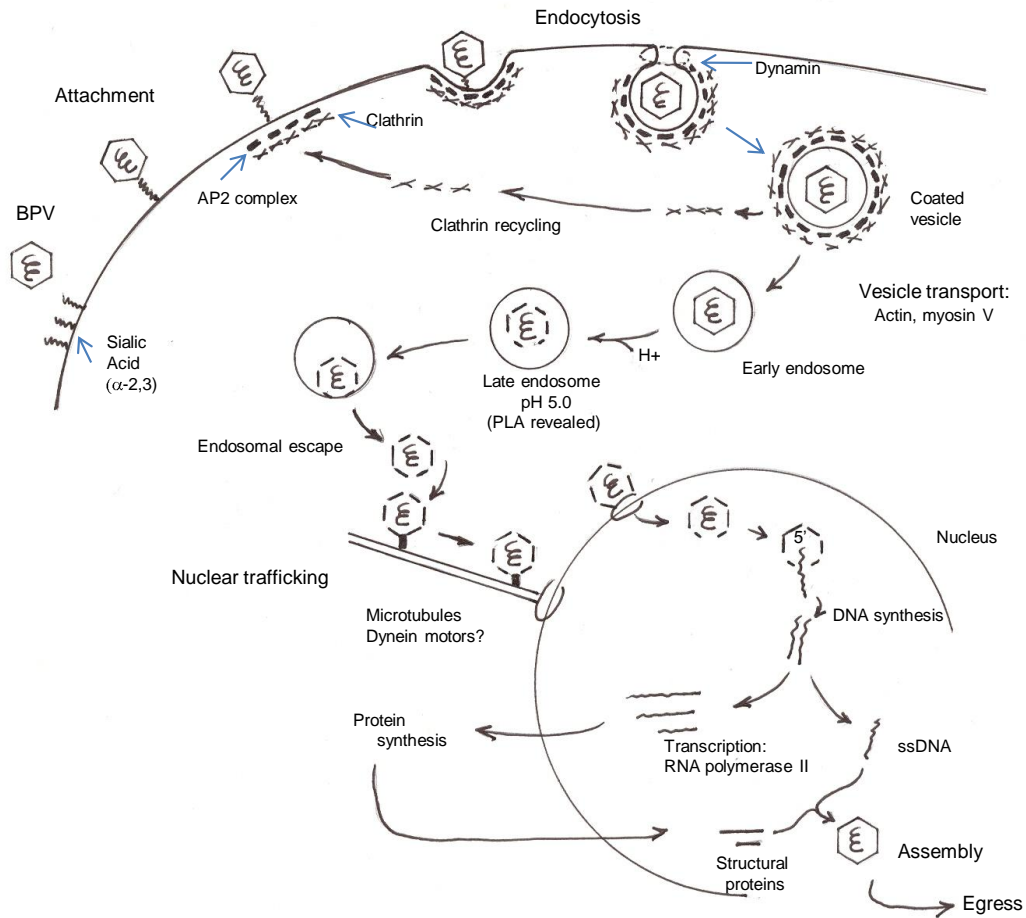


FIG. 22. A proposed model for BPV replication. Following attachment to alpha-2,3-sialic acid (O- or N-linked) the virus is endocytosed through the clathrin acid-mediated system. The actin-myosin V complex is responsible for vesicular transport. The transition from early to late endosome is accompanied by protonation which reveals the virus phospholipase moiety (PLA) which controls particle escape from the endosome. Trafficking from this cytosolic compartment to the nuclear membrane is controlled by polar microtubules and driven by the dynein motor. Penetration of the nuclear membrane allows for some capsid protein to remain attached to the 5' end of the ssDNA genome. DNA synthesis is performed by cell DNA polymerase forming a double stranded intermediate which is transcribed to the mRNAs that are formed from

a single promoter and a splicing array. The structural proteins, encoded in the CAP ORF, assemble to form empty procapsids with subsequent packaging of negative sense genome copies. Egress occurs by cell necrosis.

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